Prediction of Multiple Basal Cell Carcinomas

JORIS ARNOLDUS CORNELIS VERKOUTEREN

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Prediction of Multiple Basal Cell Carcinomas

Voorspellen van multipele basaalcelcarcinomen

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Chapter 1

General introduction

AIMS

Basal cell carcinoma (BCC) is the most common cancer in white-skinned people with increasing incidence rates, burden of disease and health care costs worldwide. In addition, a significant part (≥30%) of patients with a first BCC will develop at least a second new BCC or another ultraviolet radiation related cutaneous malignancy in time (i.e., metachronous skin cancers). Therefore it is an important topic for patients, physicians and policy makers. Unfortunately it is still unclear which patients are at risk of a metachronous BCC (mBCC) and who need follow-up in the future. Both non-genetic and genetic epidemiological studies of primary/prevalent BCCs have been conducted but studies are scarce when considering patients with mBCC. In this thesis I studied the epidemiology of mBCC using robust methodological approaches. The following main questions addressed in this thesis are presented below:

1. What is already known about the epidemiology of BCC and where are the gaps?
2. What are the non-genetic and genetic predictors of a superficial first BCC?
3. How to deal with the competing risk of death when analyzing metachronous BCCs?
4. What are the non-genetic predictors, absolute risks and cumulative incidences of metachronous BCCs?
5. What are the genetic predictors of multiple/metachronous BCCs?

OUTLINE

In **chapter 2** of this thesis a scholarly (i.e., non-systematic) review of the scientific literature on the epidemiology of BCC is presented. In this review we discussed incidences, trends and differences, burden of disease, risk factors, prevention and health policies, and gaps in existing knowledge were uncovered.

In **chapter 3** we raised the issue of a lack of well-designed and large population-based cohort studies to unravel the epidemiology of mBCC patients.

In **chapter 4** the non-genetic and genetic risk factors of the superficial subtype of BCC were investigated, because previous studies pointed out that this subtype could have a different etiology compared the other BCC subtypes and may be associated with mBCC. The reproducibility of previously found predictors was tested and potential new predictors (both non-genetic and genetic) were studied.

In **chapter 5** we discussed a common problem in survival analysis regarding multiple event data (e.g., mBCC), namely competing risk of death, and showed how to overcome this problem when calculating the probability of a new event. In chapter 6 and 7 we used this knowledge and chose models that could take competing risk into account and produce valid effect measures for the included predictors.

In **chapters 6** and **7** the primary objective was to develop a prognostic model for predicting the absolute risk of mBCC. An extensive literature search showed no other prediction models existed for mBCC. We included non-genetic predictors while adjusting for the competing risk of death. In **chapter 6** the prognostic model was developed for predicting the absolute risk of a second new BCC, whereas in **chapter 7** the follow-up was extended and a third, fourth and fifth new BCC were included as well to see whether the predictors of a second BCC were predictive for the risk of further mBCC. In addition, the frequency and timing of mBCC (i.e., cumulative incidences) was determined.

In the previous two chapters the focus was on non-genetic predictors of mBCC (i.e., patient, lifestyle and tumor-specific characteristics), but genetic predisposition could play a role as well. Therefore (**chapter 8** and **9**) we performed candidate gene approaches with known BCC loci and genome-wide association studies (GWASs) to identify single nucleotide polymorphisms associated with multiple BCC (now called "multiple" instead of "metachronous" because a small part of the included patients only had multiple BCCs on their first diagnosis date and no further BCCs in time), something which had not been done before. In **chapter 8** previously found BCC loci were tested in patients with multiple BCC and a pilot GWAS was conducted to identify susceptibility single nucleotide polymorphisms for multiple BCC. In **chapter 9** we added patients with squamous cell carcinomas to our group with BCC patients, as both tumors are keratinocyte carcinomas, to increase our power and performed both a candidate gene approach and GWAS on multiple keratinocyte carcinomas in collaboration with three different USA cohort studies.

Finally, in **chapter 10**, I answer the five research questions using results derived from this thesis. In addition, limitations of our studies are discussed and implications and future perspectives are given.

DATA SOURCES

In order to answer the five research questions formulated above I have used several data sources, which will be briefly described below. More details can be found in the corresponding chapters.

For the scholarly review in **chapter 2** and to a lesser extent for the commentary in **chapter 3** we have used different comprehensive search strategies in PubMed.

Chapters 4-8 are based on histopathologically confirmed skin cancer data gathered through a linkage between the Rotterdam Study and the Dutch Pathology Registry (PALGA). The Rotterdam Study is an ongoing prospective population-based cohort study of primarily white-skinned people aged 45 years or older living in a well-defined

district of Rotterdam, the Netherlands.[1] The Rotterdam Study started in 1989 and now comprises 14,926 participants. Detailed data were acquired by interviews and by thorough examinations of the participants in a specially built research facility in their district. These steps were repeated every 3-4 years. PALGA is the Dutch nationwide network and registry of histopathology and cytopathology, which was founded in 1971 and achieved complete national coverage in 1991.[2] The skin cancer information of the Rotterdam Study participants was obtained up to 31 December 2013 and over a 1,000 BCC patients could be included in both non-genetic and genetic analyses.

Chapter 9 has also been based upon the data described above, with the addition of data of several USA prospective cohort studies. We collaborated with the research teams of the Nurses' Health Study (NHSI and II) and the Health Professionals Follow-up Study,[3] as well as the research team of the Framingham Heart Study.[4]

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Chapter 2

Epidemiology of basal cell carcinoma: scholarly review

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ABSTRACT

Basal cell carcinoma (BCC) is the most common cancer in white-skinned individuals with increasing incidence rates worldwide. Patients with BCC place a large burden on healthcare systems, because of the high incidence and the increased risk of synchronous and metachronous BCCs and other ultraviolet radiation (UVR) related skin cancers (i.e., field cancerization). As a result, the disability-adjusted life years and healthcare costs have risen significantly in recent decades. BCC is a complex disease, in which the interplay between UVR, phenotype (UVR-sensitive) and genotype (somatic mutations and germline mutations/polymorphisms) fulfils a key role in the aetiopathogenesis. Prevention programmes with continual refinements and improvements could be of major importance in tackling the growing skin cancer problem. To provide the most appropriate BCC care, physicians should engage in shared decision-making and choose their treatments wisely.

INCIDENCE, TRENDS AND GEOGRAPHIC DIFFERENCES

Basal cell carcinomas (BCCs) do not have a precursor lesion and most likely arise from stem cells within hair follicles and interfollicular epidermis.[1,2] There are different histopathological subtypes, of which nodular is the most frequent, followed by superficial and infiltrative, and mixed types are frequently found as well.[3–5] The frequencies reported depend on the classification system used and period.[3,6] Most BCCs occur in the head and neck region (i.e., sun exposed), followed by trunk and extremities (i.e., relatively sun-unexposed).[3,4]

Incidence per region, trends and differences

BCC is the most common cancer in white-skinned people with increasing incidence rates worldwide.[7] Although reliable BCC incidence estimates are needed to monitor trends and allocate healthcare services, it is remarkable how few countries register BCCs in national/regional cancer registries. This registration gap can be explained by the tumour's high volume and low mortality, along with an inability to include nonhistopathologically confirmed BCCs and high incidence of synchronous and metachronous BCCs.

Comparisons of incidence rates between countries is difficult because different standardization methods are used. The incidence of BCC is strongly inversely related to the country's geographic latitude combined with the pigment status of its inhabitants (Table 1). The rates in Europe have increased approximately 5% annually over recent decades.[7] In the U.S.A., rates have increased about 2% per year leading to over 2.5 million patients with BCC treated annually.[7–9] The highest rates are seen in Australia, where over one in two inhabitants will be diagnosed with BCC by the time they are 70 years old, but the increasing incidence in Australia appears to be reaching a plateau, as the rates for people below 60 years of age have stabilized.[7,10,11] In non- Western regions, such as Asia and South America, incidence rates are ten to hundred-folds lower, but have also increased.[12,13]

The increase in incidence can be explained by an increased awareness in the general population and among physicians, more surgical treatments (e.g., more excisions with histopathological confirmation instead of cryotherapy or electrodessication), improved registration, an ageing population and changes in the distribution of risk factors such as ultraviolet radiation (UVR) exposure patterns. The latter is often a matter of debate, but is underlined by the observation that the incidence of UVR-related skin tumours increased significantly and more steeply compared with other cutaneous malignancies.[14]

BCC incidence increases significantly with age, but the most remarkable increase has been observed in young women in both Europe (Netherlands and Denmark) and the U.S.A., resulting in a reversed male : female ratio (female > male) in younger

Table 1. Overview of incidence rates and trends of BCC worldwide

Continent	Country	Latitude[a]	Incidence rate[b]	Standardization	Period[c]	Trends (EAPC)	Reference
Europe	Finland	61° N	♀90.2; ♂104.8	ESR	2009	-	De Vries, 2012[200]
	Scotland	56° N	♀81; ♂123	ESR	2006	-	De Vries, 2012[200]
	Denmark	56° N	♀96.6; ♂91.2	WSR	1978-**2007**	♀4.6%; ♂3.7%	Birch-Johansen, 2010[16]
	Lithuania	55° N	♀47.4; ♂46.4	ESR	1996-**2010**	♀2.6%; ♂3.3%	Jurciukonyte, 2013[201]
	United Kingdom	55° N	♀135.4; ♂172.1	ESR	2000-2011	-	Reinau, 2014[202]
	Northern Ireland	54° N	86.8	ESR	2000-2006	-	Lomas, 2012[7]
	Ireland	53° N	♀85.7; ♂98.0	WSR	1994-2003	-	Carsin, 2011[203]
	England	52° N	76.2	ESR	2000-2006	-	Lomas, 2012[7]
	Netherlands	51° N	♀157.3; ♂164.7	ESR	2002-**2009**	♀7.9%; ♂6.8%	Flohil, 2013[17]
	Germany	51° N	82.2	ESR	2006-**2010**	6.8%	Rudolph, 2015[204]
	Croatia	45° N	♀24.5; ♂33.6	WSR	2003-2005	-	Lipozenčić, 2010[205]
	Serbia	44° N	♀27.8; ♂31.0	WSR	1999-**2011**	6.1%	Videnović 2015[206]
	Spain	41° N	128.0	WSR	2006-2007	-	Bielsa, 2009[207]
	Malta	35° N	♀70; ♂84	ESR	2009	-	De Vries, 2012[200]
North America	Canada (AB)	53° N	♀119.6; ♂147.0	CAN	2000-**2006**	-0.8%	Jung, 2010[208]
	Canada (MB)	53° N	♀77.4; ♂93.9	WSR	1971-**2000**	2.4%	Demers, 2005[209]
	USA (NH)	43° N	♀165.5; ♂309.9	USA	1979-1980, **1993-1994**	♀4.4%; ♂4.4%	Karagas, 1999[210]
	USA	37° N	♀1,019; ♂1,488	ASR	2004-**2006**	-	Wu, 2013[211]
	USA (CA)	36° N	♀774; ♂1,069	USA	1998-**2012**	0.9%; ♀1.1%	Asgari, 2015[100]
	USA (NM)	34° N	♀485.5; ♂930.3	USA	1998-1999	-	Athas, 2003[212]
	USA (AZ)	34° N	♀497.1; ♂935.9	USA	1996	-	Harris, 2001[213]
Asia	Jordan	33° N	♀8.8; ♂6.2	WSR	1991-2000	-	Rawashdeh, 2004[12]
	Israel	31° N	♀158; ♂225	ESR	2006-2011	-0.7%	Sella, 2015[214]
	Singapore	1° N	4.5	-	2003-2006	-	Sng, 2009[215]

Table 1. Overview of incidence rates and trends of BCC worldwide (*continued*)

Continent	Country	Latitude[a]	Incidence rate[b]	Standardization	Period[c]	Trends (EAPC)	Reference
Africa	Kenya	0°	0.0065[d]	CIR	1968-1997	-	Munyao, 1999[216]
	South Africa	30° S	♀1.7[d]; ♂3.0[d]	ASR	2000-2004	-	Norval, 2014[217]
South America	Brazil	27° S	295.2	CIR	2008	-	Custódio, 2010[218]
	Chile	53° S	3.9	CHL	1994-2000	-	Abarca, 2002[13]
Oceania	Papua NG	6° S	0.3	CIR	1960-1980	-	Foster, 1988[219]
	Australia	25° S	♀745; ♂1,041	WSR	2002	-	Staples, 2006[10]
	Australia (QLD)	26° S	♀1,269; ♂1,813	WSR	1997-2006	-	Richmond-Sinclair, 2009[220]
	New Zealand	40° S	♀215; ♂383	WSR	1997-2006	♀4.4%; ♂3.1%	Brougham, 2011[221]

BCC, basal cell carcinoma; EAPC, estimated annual percentage change; ESR: European Standardized Rate; WSR: World Standardized Rate; AB, Alberta; CAN: Canadian standardized rate; MB, Manitoba; USA (country), United States of America; NH, New Hampshire; USA, USA standardized rate; ASR, Age Standardized Rate; CA, California; NM, New Mexico; AZ, Arizona; CIR: Crude Incidence Rate; CHL: Chile standardized rate; Papua NG, Papua New Guinea; QLD, Queensland.

[a] Estimate (rounded) of the latitude (N, Northern Hemisphere; S, Southern Hemisphere), based on latitudes from www.worldatlas.com (visited at 18-04-2016).

[b] Per 100,000 person years, both sexes combined or separated.

[c] The year(s) represent the period to which the incidence rates belong, if in **bold**, the incidence rate belongs to that specific year.

[d] Incidence rate for native Africans.

populations compared with older populations (male > female).[15–17] This discrepancy between men and women could be a result of the higher use of tanning beds by young women[18,19] and of women paying closer attention to their appearance and the health of their skin, which may result in more medical visits.[20]

Multiple basal cell carcinomas

In line with the concept of field cancerization,[21] patients diagnosed with a first BCC have an increased risk of developing a second BCC and other UVR-related skin cancers.[22,23] Patients with a BCC have a 17-fold increased risk of a subsequent BCC compared with the general population, followed by a threefold increased risk of a subsequent squamous cell carcinoma (SCC) and a twofold increased risk of a melanoma.[23] The majority of patients with skin cancer are prone to develop the same type (i.e., BCC or SCC) of skin cancer.[24] Approximately one-third of all patients with a first BCC will develop at least a second BCC, 4% an SCC and 0.5% a melanoma, but these elevated risks also vary geographically and reflect the underlying incidence rates.[23] The likelihood of subsequent UVR-related cancers supports the concept that skin cancer shows similarities with other chronic conditions, something which has been coined 'actinic neoplasia syndrome' by Weinstock et al.[25]

BURDEN OF DISEASE

Global skin cancer burden

The World Health Organization (WHO) quantifies the burden of a disease with the disability-adjusted life year (DALY).[26] This time-based measure aggregates years of life lost through premature death (YLL) and years lived with disability (YLD). One DALY equals the loss of 1 year of life lived in full health.

The mortality of BCC is extremely low as it rarely metastasizes, with rates ranging from 0.0028% to 0.55%, and will therefore hardly affect YLL.[27] However, the high and increasing BCC incidence, the decreasing age at first BCC, and the high occurrence of multiple BCCs (mBCC) and other UVR-related skin cancers puts a strain on healthcare services. The WHO Global Burden of Disease (GBD) project showed that the age-standardized YLD rates for nonmelanoma skin cancers (NMSCs) increased significantly between 1990 and 2013 (42.5%, to 126 200) and are comparable with the rates of oesophageal, ovarian or thyroid cancer.[28] Unfortunately, the GBD project does not differentiate between the various NMSC subtypes.

A GBD on UVR exposure computed a BCC-specific DALY and estimated that 58 000 DALYs were lost globally in 2000.[29] A Dutch study of keratinocyte cancer (KC; both BCC and SCC) burden showed that the world standardized DALY rates for BCC

in both sexes doubled between 1989 and 2008, from two to four per 100 000 person-years.[30] However, both studies included only the first BCC and therefore will have underestimated the true burden.

Healthcare costs

Results of cost analysis studies of different BCC treatments are usually not generalizable, because of the different healthcare systems between countries. Nonetheless, a 2015 systematic review summarized the healthcare expenditure for different countries and national cost estimates were adjusted for country-specific inflation and presented in 2013 euros.[31] In absolute terms, the U.S.A. spends the most money on KC (~ 600 million), followed by Australia (> 350 million), Germany (> 150 million) and the U.K. (> 100 million).[31] However, the KC costs relative to the size of the population were highest for Australia, followed by New Zealand, Sweden and Denmark, whereas Brazil and Canada had the lowest.[31]

A U.S.A. Medicare expenditure study showed that NMSC was the fifth most costly cancer between 1992 and 1995.[32] One U.S.A. study estimated the productivity loss per BCC case and reported an estimated cost of $1235.[33] A recent report estimated the average annual cost of treating NMSC in the U.S.A. at $4.8 billion from 2007 to 2011, which is a 74% increase compared with the 2002–06 estimate.[34] A relatively large part of the U.S.A. treatment costs (> $2 billion) comprise Mohs micrographic surgery (MMS), a treatment which has grown exponentially in recent decades.[35,36] MMS is a cost-effective treatment as long as it is performed by skilled physicians and used in properly selected patients, such as patients with recurrent or aggressive histological BCCs in the H-zone (temporal, retro- and pre-auricular, orbital and infranasal areas, ears and nose).[35,37,38] From at least a cost perspective, usage of MMS should be monitored to prevent over-usage.

Another potential cost driver of BCC care is methylaminolaevulinate photodynamic therapy (MAL-PDT). In the Netherlands, MAL-PDT was used very frequently, in part due to a very profitable reimbursement.[39] This changed after a Dutch single-blind, noninferiority, randomized controlled trial (RCT) demonstrated that the much less costly topical fluorouracil and imiquimod were not inferior to MAL-PDT for clearance of superficial BCC after 12 months.[40,41]

RISK FACTORS

BCC is a complex disease because the likelihood of developing this tumour depends on the interplay between constitutional predisposition (genotypic and phenotypic characteristics) and subsequent exposure to environmental risk factors. Figure 1

shows the most important genetic, phenotypic and environmental risk factors for the development of BCC (see Supporting Information, Table S1, for more details). Because BCC is a complex disease, most risk factors studied have small effect sizes and it is very possible that several of the observed associations are false-positive and/or clinically irrelevant.[42]

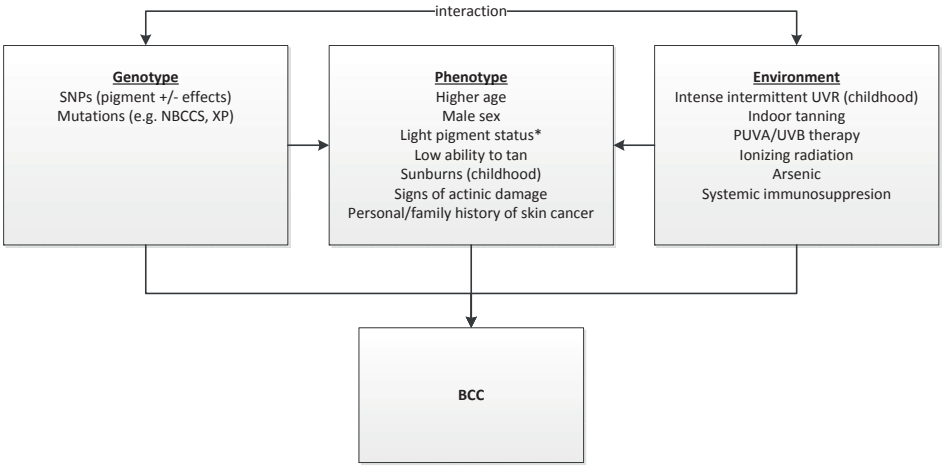

Figure 1. Main nongenetic risk factors of BCC
This flowchart shows the main genotypic, phenotypic and environmental risk factors for BCC. The arrows show how the different risk factor categories exert effects on each other and on BCC. BCC, basal cell carcinoma; SNPs, single nucleotide polymorphisms; NBCCS, naevoid basal cell carcinoma syndrome; XP, xeroderma pigmentosum; UVR, ultraviolet radiation; PUVA, psoralen plus ultraviolet-A radiation; UVB, ultraviolet B radiation. * Consists of complexion, hair colour and eye colour.

Complex disease: environmental risk factors

Ultraviolet radiation

UVR is the major environmental risk factor for BCC (population attributable fraction > 90%[43]), but its assessment is problematic (i.e., exposure pattern, timing and amount), its exposure varies but is universal and its effect sizes are small. Nevertheless, it seems that intense intermittent UVR exposure (e.g., outdoor recreational activities and beach holidays), in particular during childhood and adolescence, leads to a significant increase in the risk of BCC.[44–49] The amount of UVR exposure is positively associated with BCC risk, but this effect levels off or even decreases after a certain amount of exposure.[46,47] The skin's ability to tan modulates the UVR-induced risk.

A systematic review and meta-analysis showed that indoor tanning is significantly associated with an increased risk of BCC [relative risk 1.29; 95% confidence interval

(CI) 1.08–1.53; I^2 = 37%; no evidence of publication bias], especially if used early in life.[50] Patients with psoriasis who have had a high number (> 100–200) of psoralen plus UVA radiation (PUVA) treatments develop significantly more BCCs than expected and this risk seems to persist over time.[51–53] UVB therapy (> 300 treatments) has also been associated with modest risk increases in the risk of developing BCC.[51,54]

Photosensitizing drugs

Photosensitizing medication has the ability to induce a phototoxic and/or photo-allergic reaction upon UVR exposure. In addition to psoralen, other photosensitizing medications (e.g., diuretics, tetracyclines and nonsteroidal anti-inflammatory drugs) were shown to be positively associated with BCC in several pharmaco-epidemiological studies.[55–57] However, most of these studies suffered from important limitations and no dose–response relationships were observed.

Ionizing radiation

Patient groups at risk are those irradiated in the past for benign disorders such as tinea capitis, acne and otitis serosa,[58–61] and those irradiated for different types of cancer, including childhood cancer survivors and haematopoietic cell transplantation survivors.[62,63] Nonmedical groups at risk are atomic bomb survivors and occupational groups such as radiological technologists.[64,65] The elevated BCC risks are confined to the site of radiation exposure.[66]

Ionizing radiation (IR)-induced BCC risk appears to increase with a person's skin susceptibility to UVR and younger age at exposure (i.e., basal layer more sensitive to radiation carcinogenesis). [58,59,61] The development of mBCC in irradiated skin occurs frequently as well.[61]

Chemicals

Arsenic is a carcinogen that appears naturally (i.e., well water), medicinally and in the workplace (e.g., mining and agriculture).[67] Chronic exposure to arsenic can induce BCC formation, especially on the trunk, and BCC multiplicity occurs frequently as well.[67–69]

Smoking

A systematic review and meta-analysis showed that smoking is not significantly associated with BCC, with no evidence of publication bias [odds ratio (OR) 0.95; 95% CI 0.82–1.09; I^2 = 59%].[70] A less rigorous meta-analysis suggested that 'ever smokers' compared with 'never smokers' had slightly elevated risks of BCC (OR 1.02; 95% CI 1.00–1.04; I^2 = 84%).[71] Overall, it seems that smoking has little to no effect on BCC development.

Human papillomaviruses

In contrast to SCC, some observational studies have found a significant positive association between human papillomavirus (HPV) DNA or seropositivity and BCC.[72–74] However, most case–control studies did not find a clear association between different cutaneous oncogenic HPV types and BCC.[75–77] For now, the evidence that viral oncogenesis plays a role in BCC development is far from conclusive.[78]

Diet and drinks

The epidemiological literature on the role of dietary factors in the development of BCC is inconsistent and insufficient for most of the factors studied.[79] The evidence for protective effects of selenium, carotenoids and vitamins on BCC development is inconsistent.[80–84]

Several studies on the relationship between alcohol and BCC have been conducted, showing conflicting evidence and beverage-dependent relations.[85–89] Caffeine intake (e.g., coffee) has been associated with a reduced risk of BCC and mBCC.[90–92] Whether caffeine really inhibits photocarcinogenesis or is just a proxy for global health and lifestyle needs to be differentiated.[93]

Systemic immunosuppression

Over recent decades, the number of chronic immune-suppressed patients, who are at an elevated risk of SCC and to a lesser extent of BCC, has grown consistently as a result of the increasing number of organ transplant recipients, the immunosuppressive agents used in different diseases (e.g., inflammatory bowel disease and non-Hodgkin lymphoma) and the increased longevity of these chronic immune-suppressed patients.[94–96] The overall BCC incidence in renal transplant recipients was[7–16] (depending on geographic location) times greater than in the general population.[94,97,98] The extent of UVR exposure and UVR-induced DNA damage prior to transplantation (i.e., UVR-induced DNA mutations) combined with an impaired cutaneous immune surveillance results in an elevated field risk and metachronous BCCs and SCCs.[99]

Complex disease: phenotypic risk factors

Increasing age and male sex (at older age) are well-known host characteristics that increase the risk of BCC.[17,100] The ability to repair (UVR-induced) DNA damage reduces with age, which leads to an accumulation of damage and an increased incidence of BCC in older people.[101,102]

The highest BCC risks can be found in people with a personal and/or family history of skin cancer, who are (highly) sensitive to UVR exposure and are exposed to intense intermittent UVR. This sensitivity is determined by the combination of a fair complexion, light hair colour and light eye colour, and low ability to tan.[47–49,103–108]

Acute effects of excessive UVR exposure such as (childhood) sunburns and more long-lasting signs of actinic damage such as melanocytic naevi, freckles, solar elastosis, solar lentigines and actinic keratoses are also significant predictors of an increased BCC risk.[44,48,109–111] These manifestations of photodamage could be a warning sign of field cancerization.

Risk factors for different histopathological subtypes

Multiple observational studies have found body area, age and sex preferences for certain histopathological BCC subtypes (see Supporting Information, Table S1). Superficial BCCs are predominantly located on the trunk and patients diagnosed with a superficial BCC are significantly younger and more often female than patients with other subtypes.[3,4,6] These results could indicate that the different subtypes have other aetiologies with respect to UVR exposure and the interaction between constitutional characteristics and other environmental risk factors. In addition, truncal BCCs have been associated with acute intense intermittent exposure patterns.[112,113]

Risk factors for multiple basal cell carcinomas

Higher age at initial BCC, male sex and a history of BCC have all been found to be positively associated with metachronous BCCs (see Supporting Information, Table S1).[22,114,115] The value of other phenotypic (e.g., skin type) and environmental (e.g., UVR) characteristics in predicting a new BCC is under debate[22,114,116,117] and studies may be hindered by the index event bias.[118]

A recently developed prediction model for a second BCC showed that the risk factor profile differs between a first and second BCC.[92] The most discriminating predictor was the presentation of mBCC at first BCC diagnosis.[92] Other factors associated with a second BCC were age at first BCC (parabolic relation with maximum risk at 68 years), male sex, superficial subtype of the first BCC and coffee consumption.[92] An update of this prediction model, including up to five metachronous BCCs, is in preparation.

GENETIC PREDISPOSITION

Somatic mutations

UVR-induced cancers such as BCC and melanoma exhibit the highest prevalence of somatic mutations, of which the majority show 'UV signatures', of all cancers.[119,120] Acquired mutations in RAS oncogenes do not seem to play an important role in BCC pathogenesis.[121–123] However, two tumour suppressor genes are important in sporadic BCC carcinogenesis, namely patched 1 (PTCH1) and tumour protein p53 (TP53).

The key evidence of a crucial role of PTCH1 in BCC development came from patients with naevoid basal cell carcinoma syndrome (NBCCS). PTCH1 (chromosome 9q22) encodes a protein that is the receptor for sonic hedgehog, a secreted molecule implicated in the formation of embryonic structures and in tumorigenesis.[124,125] Loss of heterozygosity on chromosome 9q22 is the most frequent (58-69%) genetic alteration in sporadic BCCs.[126–128] Inactivation of PTCH1 and upregulation of hedgehog signalling are most likely pivotal events in BCC carcinogenesis.[129,130]

TP53 (chromosome 17p13) encodes a tumour suppressor protein that can induce several processes, such as cell cycle arrest, senescence, apoptosis and DNA repair.[131] Mutations in this gene play a role in carcinogenesis in a wide variety of tissues.[132] Direct DNA sequencing of the TP53 gene in BCCs revealed mutations in approximately 44–65% of tumours.[127,133,134]

Germline polymorphisms

The melanocortin 1 receptor gene (MC1R) is a major determinant of skin colour and hair colour, and MC1R variants are significantly associated with BCC risk, even after correcting for skin pigmentation.[135–137] This pleiotropy suggests that MC1R variants exert carcinogenic pigmentation independent effects. Pigmentation pathway single-nucleotide polymorphisms (SNPs) in tyrosinase (TYR) and agouti signalling protein (ASIP) confer risk of BCC as well.[138] Studies investigating a possible link between defects in DNA repair genes and BCCs have yielded conflicting results.[130]

The first genome-wide association study in patients with BCC was conducted in 2008 and since then, six have been performed in total, finding 17 different risk-increasing SNPs mapped to 16 different chromosomal regions (Table 2).[139– 144] The ORs found are small overall, between 1.15 and 1.55 (except for TP53 variants), and it is not surprising that much of the genetic variability is still unexplained. New approaches such as exome sequencing and epigenetic studies will further explain heritability.

The genetic predisposition of mBCC is not well documented and may involve genetic changes different from those associated with a primary BCC.[145] The cytochrome (CYP) supergene family and the glutathione S-transferase (GST) supergene family are involved in different metabolizing and detoxification processes, such as detoxification of products of oxidative stress.[146] Polymorphisms in these genes have been associated with increasing BCC numbers.[147–149]

Germline mutations

NBCCS is an autosomal dominant disorder characterized by mBCC, odontogenic keratocysts of the jaws, palmar and/or plantar pits and skeletal abnormalities.[150–152] The majority of patients with NBCCS start developing their BCCs from puberty onwards and affected individuals may develop from a few up to over a thousand BCCs.[150,151]

Patients exposed to IR or high levels of UVR become even more susceptible to BCC formation.[150,151] Using family-based linkage studies of NBCCS kindreds, the causative locus was first mapped to 9q22 and then to the PTCH1 gene.[124–126]

Patients suffering from xeroderma pigmentosum (XP) have germline mutations in their nucleotide excision repair genes, which are of crucial importance for removing UVR-induced DNA damage.[153] They have high risks of developing mBCC and other skin cancers during childhood.[154]

A few other genodermatoses can also cause development of mBCC early in life, namely Bazex Dupre–Christol syndrome[155,156] and Rombo syndrome.[157,158]

Table 2. Genome-wide significant risk SNPs for BCC[a]

SNP[b]	Risk allele	Frequency	Context	Region	Mapped gene[b]	OR (95% CI)
rs7538876	A	0.35	intron	1p36.13	PADI6	1.28 (1.19-1.37)
rs801114	G	0.33	downstream gene	1q42.13	RHOU, LOC105373143	1.28 (1.19-1.37)
rs401681	C	0.56	intron	5p15.33	CLPTM1L	1.25 (1.18-1.34)
rs7335046	G	0.12	downstream gene	13q32.3	UBAC2, LINC01232	1.26 (1.18-1.34)
rs1805007	T	0.07	missense	16q24.3	MC1R	1.55 (1.45-1.66)
rs12210050	T	0.17	intergenic	6p25.3	LOC105374875	1.24 (1.17-1.31)
rs78378222	C	NR	3′ UTR	17p13.1	TP53	2.16 (1.83-2.54)
rs214782	G	0.17	intron	20p13	LOC105372503, TGM3	1.29 (1.22-1.37)
rs7006527	A	0.86	intron	8q22.2	RGS22	1.3 (1.22-1.41)
rs59586681	T	0.61	intergenic	20p13	LOC388780	1.16 (1.11-1.22)
rs2151280	G	NR	intron	9p21.3	CDKN2B-AS1	1.2 (1.14-1.27)
rs157935	T	NR	intron	7q32.3	LINC-PINT	1.23 (1.15-1.31)
rs57244888	T	0.90	intergenic	2p24.3	LOC105373443, LOC105373444	1.32 (1.22-1.43)
rs13014235	C	0.46	missense	2q33.1	ALS2CR12	1.15 (1.10-1.20)
rs28727938	C	0.94	intron	8q21.13	LINC01111, MRPL9P1	1.43 (1.30-1.59)
rs73635312	G	0.87	Upstream gene	10p14	LOC105376400	1.35 (1.25-1.45)
rs11170164	T	0.09	missense	12q13.13	KRT5	1.29 (NR)

BCC, basal cell carcinoma; SNP, single nucleotide polymorphism; OR, odds ratio; 95% CI, 95% confidence interval; NR, not reported; 3′ UTR, three prime untranslated region.

[a] This table has been based on data available at www.ebi.ac.uk/gwas, accessed 20-02-2016 with the search term "basal cell carcinoma".[222]

[b] Mapped to dbSNP Build 146 and Genome Assembly GRCh38.p5.

PREVENTION

Primary prevention

The goal of primary prevention is to reduce the incidence of a first BCC. Even though UVR exposure is not solely responsible for the development of BCC, a considerable risk reduction is expected by adequate sun protection.[159] However, an Australian community-based RCT demonstrated that the daily application of sunscreen did not reduce the risk of BCC.[160] This finding could be partly explained by the occurrence of BCCs on sites that were not treated with sunscreen and the relatively high age of the included participants, and underlines the complex association between UVR and BCC.[160] Multiple national campaigns have been initiated to create public awareness, improve professional education and start behavioural change, such as the SunSmart programme in Australia.[161,162] Initially, these campaigns focused on informing people about the harmful effects of UVR exposure but now more actively try to influence behaviour. In addition, they target children and adolescents at schools, because minimizing (excessive) UVR exposure at an early age is a very important preventive measure.[161,163] At a legislative level, local governments were encouraged to adopt sun protection policies such as sales tax exemption for approved sunscreens and the creation of sufficient shade at schools and other public open spaces.[161,162] Commercial indoor tanning salons in Australia were banned completely as of 1 January 2015 and multiple other countries have restricted the use of indoor tanning as well.[164]

Although the awareness of the hazardous effects of excessive UVR exposure has increased over time, the incidence of most UVR-related skin cancers is still increasing, suggesting that people have not fully adopted this knowledge in their behavior (i.e., 'knowledge–behaviour gap'). Nevertheless, Australian studies reported the stabilization of NMSC rates for people younger than 60 years[10] and also showed a significant decline in excision rates for KCs in men and women younger than 45 years.[165] The positive effects of primary prevention programmes might become more evident over time, as the follow-up is still relatively short since the initiation of these programmes.

In addition to behavioural changes, the use of natural, synthetic or biological chemical agents to reverse, suppress or prevent carcinogenic progression to invasive cancer (i.e., chemoprevention) could be promising in reducing the BCC burden as well.[166] Chemoprevention could be used as both a primary and secondary prevention measure. Whether an agent is a good chemoprophylactic candidate is determined by the risk : benefit ratio. Many agents, such as beta carotene, selenium, synthetic retinoids (tretinoin, isotretinoin) and nonsteroidal anti-inflammatory drugs have been tested but showed no chemopreventive effect on BCC development.[160,167–172] However, when retinoids were used in patients with genodermatoses (NBCCS, XP) a more promising protective effect was seen on BCC development.[173–175] Another systemic

chemoprophylactic that works well in patients with NBCCS is the hedgehog pathway inhibitor vismodegib, but adverse events occur frequently.[176]

Secondary prevention

The goal of secondary prevention is to detect skin cancer at an early stage (screening) and to prevent metachronous skin cancers. Taking the Wilson and Jungner principles for population-based disease screening into consideration, BCC screening by itself is not likely to be cost-effective because the costs of case-finding (including diagnosis and treatment) are most likely in a nonacceptable relation to the overall healthcare costs.[177] In addition, the U.S. Preventive Services Task Force recently (2016) concluded that: 'the current evidence is insufficient to assess the balance of benefit and harms of screening for skin cancer in adults with a clinical visual skin examination'.[178] However, the German skin cancer screening programme showed that the skin cancer incidence went up during the screening period, but this does not necessarily mean that it was (cost) effective.[179]

A way of increasing the cost-effectiveness of screening is restricting screening to high-risk patients such as those with a history of BCCs. Recently, a prediction was developed that could reasonably assess the absolute risk of a second BCC using simple phenotypic, lifestyle and tumour-specific characteristics.[92] Further improving these prediction models in the coming years could help physicians identify these high-risk patients and give them the right follow-up. The downside of targeted screening approaches in high-risk patients is the so-called 'prevention paradox' in which you address the high-risk individual but not the overwhelming majority of low-risk patients that develop BCCs.[180]

Tertiary prevention

The goal of tertiary prevention is to soften the impact of (advanced/metastatic) BCC on patients' lives. A small group (about 1%) of patients have BCCs that have progressed to an inoperable stage or have metastasized, and these advanced BCCs were associated with a significant disease burden.[181,182] In order to improve their ability to function, their quality of life and their life expectancy, MMS, radiotherapy and vismodegib could be used.

IMPLICATIONS FOR HEALTH POLICIES

Overall impact

Although BCC-related mortality is low, both tumour growth and treatment can cause considerable functional and cosmetic morbidity. The recent U.S.A. initiative to rename

BCC to 'indolent lesion of epithelial origin (IDLE)' may be understandable from a public health perspective, but is inappropriate on an individual level because it falsely reassures patients.[183] In addition, the lay press recently minimized the consequences that BCC can have on the well-being of a patient, confirming the downgrading of BCC as a nonissue.[184] These controversial opinions could be a warning sign that policy-makers are developing a different view on BCC care.

Treatment-related impact

To provide the most appropriate BCC care, physicians should individualize the management of BCCs, taking tumour, patient and treatment characteristics into account, and combine this with patient preferences and needs (i.e., shared decision-making).[185] The dermatologist should be the lead of skin cancer management, but needs to combine diagnostic expertise with a high level of surgical skills to provide the optimal care. In addition to dermatologists, the general practitioner (GP) also can play an important role in BCC management. In countries such as Australia, where skin cancer poses a large burden on the healthcare systems, trained GPs with a special interest in skin cancer are functioning as specialized primary care physicians to detect and treat skin cancer. A Dutch study showed that the majority of GPs questioned were willing to extend their role in skin cancer care, including surgical excision of low-risk BCCs, but that they requested additional skin cancer training.[186]

Choosing the most cost-effective treatment for BCC wisely becomes increasingly important.[187] The positioning and appropriate use of MMS in the management strategy of BCC is crucial, because it drives the increment in costs related to BCC care.[35,188] Appropriate use of more costly treatments is warranted to ensure access to this more expensive treatment over the long term. Linos et al. have raised another controversial issue in BCC management among patients with limited life expectancy.[189] In a U.S.A. prospective cohort study, they showed that most NMSCs were treated surgically, regardless of the patient's life expectancy.[189] Although it remains a controversial topic, it should stimulate clinicians to provide individualized care in line with patients' needs, especially for certain subgroups of patients with BCC.

Follow-up-related impact

The underlying rationale to monitor patients with BCC is to identify recurrences and new tumours, educate and psychologically support and reassure patients.[190] This multidimensional rationale makes it difficult to generate consensus about frequency and duration of follow-up. For example, most clinical recurrences appear within 3 years, but up to 20% may occur within 5–10 years,[191,192] whereas the psychological stress often peaks in the first years after a cancer diagnosis.[193,194] In addition, the risk of metachronous BCCs is highest in the first 3 years after diagnosis, but remains elevated

over time.[115,195,196] The Dutch BCC guideline differentiates between high- and low-risk BCCs and recommends annual follow-up for high-risk BCCs.[197] In contrast, the U.K. guideline concludes, 'Clearly, within the British health care system it is not possible to offer long-term follow-up to all patients who have had their first and only primary BCC treated'.[198] Again, there is very little data to support both recommendations, but the costs of annual monitoring by dermatologists is a very expensive surveillance method because of the tumour's high incidence.

In contrast, there exists enough data that support that both the dermatologist and the GP should perform total body skin examinations in patients presenting with a primary BCC, because the chance of finding another synchronous BCC is significant.[115,199] Clinicians should also be aware of the increase in BCC incidence in younger (female) patients,[15] which could lead to an exponential increase in its occurrence in the future elderly population, because those with a history of BCC are likely to develop more of these tumours.[23]

A more cost-effective approach could be to invest in providing personalized information on BCC and its treatment, and educate patients on important risk factors, risks of metachronous skin cancer, sun avoidance measures, skin self-examination, and train GPs in after care of patients with skin cancer, but this needs to be studied in more detail as has been done for other cancers.

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SUPPORTING INFORMATION

Table S1. Main nongenetic risk factors for basal cell carcinoma (BCC), superficial BCC and multiple BCCs

	BCC	Superficial BCC	Multiple BCCs
Phenotypic factors			
Age	+ (higher)[1-8]	+ (lower)[9-12]	+ (higher)[13-22]
Sex[a]	+ (male)[4]	+ (female)[10,11]	+ (male)[13,17,19,20,23-26]
Light pigment status[b]	+[1-3,6-8,27-43]		
Low ability to tan (i.e., burn easily)	+[1-3,7,27,31,34-39,41,43-47]		
Painful/blistering sunburns	+[6,8,30,31,34,40,43,46,48]		
Childhood painful/blistering sunburns	+[28,29,35,36,38,39,49,50]		
Signs of actinic damage	+[3,4,6-8,28-31,33-35,38,41,44,45,51-53]		
Personal history of skin cancer	+[6,35,41]		+[13,21,54]
Family history of skin cancer	+[6,7,28,35,45,55,56]		
Truncal (first) BCC		+[9-12,57]	+[18,19,37,58,59]
Multiple BCCs at (initial) presentation			+[22,60]
Environmental factors			
Childhood intense intermittent UVR exposure	+[29,34,35,39,46,55,61]		
(Adult) intense intermittent UVR exposure	+[8,35,38-41,46,50,56,62]		
Indoor tanning	+[63-68]		
PUVA therapy	+[69-74]		+[75]
UVB therapy	+[71,73,76,77]		
Medical ionizing radiation	+[35,71,78-88]		+[84]
Non-medical ionizing radiation	+[89-92]		
Arsenic	+[93,94]		+[95]
Organ transplant recipients	+[96-100]		+[101]
Immunosuppressive agents	+[102-105]		

"+" means positively associated with the outcome and an empty cell means no (clear) association. References are shown in superscript. BCC, basal cell carcinoma; AK, actinic keratosis; PUVA, psoralen and ultraviolet-A; UVB, ultraviolet B.

[a] analyses were frequently adjusted for age and sex, but papers often don't report the effect sizes. However, from incidence studies it is clear that both age and sex are significantly associated with BCC.

[b] Consists of complexion, hair colour and eye colour.

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Chapter 3

Cohort studies (and skin cancer) never come alone

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ABSTRACT

A previous keratinocyte carcinoma is probably the strongest predictor of developing new keratinocyte carcinomas, which makes these patients an interesting population for prevention interventions. Investing in large cohort studies and consortia might increase the validity of observational findings and should stimulate scientists to investigate the underlying mechanisms in detail.

It is well known that the risk of a subsequent cutaneous malignancy is increased in patients with a previous keratinocyte carcinoma (KC). A recent meta-analysis showed that 29% of patients with a history of basal cell carcinoma (BCC) developed a subsequent BCC and 4% a subsequent squamous cell carcinoma (SCC), whereas 13% of patients with a history of SCC developed a subsequent SCC and 16% a subsequent BCC (Flohil *et al.,* 2013). The majority of studies on multiple cutaneous malignancies calculated risks of a subsequent or second primary skin cancer but did not calculate risks of additional skin cancers. In this issue, Adèle Green's research group selected a cohort of 1,191 white-skinned Australian residents from their Nambour skin cancer prevention trial, without KC, before or at the start of this trial, to determine the proportion who developed a BCC exclusively, SCC, or both (Keim *et al.,* 2014). The original cohort consisted of 1,621 residents of the subtropical city Nambour, who were selected at random in 1986, and therefore the cohort reflects a general population sample from Australia followed prospectively between 1992 and 2007. Besides the type of skin cancer, the investigators also assessed anatomic site distributions and other clinical features such as pigmentary characteristics and signs of actinic damage. This study demonstrated that about 21% of the study population developed a first KC and 47% of this group developed at least a second KC. The majority of this latter group developed exclusively BCCs (56%), 28% developed both, 16% developed SCCs exclusively, with age as the most important predictor of increasing incidence rates (Keim *et al.,* 2014). Participants who developed SCC exclusively were the most distinct group, because they had significantly higher prevalences of easily sunburned skin, propensity to tan without burning, and freckling of the back than did the BCC only and mixed groups. The skin, eye, and hair color characteristics showed no significant differences among the three groups. In those with BCCs exclusively or both BCC and SCC, the head and neck area were the predominant sites of development, whereas in the SCC only group the limbs were the predominant sites of development. These differences may be the result of differences in UVR exposure or genetic susceptibilities, and they suggest different tumor biologies.

Major strengths of this study are 16 years of follow-up, a clear case definition (i.e., histopathologically confirmed tumors), full-body skin examinations, and detailed information on clinical features. However, the main limitation lies in the small sample of patients with multiple cutaneous malignancies, especially the group who developed SCCs exclusively (n = 28). Small sample sizes result in wide confidence intervals and a possible type II error (i.e., no power calculation shown). Although the cohort was followed for 16 years, the study population was young (mean age 46 years) at enrollment, suggesting that the majority of the patients had not yet reached the age in which the incidence of cutaneous malignancy is highest.

ACTINIC NEOPLASIA SYNDROME

Martin Weinstock coined the term ''actinic neoplasia syndrome'' to emphasize that cutaneous (pre-)malignancies are not a single event but often reflect a field dysplasia from which patients suffer chronically (Weinstock *et al.,* 2009). After the 1992 landmark study on this subject (Karagas *et al.,* 1992), many observational studies of different populations demonstrated that almost half of patients with cutaneous malignancy will develop at least a second KC, and even more will show other signs of chronic actinic skin damage (e.g., actinic keratosis, solar elastosis) due to the relatively high levels of acute, intermittent, and/or cumulative UVR exposure during their lives. Therefore, a previous cutaneous malignancy is probably the strongest predictor of developing subsequent malignancies, making this an interesting population for studies of prevention intervention. One might argue that the occurrence of multiple malignancies might pose a greater problem to both patients and health-care systems compared with disease progression or recurrence.

The benefits of primary prevention programs should become evident only after decades (Staples *et al.,* 1998). Even though people become more and more aware of the harmful effects of UVR, they do not seem to change their attitude toward it (i.e., knowledge–behavior gap; Ma *et al.,* 2007). For now, it seems that primary prevention is not meeting its expectations, as the incidence of skin cancer continues to increase worldwide, with the possible exception of Australia, which has a highly active public education campaign (Lomas *et al.,* 2012). As primary prevention falls short, secondary prevention offers a good alternative strategy. This prevention method will be most successful when high-risk populations are defined and screening strategies for specific patient groups constructed. Well-calibrated, discriminating, and validated prediction models could provide physicians with a tool to find high-risk patients, such as patients with histories of skin cancer, and give them appropriate right follow-up and tailored instructions. If there indeed exists a type-specific skin cancer susceptibility, as suggested by Keim *et al.* (2014), different prediction models should be developed, combining environmental, phenotypic, and genotypic risk factors. However, there also exists a significant group of patients who develop both BCCs and SCCs, which is not surprising, as they share many risk factors (Figure 1). Although the risk factor profiles of the different cutaneous (pre-)malignancies are well documented, the extent to which these risk factors are applicable to subsequent tumors is not certain. On the basis of Rothman's sufficient- component cause model, it could be argued that the contribution of the conventional risk factors for a first event is not applicable to subsequent events, defined as the index event bias (Dahabreh and Kent, 2011). In recent decades, huge steps have been made in understanding the genetic predisposition (germline and somatic mutations) for BCC and to a lesser extent for SCC and actinic keratosis.

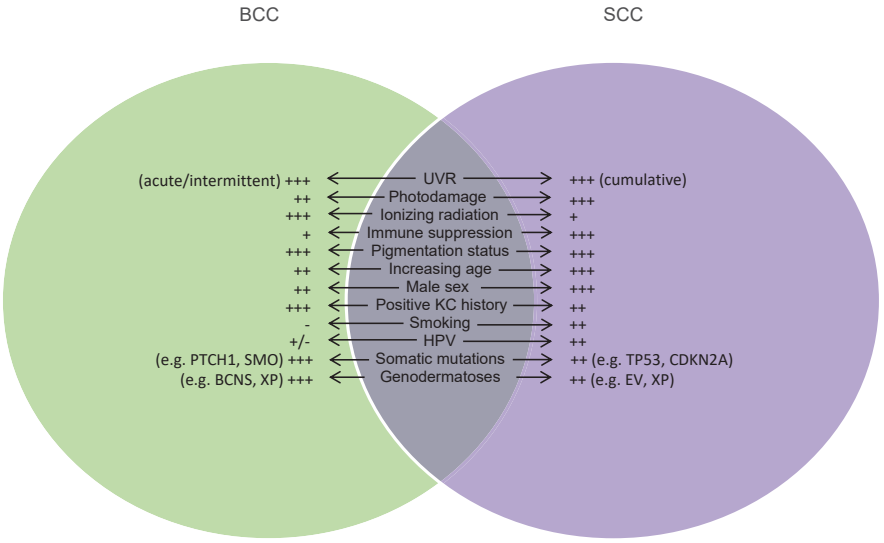

Figure 1. Risk factor profiles of basal cell carcinoma (BCC) and squamous cell carcinoma (SCC) UVR, ultraviolet radiation; HPV, human papilloma virus; BCNS, basal cell nevus syndrome; EV, epidermodysplasia verruciformis; XP, xeroderma pigmentosum.

However, our genetic understanding of these very common keratinocyte malignancies lags behind melanoma. Except for a few candidate gene studies and a genome-wide association study, no studies have investigated the common or rare genetic variants found in patients with multiple keratinocyte malignancies. There is hope, because an international consortium has been established to explore the genetics of patients with multiple skin cancers and to develop prediction models that include genetic variation.

PROSPECTIVE FOLLOW-UP STUDIES

As dermato-epidemiologists, we noticed another important element in this study (Keim *et al.,* 2014), which is the enormous return on investment seen in this prospective Nambour skin cancer study. Clinical epidemiology includes experimental and observational research that might aid our understanding of diseases through a quantitative approach of clinical problems. The Nambour skin cancer trial started as an experimental study (a randomized field trial) but extended its follow-up as a prospective cohort study. The advantages of that type of design are the possibility of calculating risk measures (absolute and relative risk) and a relatively low risk of bias compared with other observational designs. The classical argument against cohort studies is that they are too expensive, but large (population-based) prospective cohort studies such as the

Nambour Skin Cancer Study, the Rotterdam Study, Nurses' Health Study, the Health Professionals Follow-up Study, and the PUVA Follow-up Study have a tremendous scientific return on investment in many diseases, including skin cancer (Nan *et al.,* 2011; Stern and Study PF-U, 2012; Hofman *et al.,* 2013; Keim *et al.,* 2014). We are strong advocates of investing in well-designed and large cohort studies (including drug or disease specific registries), but at the same time we encourage investigators to form a consortia to increase sample size and to replicate each other's findings. Collaborative efforts increase the validity of the observational findings and should stimulate laboratory scientists even more strongly to investigate the underlying mechanisms in detail.

In conclusion, good research raises more questions than it answers, and it lifts the bar for scientific progress.

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Chapter 4

Predictors of a superficial first basal cell carcinoma

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ABSTRACT

Background: several observational studies have suggested differences in the risk factor profile between patients with superficial basal cell carcinomas (BCCs) and non-superficial BCCs.

Objective: to test the reproducibility of previous study findings and to find new genetic and non-genetic predictors for patients with a superficial first BCC.

Methods: 14.628 participants of northwestern European descent aged 45 years or older from a prospective population-based cohort study (Rotterdam Study) were linked with the Dutch Pathology Registry (PALGA) of whom 1,528 were identified as BCC patients. After exclusion, 948 eligible BCC patients remained for further non-genetic analyses and 1,014 for genetic analyses. We included 11 phenotypic, environmental and tumor-specific characteristics, and 20 candidate single nucleotide polymorphisms (SNP) as potential predictors for patients with a superficial first BCC. We performed binary logistic multivariable regression analyses.

Results: we found that patients with a superficial first BCC were significantly younger, almost two times more often female and 12-18 times more likely to have their BCC on the trunk or extremities than patients with a non-superficial first BCC. One SNP (rs12203592), mapped to IRF4, looked promising (OR 1.83, 95% CI 1.13-2.97, p-value <0.05), but after adjustment for multiple testing, no significant differences in genetic make-up between superficial BCC and non-superficial BCC patients were found.

Conclusion: we conclude that patients with a superficial BCC differ from non-superficial BCC patients with respect to environmental factors (tumor localization as a proxy for UVR exposure) and phenotypic characteristics (age and sex), but we found no difference in genotype. As superficial BCC patients develop their first BCCs at a younger age, they could be at higher life-time risk for subsequent skin cancers and therefore be an interesting group for secondary prevention.

INTRODUCTION

Patients with basal cell carcinoma (BCC) put a strain on health care services worldwide, as a result of the high and increasing BCC incidence, especially in young white-skinned women, and the increased risk of synchronous and metachronous BCCs and other ultraviolet radiation (UVR) related skin cancers (i.e., field cancerization).[1-3] In addition, the disability adjusted life years and health care costs for BCC have risen significantly as well.[4,5]

There are different histopathological subtypes of BCC, based on the growth pattern(s) found within the tumor tissue. The nodular pattern is most frequently found histological subtype (>50%), followed by superficial (~20%) and infiltrative (~10%) and about 20% of the tumors show a mixed type.[6-11] The frequencies reported depend on the used pathological classification system and period of the study, because classification systems and subtype incidences changed over time.[12,13] BCCs mostly occur on the head and neck area (i.e., chronically sun exposed; >70%), followed by the trunk (~20%) and extremities (~10%), which are both areas intermittently exposed to UVR.[7-10,14] Several observational studies have identified associations between age, sex and anatomical site, and BCC subtypes.[6-9,13] Patients with a superficial BCC have more often their BCC on the trunk and extremities than in the head and neck region,[6-9,13] are younger[7-9,13] and more often female.[8,9] In addition, patients with an initial truncal superficial BCC developed metachronous BCCs at a faster rate than patients with other anatomical site and histology combinations.[15]

These results could indicate that different BCC subtypes, in particular superficial, have other etiologies with respect to environmental factors (e.g., UVR exposure), phenotypic characteristics (e.g., age and sex) and genetic predisposition. However, only a few studies have studied other predictors than age, sex and anatomical site, with conflicting results.[10,16,17]

The objective of this study is to test the reproducibility of these findings and to find potentially new predictors for patients with a superficial first BCC (sBCC). We hereto analyzed the data of almost 1,000 white-skinned participants with a BCC of a prospective population-based cohort study (Rotterdam Study).

MATERIALS AND METHODS

Study population

The Rotterdam Study is a prospective population-based cohort study of 14,926 participants (divided over three cohorts) aged 45 years or older, living in a well-defined suburb of Rotterdam, the Netherlands.[18] The cohorts predominantly consist of people

of northwestern European descent. All the participants were interviewed and examined at baseline and these examinations were repeated about every 4 years. The Rotterdam Study has been approved by the Medical Ethics Committee of the Erasmus MC and by the Ministry of Health, Welfare and Sport of the Netherlands , implementing the Wet Bevolkingsonderzoek: ERGO (Population Studies Act: Rotterdam Study) and it was conducted in accordance with the Declaration of Helsinki. All participants provided written informed consent to participate in the study and to obtain information from their treating physicians.

Phenotype / Case definition

The method by which we identified BCCs has been described in detail previously.[19] In short, the study database was linked to the Dutch nationwide network and registry of histopathology and cytopathology (PALGA) to retrieve medical history of all participants on histopathologically confirmed BCCs between 1 July 1989 and 31 December 2013.[20] Of the 14,926 RS participants, 298 did not sign informed consent for a linkage and could not be linked to PALGA. The pathology excerpts we received, contained information on date of diagnosis, anatomical location, body side, type of procedure (i.e., biopsy or excision), radicality, and diagnosis. The majority of these excerpts showed a subtyping of the BCC and these subtypes were coded based on the World Health Organization's histological classification of keratinocytic skin tumors.[21] If there was a subtype discrepancy between a biopsy and an excision or a biopsy/excision included more than one subtype, we coded it as a mixed type BCC and noted the concerned subtypes. Patients with a missing subtype were excluded and patients with a mixed type first BCC with a superficial component were excluded as well, because it was unclear to which subtype these belong (i.e., superficial or non-superficial). Metachronous BCCs that occurred within 6 months of the first BCC were counted as additional tumors at the date of the initial diagnosis, as those BCCs were most likely present at this earlier date. We randomly selected a BCC for participants with synchronous BCCs on their first diagnosis date.

Selection of non-genetic candidate predictors

A literature search up to May 2016 for English publications on phenotypic, environmental and tumor specific factors previously involved in BCC subtypes was done in PubMed. Four phenotypic factors were included, namely age at first BCC, sex, pigment status and tendency to develop sunburn.[7-10,13] The latter was a combination of eye color and hair color when young (e.g., a participant with blue eyes and red hair was scored as light). Five environmental characteristics were chosen and concerned a history of being outdoor for over 4 hours per day during more than 25 years, sun protective behavior measured by wearing sunglasses or a hat, smoking, alcohol consumption and

coffee consumption.[10,22,23] Finally, two tumor related variables were included, namely localization of the first BCC and the number of BCCs at first date of diagnosis.[6-9,13,15] All selected variables (except tumor-specific characteristics) were measured at study entry or at a study visit closest to study entry.

Selection of candidate single nucleotide polymorphisms

A literature search up to May 2016 for English genome-wide association study (GWAS) publications of loci that confer risk of BCC or non-melanoma skin cancer was done in PubMed. There was no GWAS of the histopathological subtypes of BCC. To reduce the burden of multiple testing, all selected single nucleotide polymorphisms (SNPs) had to be at least borderline genome-wide significant (p-value < $7.0x10^{-8}$) and had to be replicated in another cohort. This resulted in a list of 20 candidate SNPs located in 17 different chromosomal regions (eTable 1).[24]

Genotype

DNA was isolated from whole blood, further processed and quality checked following standard protocols.[18] The Illumina Infinium II HumanHap550 BeadChips and the Illumina Human610-Quad BeadChips were used to genotype the RS participants.

Quality control criteria included removing SNPs with Hardy-Weinberg equilibrium deviations (p-value < 0.0001), genotyping call rate < 97%, gender mismatch and a high mean autosomal heterozygosity. SNPs were not included if they had a minor allele frequency of less than 1% and/or an imputation r^2 of less than 0.3.

For the candidate SNP approach we used genotypes that were estimated from the imputed 1000Genomes, GIANT Phase I version 3 dosage data[18] using the Genome-wide Complex Trait Analysis (GCTA) software with default parameters.[25] All selected candidate SNPs were included in our genetic database.

Statistical analysis

Non-genetic binary logistic regression analysis of sBCC vs non-superficial BCC (nsBCC)

All the assumptions of a binary logistic regression analysis were tested and we found no violations. There existed no strong (multi)collinearity between the selected non-genetic candidate predictors. A few outliers in the coffee consumption and alcohol consumption variables were found using the outlier labeling rule,[26] but all values were realistic. There was sufficient power to include the 11 selected candidate predictors in the multivariable binary logistic regression analysis.

We could safely assume that missing predictor values were missing at random (i.e., missing data points were not related to the missing data itself, but to the observed

data). Missing predictor values could therefore be imputed using multiple imputation (30 times) by an iterative Markov Chain Monte Carlo method. The imputation model included all candidate predictors, the outcome, the body mass index (kg/m^2), the level of education, the side of the first BCC and the Rotterdam Study cohort number. After the imputations we did both univariable and multivariable binary logistic regression analyses. No selection methods were used for the multivariable analysis.

All of the data management and the non-genetic binary logistic regression analyses were done in IBM® SPSS® Statistics for Windows version 21 (Chicago, IL).

Genetic (SNP-based) binary logistic regression analysis of sBCC vs nsBCC

All the assumptions of a binary logistic regression analysis were tested and we found one violation, namely collinearity between two selected candidate SNPs. A bivariate correlation matrix showed a Pearson correlation coefficient of 0.86 between rs12210050 and rs12202284, which means that these predictors were highly correlated. A few outliers in the age and principal component variables were found using the outlier labeling rule,[26] but all values were realistic. There was insufficient power to include the 20 selected candidate SNPs, age, sex and four principal components (PCs) in the multivariable binary logistic regression analysis. Therefore, we adjusted our analyses for multiple testing using the false discovery rate (FDR).[27] PCs were included to adjust for possible population stratification.

The SNP-based association analyses were performed on the imputed dosage data using a binary logistic regression with an additive model. The multivariable logistic regression analysis was adjusted for age at BCC diagnosis, sex and four PCs. No selection methods were used for the multivariable analysis.

The genetic data were prepared on our genetic servers and IBM® SPSS® Statistics for Windows version 21 (Chicago, IL) was used for the analyses.

Sensitivity analyses of sBCC vs. nodular BCC

We performed sensitivity analyses by doing the same non-genetic and genetic regression analyses as for sBCC versus nsBCC, but now including only patients with superficial or nodular first BCC.

RESULTS

Study population for non-genetic analyses

Of the 14,628 RS participants linked to PALGA, 1,528 had at least one BCC. After the exclusion of patients with a missing subtype (n = 71), patients with a mixed superficial first BCC (n = 58) and patients who developed at least one BCC before study entry (n =

451), 948 eligible BCC patients remained for further analyses. We randomly selected a BCC for participants with synchronous BCCs on their first diagnosis date (n = 125). Of the included patients, 137 (14%) had a superficial first BCC, 496 (52%) a nodular first BCC and the remaining 315 (33%) another subtype (infiltrative, micronodular or non-superficial mixed type; Table 1 and eTable 2).

Patients with a superficial first BCC were younger than patients with a non-superficial first BCC (median age 70.2 vs 75.5 years) and the proportion females (64%) was higher in sBCC patients than in nsBCC patients (54%; Table 1). Approximately 4 out of 5

Table 1. Non-genetic characteristics of 948 Rotterdam Study patients with a first BCC

Patient and tumor characteristics	Coding	Overall[1]	Superficial BCC	Non-superficial BCC
Number of patients		948 (100%)	137 (100%)	811 (100%)
Age at first BCC (years)	Median (IQR)	74.6 (67.9-81.2)	70.2 (64.3-76.0)	75.5 (68.9-81.8)
Sex	Female	526 (55%)	87 (64%)	439 (54%)
Pigment status	Dark	153 (16%)	24 (18%)	129 (16%)
	Intermediate	447 (47%)	70 (51%)	377 (46%)
	Light	213 (22%)	30 (22%)	183 (23%)
	Missing	135 (14%)	13 (9%)	122 (15%)
Easily sunburned	Yes	319 (34%)	53 (39%)	266 (33%)
	Missing	65 (7%)	5 (4%)	60 (7%)
Outdoor work	Yes	124 (13%)	14 (10%)	110 (14%)
	Missing	274 (29%)	42 (31%)	232 (29%)
Sun protection	No, never or hardly ever	357 (38%)	44 (32%)	313 (39%)
	Missing	60 (6%)	4 (3%)	56 (7%)
Smoking	Current or former	623 (66%)	92 (67%)	531 (65%)
	Missing	17 (2%)	1 (1%)	16 (2%)
Alcohol consumption (glasses/day)	Median (IQR)	0.6 (0.1-1.7)	0.6 (0.1-1.4)	0.6 (0.1-1.8)
	Missing	215 (23%)	18 (13%)	197 (24%)
Coffee consumption (cups/day)	Median (IQR)	3.3 (2.0-4.5)	3.3 (1.5-4.0)	4.0 (2.0-5.0)
	Missing	215 (23%)	18 (13%)	197 (24%)
>1 BCC at initial diagnosis	Yes	125 (13%)	24 (18%)	101 (12%)
Localization of first BCC	Head and neck	630 (66%)	24 (18%)	606 (75%)
	Extremities	128 (14%)	54 (39%)	74 (9%)
	Trunk	184 (19%)	58 (42%)	126 (16%)
	Missing	6 (1%)	1 (1%)	5 (1%)

[1] Participants with a mixed-type BCC with a superficial component were excluded.
BCC, basal cell carcinoma; IQR, interquartile range.

sBCCs were located on the extremities (39%) or trunk (42%) as opposed to 1 in 4 of the nsBCCs.

Non-genetic binary logistic regression analyses of sBCC vs. nsBCC

Of the 11 candidate predictors, 3 were significantly associated with a superficial first BCC in the univariable binary logistic regression analyses, namely a younger age at first BCC diagnosis (OR: 0.94, 95% CI: 0.92-0.96 per year), female gender (OR: 1.47, 95% CI: 1.01-2.14) and localization on the trunk (OR: 11.44, 95% CI: 6.85-19.10) or extremities (OR: 18.07, 95% CI: 10.56-30.93; Table 3).

These associations remained strongly significant after the multivariable binary logistic regression analysis and no other predictors became significant (Table 3). Female gender gave an even stronger risk increase for sBCC (OR: 1.88, 95% CI: 1.16-3.03, p-value < 0.05), but localization remained the strongest predictor (truncal OR: 12.20, 95% CI: 7.08-21.03, p-value < 0.001; extremities OR: 17.57, 95% CI: 10.06-30.70, p-value < 0.001). The 11 predictors together explained 19.7% (Cox and Snell R^2) of total variability of a superficial first BCC compared to a non-superficial first BCC.

Study population for genetic analyses

Of the 14,628 RS participants linked to PALGA, 1,257 were genotyped and had at least one BCC. After the exclusion of patients with a missing subtype (n = 181) and patients with a mixed superficial first BCC (n = 62), 1,014 eligible BCC patients remained for further analyses. We randomly selected a BCC for participants with synchronous BCCs on their first diagnosis date (n = 126). Of the included patients, 159 (16%) had a superficial first BCC, 522 (51%) a nodular first BCC and the remaining 333 (33%) another subtype (infiltrative, micronodular or non-superficial mixed type; Table 2 and eTable 3).

Patients with a superficial first BCC were younger than patients with a non-superficial first BCC (median age 68.0 vs 73.5 years) and the proportion females (65%) was higher in sBCC patients than in nsBCC patients (53%).

Table 2. Genetic characteristics of 1,014 Rotterdam Study patients with a first BCC

Patient and tumor characteristics	Coding	Overall[1]	Superficial BCC	Non-superficial BCC
Number of patients		1,014 (100%)	159 (100%)	855 (100%)
Age at first BCC (years)	Median (IQR)	72.9 (64.4-79.8)	68.0 (60.8-75.6)	73.5 (65.5-80.5)
Sex	Female	556 (55%)	103 (65%)	453 (53%)

[1] Participants with a mixed-type BCC with a superficial component were excluded.
BCC, basal cell carcinoma; IQR, interquartile range.

Table 3. Associations between predictors and occurrence of superficial first BCC (n = 948)[1]

Patient and tumor characteristics	Coding	Univariable models[2]	Multivariable model[2,3]
Age at first BCC (years)	Continuous	0.94 (0.92-0.96)***	0.95 (0.93-0.98)***
Sex	Female	1.47 (1.01-2.14)*	1.88 (1.16-3.03)*
Pigment status	Dark	Reference	Reference
	Intermediate	1.01 (0.61-1.67)	0.91 (0.50-1.64)
	Light	0.92 (0.51-1.65)	0.80 (0.40-1.61)
Easily sunburned	Yes	1.22 (0.83-1.78)	1.13 (0.70-1.81)
Outdoor work	Yes	0.77 (0.42-1.38)	0.85 (0.43-1.69)
Sun protection	No or hardly ever	0.70 (0.48-1.04)	0.80 (0.51-1.26)
Smoking	Current or former	1.03 (0.70-1.52)	1.41 (0.85-2.33)
Alcohol consumption (glasses/day)	Continuous	0.90 (0.77-1.05)	0.84 (0.70-1.01)
Coffee consumption (cups/day)	Continuous	0.92 (0.82-1.02)	0.90 (0.79-1.02)
>1 BCC at initial diagnosis	Yes	1.49 (0.92-2.43)	1.41 (0.79-2.52)
Localization of first BCC	Head and neck	Reference	Reference
	Extremities	18.07 (10.56-30.93)***	17.57 (10.06-30.70)***
	Trunk	11.44 (6.85-19.10)***	12.20 (7.08-21.03)***

[1] Compared to nodular, micronodular, infiltrative and mixed-type BCCs; all mixed-type BCCs with a superficial component were excluded.
[2] Pooled ORs with 95% CIs between parentheses.
[3] Full model, no selection procedures used.
* P-value < 0.05; ** P-value < 0.01; *** P-value < 0.001.
BCC, basal cell carcinoma.

Genetic (SNP-based) binary logistic regression analyses of sBCC vs. nsBCC

Of the 20 candidate SNPs, 2 were borderline significantly associated with a first sBCC in the univariable SNP-based binary logistic regression analyses, namely rs8015138 (OR: 0.76, 95% CI: 0.60-0.97) and rs12203592 (OR: 1.55, 95% CI: 1.01-2.37; Table 4).

Before the multivariable SNP-based binary logistic regression analyses, we excluded rs12210050 because it was highly correlated (Pearson's r: 0.86) with rs12202284 and both SNPs were also in strong linkage disequilibrium (r^2: 0.73) with each other. The multivariable analysis resulted in 1 promising SNP, namely rs12203592 (OR: 1.83, 95% CI: 1.13-2.97, p-value 0.014) mapped to pigmentation gene IRF4, but after adjustment for multiple testing (FDR) this SNP lost its significance as well. No other SNPs were significantly associated to sBCC (Table 4).

The 19 candidate SNPs together explained 1.6%, of which rs12203592 explained 0.4% (Cox and Snell R^2), of the total variability of a superficial first BCC compared to a non-superficial first BCC.

Table 4. Associations between predictors and occurrence of superficial first BCC (n = 1,014)[1]

Patient and tumor characteristics	Coding	Univariable models[2]	Multivariable models[2,3]	Multivariable model[2,4]
Age at first BCC (years)	Continuous	0.97 (0.95-0.98)***		0.96 (0.95-0.98)***
Sex	Female	1.63 (1.15-2.32)**		1.67 (1.16-2.40)**
rs73635312	Yes	1.11 (0.74-1.67)	1.08 (0.71-1.64)	1.07 (0.70-1.63)
rs11170164	Yes	0.88 (0.57-1.35)	0.93 (0.59-1.45)	0.95 (0.61-1.49)
rs7335046	Yes	0.95 (0.67-1.36)	0.92 (0.64-1.32)	0.89 (0.61-1.29)
rs8015138	Yes	0.76 (0.60-0.97)*	0.78 (0.61-1.00)	0.79 (0.62-1.02)
rs1805007	Yes	0.96 (0.60-1.55)	0.96 (0.59-1.57)	0.95 (0.58-1.56)
rs78378222	Yes	0.89 (0.38-2.10)	0.95 (0.40-2.52)	0.98 (0.41-2.36)
rs7538876	Yes	1.06 (0.82-1.36)	1.02 (0.79-1.32)	1.02 (0.78-1.32)
rs801114	Yes	0.96 (0.75-1.23)	1.03 (0.80-1.33)	1.00 (0.77-1.30)
rs214782	Yes	0.89 (0.66-1.19)	0.91 (0.68-1.22)	0.93 (0.69-1.26)
rs13014235	Yes	1.07 (0.84-1.36)	1.07 (0.83-1.37)	1.07 (0.83-1.38)
rs57244888	Yes	1.06 (0.67-1.67)	1.11 (0.70-1.78)	1.12 (0.70-1.80)
rs401681	Yes	1.05 (0.82-1.33)	1.00 (0.78-1.28)	0.98 (0.76-1.26)
rs12203592	Yes	1.55 (1.01-2.37)*	1.55 (1.01-2.39)*	1.83 (1.13-2.97)*
rs12202284	Yes	0.92 (0.64-1.32)	0.92 (0.63-1.33)	0.72 (0.47-1.08)
rs12210050	Yes	1.00 (0.71-1.40)	1.03 (0.73-1.46)	
rs157935	Yes	0.95 (0.73-1.24)	0.94 (0.72-1.23)	0.95 (0.72-1.26)
rs28727938	Yes	0.89 (0.52-1.53)	0.78 (0.45-1.34)	0.75 (0.43-1.32)
rs7006527	Yes	0.81 (0.58-1.13)	0.79 (0.56-1.12)	0.79 (0.55-1.12)
rs2151280	Yes	1.09 (0.86-1.38)	1.13 (0.89-1.44)	1.14 (0.89-1.46)
rs59586681	Yes	1.09 (0.85-1.40)	1.13 (0.88-1.46)	1.10 (0.85-1.44)

[1] Compared to nodular, micronodular, infiltrative and mixed-type BCCs; all mixed-type BCCs with a superficial component were excluded.
[2] Odds ratios with 95% confidence intervals between parentheses.
[3] Included one SNP at a time, adjusted for age at first BCC, sex and first 4 principal components.
[4] Full model, adjusted for age at first BCC, sex and first 4 principal components. No selection procedures used.
* P-value < 0.05; ** P-value < 0.01; *** P-value < 0.001.
BCC, basal cell carcinoma.

Sensitivity analyses of sBCC vs. nodular BCC

After the non-genetic multivariable binary logistic regression analysis comparing sBCC to nodular BCC, the same predictors (age at first BCC diagnosis, sex and a localization on the trunk or extremities) were significantly associated with a superficial first BCC with similar effect sizes (eTable 4). The explained variability increased by 4.3% to 25.0% (Cox and Snell R^2).

The multivariable SNP-based binary logistic regression analysis comparing sBCC to nodular BCC resulted in 2 promising SNPs, namely rs12203592 (OR: 2.11, 95%

CI: 1.25-3.58, p-value 0.005) and rs12202284 (OR: 0.55, 95% CI: 0.35-0.88, p-value 0.012), both mapped to the IRF4 – EXOC2 region, but were not in strong linkage disequilibrium (r^2: 0.18) with each other (eTable 5). However, after adjustment for multiple testing (FDR) both SNPs lost their significance.

DISCUSSION

This prospective population-based cohort study replicates some previous non-genetic findings and shows that there are significant differences between patients with a superficial first BCC and a non-superficial first BCC. Patients who presented with a sBCC were younger, more often female and had their BCCs more frequently on the extremities and trunk than patients with nsBCCs. This study also looked into potential genetic differences. One SNP, mapped to IRF4, looked promising, but after adjustment for multiple testing, no significant differences in genetic make-up between sBCC and nsBCC patients were found.

The associations found between the non-genetic predictors and the occurrence of a superficial first BCC were in line with several other older and more recent observational studies from Europe and Australia.[6-10,13] However, most of these non-genetic studies on histopathological BCC subtypes did not adjust for potential confounders.[6-9,13] Therefore, it is possible that the associations found, were spurious. We included 11 potential confounders in our non-genetic multivariable model and found that patients with a superficial first BCC were significantly younger, almost twice as likely to be female and 12-18 times more likely to have their BCC on the trunk or extremities than patients with a non-superficial first BCC. These differences in age, sex and localization could suggest that a different pattern of UVR exposure, namely intense intermittent, plays a role in the etiology of sBCC as compared to nsBCC. A British and Australian cohort study showed that excessive recreational UVR exposure significantly increased the risk of truncal (superficial) BCCs,[17,28] whereas Dutch and Italian case-control studies showed no relation between cumulative lifetime UVR exposure and sBCC.[10,16]

Another potential explanation for the significantly higher risk of sBCC in younger women could be behavior. Women tend to use tanning beds more often than men[29,30] and pay closer attention the their health and physical appearance than men, which may lead to more medical visits.[31]

It is also possible that tumor biology differs at various anatomical sites. A Dutch renal transplant study showed that transplant recipients more often developed sBCCs and that their BCCs were located more frequently on the trunk and extremities than in the non-immunosuppressed, which may point at role for the immune system.[8]

Superficial first BCC patients were significantly younger (approximately 5 years) than non-superficial first BCC patients and developed their BCCs more often on relatively sun-unexposed sites, which could mean that they have a different genetic predisposition which makes them more vulnerable to develop (superficial) BCC. It is possible that they, for example, have a reduced DNA repair capacity or other risk increasing DNA differences.[32] Hence, we compared carefully selected BCC candidate SNPs between these two patient groups. Of the 19 included candidate SNPs in the multivariable regression analysis, rs12203592 looked most promising (OR: 1.83, 95% CI: 1.13-2.97, p-value 0.014), but lost its significance after adjusting the FDR. This SNP is an intron variant mapped to the interferon regulatory factor 4 (IRF4) gene, which belongs to a well-known family of transcription factors that are important in the regulation of the immune system. It is possible that certain SNPs downregulate the immune system which could lead to the formation of sBCC in relatively sun-unexposed areas earlier in life. A recent genetic analysis of melanoma patients showed a significant association with the bimodal (early- and late-onset) age distribution of melanoma for different rs12203592 genotypes.[33] In addition, IRF4 also plays a key role in the pigmentation pathway and in the formation of (pre)malignancies of the skin.[34-36] These pre-malignancies (i.e., actinic keratosis) have a superficial growth pattern which is comparable to that of sBCCs.

Limitations

Misclassification of BCC subtypes by pathologists most likely occurred throughout the study period, but it is unlikely that this misclassification was differential. However, we could not check the tissue samples as we only received excerpts from PALGA.

The total number of BCCs could have been underestimated, since we only included histopathologically confirmed BCCs. This underestimation will be most pronounced for superficial BCCs, because physicians could diagnose these BCCs visually and treat them non-invasively. However, a recent Dutch observational study showed that only a small percentage (ca. 7%) of patients with metachronous BCCs had subsequent non-histologically confirmed BCCs.[37] In addition, the evidence-based BCC guideline from the Dutch Society for Dermatology and Venereology states that histopathological verification is needed for all for BCC suspicious lesions.[38] Finally, the distribution pattern of the histopathological subtypes in our study population is in line with other studies, with the nodular type being the most common, followed by the superficial type and infiltrative type, while mixed types were frequently found as well.[6-11] Our candidate SNP approach likely lacked sufficient power (26 degrees of freedom used and 159 patients with a superficial first BCC) despite the FDR approach taken. Detailed information about other limitations of the Rotterdam Study, the phenotype collection and the non-genetic and genetic predictors can be found in two earlier publications.[19,39]

Conclusion

Patients with a superficial first BCC differ from non-superficial first BCC patients with respect to environmental factors (tumor localization as a proxy for UVR exposure) and phenotypic characteristics (age and sex), but (as far as we could find) not in genotype. As sBCC patients develop their first BCCs at a younger age, they could be at higher risk for subsequent skin cancers. Further study of the interplay between environmental, phenotypic and genotypic predictors and BCC subtypes may provide useful knowledge for BCC pathogenesis and the design of programs for prevention and early detection of BCC.

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eTable 1. Candidate single nucleotide polymorphisms for basal cell carcinoma or non-melanoma skin cancer

First Author	Date	Disease/Trait	Region	Reported Gene(s)	Mapped gene	Strongest SNP-Risk Allele	Context	Risk Allele Frequency	p-Value	OR or beta	95% CI
Stacey SN	11-10-2008	Basal cell carcinoma	1p36.13	RCC2, ARHGEF10L, PADI4, PADI6	PADI6	rs7538876-A	intron	0,35	4,00E-12	1,28	[1.19-1.37]
Stacey SN	11-10-2008	Basal cell carcinoma	1q42.13	RHOU	FTH1P2 - ISCA1P2	rs801114-G		0,33	6,00E-12	1,28	[1.19-1.37]
Rafnar T	18-01-2009	Basal cell carcinoma	5p15.33	TERT, CLPTM1L	CLPTM1L	rs401681-C	intron	0,56	4,00E-12	1,25	[1.18-1.34]
Nan H	22-06-2011	Basal cell carcinoma	13q32.3	UBAC2	HMGB3P4 - CCR12P	rs7335046-G		0,12	3,00E-08	1,26	[1.18-1.34]
Nan H	22-06-2011	Basal cell carcinoma	16q24.3	MC1R	MC1R	rs1805007-T	missense	0,07	4,00E-17	1,55	[1.45-1.66]
Nan H	22-06-2011	Basal cell carcinoma	6p25.3	EXOC2	IRF4 - EXOC2	rs12210050-T		0,17	1,00E-09	1,24	[1.17-1.31]
Stacey SN	24-09-2011	Basal cell carcinoma	17p13.1	TP53	TP53	rs78378222-C	UTR-3	NR	2,00E-20	2,16	[1.83-2.54]
Zhang M	31-03-2013	Non-melanoma skin cancer	6p25.3	IRF4	IRF4	rs12203592-T	intron	NR	7,00E-14	0,21	[0.15-0.27]
Zhang M	31-03-2013	Non-melanoma skin cancer	6p25.3	EXOC2, IRF4	IRF4 - EXOC2	rs12202284-A		NR	5,00E-08	0,15	[0.091-0.209]
Zhang M	31-03-2013	Non-melanoma skin cancer	14q22.1	GNG2	OR7E159P - GNG2	rs8015138-C		NR	7,00E-08	0,11	[0.071-0.149]
Stacey SN	08-01-2014	Basal cell carcinoma	20p13	TGM3	TGM3	rs214782-G	intron	0,17	6,00E-17	1,29	[1.22-1.37]
Stacey SN	08-01-2014	Basal cell carcinoma	8q22.2	RGS22	RGS22	rs7006527-?	intron	0,86	9,00E-13	1,3	[1.22-1.41]
Stacey SN	08-01-2014	Basal cell carcinoma	7q32.3	KLF14	LINC-PINT	rs157935-T	intron		9,00E-11	1,23	[1.15-1.31]

eTable 1. Candidate single nucleotide polymorphisms for basal cell carcinoma or non-melanoma skin cancer (*continued*)

First Author	Date	Disease/Trait	Region	Reported Gene(s)	Mapped gene	Strongest SNP-Risk Allele	Context	Risk Allele Frequency	p-Value	OR or beta	95% CI
Stacey SN	08-01-2014	Basal cell carcinoma	20p13	TGM3	STK35 - TGM3	rs59586681-T		0,61	3,00E-09	1,16	[1.11-1.22]
Stacey SN	08-01-2014	Basal cell carcinoma	9p21.3	CDKN2A, CDKN2B	CDKN2B-AS1	rs2151280-G	intron		3,00E-10	1,2	[1.14-1.27]
Stacey SN	08-04-2015	Basal cell carcinoma	2q33.1	ALS2CR12, CASP8, CASP10	ALS2CR12	rs13014235-C	missense	0,456	2,00E-09	1,15	[1.10-1.20]
Stacey SN	08-04-2015	Basal cell carcinoma	8q21.13	ZFHX4, ZFHX4-AS1	LINC01111 - MRPL9P1	rs28727938-C		0,938	4,00E-12	1,43	[1.30-1.59]
Stacey SN	08-04-2015	Basal cell carcinoma	12q13.13 / 5q11.2	KRT5	KRT5	rs11170164-T	missense	0,087	9,00E-09	1,29	[NR]
Stacey SN	08-04-2015	Basal cell carcinoma	2p24.3	MYCN, FAM49A	MYCN - FAM49A	rs57244888-T		0,898	5,00E-12	1,32	[1.22-1.43]
Stacey SN	08-04-2015	Basal cell carcinoma	10p14	GATA3, RP11-428L9.1, RP11-428L9.2	RNA5SP299 - LINC00709	rs73635312-G		0,874	2,00E-16	1,35	[1.25-1.45]

SNP, single nucleotide polymorphism; OR, odds ratio; CI, confidence interval.

eTable 2. Non-genetic characteristics of 633 Rotterdam Study patients with a primary BCC

Patient and tumor characteristics	Coding	Overall[1]	Superficial BCC	Nodular BCC
Number of patients		633 (100%)	137 (100%)	496 (100%)
Age at first BCC (years)	Median (IQR)	74.0 (67.3-80.4)	70.2 (64.3-76.0)	75.4 (68.2-81.5)
Sex	Female	339 (54%)	87 (64%)	252 (51%)
Pigment status	Dark	106 (17%)	24 (18%)	82 (17%)
	Intermediate	296 (47%)	70 (51%)	226 (46%)
	Light	151 (24%)	30 (22%)	121 (24%)
	Missing	80 (13%)	13 (9%)	67 (14%)
Easily sunburned	Yes	219 (35%)	53 (39%)	166 (33%)
	Missing	38 (6%)	5 (4%)	33 (7%)
Outdoor work	Yes	83 (13%)	14 (10%)	69 (14%)
	Missing	173 (27%)	42 (31%)	131 (26%)
Sun protection	No, never or hardly ever	234 (37%)	44 (32%)	190 (38%)
	Missing	34 (5%)	4 (3%)	30 (6%)
Smoking	Current or former	422 (67%)	92 (67%)	330 (67%)
	Missing	9 (1%)	1 (1%)	8 (2%)
Alcohol consumption (glasses/day)	Median (IQR)	0.5 (0.04-1.8)	0.6 (0.1-1.4)	0.5 (0.03-2.1)
	Missing	126 (20%)	18 (13%)	108 (22%)
Coffee consumption (cups/day)	Median (IQR)	3.3 (2.0-4.0)	3.3 (1.5-4.0)	4.0 (2.0-4.5)
	Missing	126 (20%)	18 (13%)	108 (22%)
>1 BCC at initial diagnosis	Yes	86 (14%)	24 (18%)	62 (13%)
Localization of first BCC	Head and neck	393 (62%)	24 (18%)	369 (74%)
	Extremities	101 (16%)	54 (39%)	47 (9%)
	Trunk	135 (21%)	58 (42%)	77 (16%)
	Missing	4 (1%)	1 (1%)	3 (1%)

[1] Participants with a mixed-type BCC with a superficial component were excluded.
BCC, basal cell carcinoma; IQR, interquartile range.

eTable 3. Genetic characteristics of 681 Rotterdam Study patients with a primary BCC

Patient and tumor characteristics	Coding	Overall[1]	Superficial BCC	Nodular BCC
Number of patients		681 (100%)	159 (100%)	522 (100%)
Age at first BCC (years)	Median (IQR)	72.2 (63.1-79.0)	68.0 (60.8-75.6)	73.3 (64.8-80.3)
Sex	Female	363 (53%)	103 (65%)	260 (50%)

[1] Participants with a mixed-type BCC with a superficial component were excluded.
BCC, basal cell carcinoma; IQR, interquartile range.

eTable 4. Associations between predictors and occurrence of superficial primary BCC (n = 633)[1]

Patient and tumor characteristics	Coding	Univariable models[2]	Multivariable model[2,3]
Age at first BCC (years)	Continuous	0.95 (0.93-0.97)***	0.96 (0.94-0.99)**
Sex	Female	1.69 (1.14-2.49)**	2.29 (1.36-3.83)**
Pigment status	Dark	Reference	Reference
	Intermediate	1.07 (0.63-1.80)	0.99 (0.52-1.86)
	Light	0.89 (0.49-1.63)	0.73 (0.35-1.52)
Easily sunburned	Yes	1.19 (0.80-1.77)	1.17 (0.71-1.95)
Outdoor work	Yes	0.77 (0.41-1.42)	0.99 (0.47-2.10)
Sun protection	No or hardly ever	0.72 (0.48-1.08)	0.89 (0.54-1.45)
Smoking	Current or former	1.00 (0.66-1.49)	1.33 (0.77-2.29)
Alcohol consumption (glasses/day)	Continuous	0.88 (0.75-1.04)	0.85 (0.70-1.04)
Coffee consumption (cups/day)	Continuous	0.92 (0.81-1.03)	0.92 (0.80-1.05)
>1 BCC at initial diagnosis	Yes	1.49 (0.89-2.49)	1.34 (0.72-2.52)
Localization of first BCC	Head and neck	Reference	Reference
	Extremities	17.34 (9.83-30.59)***	15.92 (8.80-28.80)***
	Trunk	11.38 (6.67-19.43)***	13.28 (7.40-23.82)***

[1] Compared to nodular BCCs only; all mixed-type BCCs with a superficial component were excluded.
[2] Pooled ORs with 95% CIs between brackets.
[3] Full model, no selection procedures used.
* P-value < 0.05; ** P-value < 0.01 ; *** P-value < 0.001.
BCC, basal cell carcinoma.

Model fit

Cox and Snell R^2:	25.0%
Nagelkerke R^2:	38.6%
Hosmer and Lemeshow Test:	0/30 had a p-value < 0.05

eTable 5. Associations between predictors and occurrence of first superficial BCC (n = 681)[1]

Patient and tumor characteristics	Coding	Univariable models[2]	Multivariable models[2,3]	Multivariable model[2,4]
Age at first BCC (years)	Continuous	0.97 (0.95-0.98)***		0.97 (0.95-0.98)***
Sex	Female	1.85 (1.28-2.68)**		1.95 (1.32-2.87)***
rs73635312	Yes	1.02 (0.67-1.56)	0.99 (0.64-1.53)	0.97 (0.63-1.51)
rs11170164	Yes	0.94 (0.59-1.48)	0.95 (0.59-1.53)	0.99 (0.60-1.61)
rs7335046	Yes	0.83 (0.57-1.22)	0.79 (0.53-1.18)	0.78 (0.51-1.19)
rs8015138	Yes	0.78 (0.61-1.01)	0.81 (0.63-1.06)	0.83 (0.64-1.08)
rs1805007	Yes	0.97 (0.59-1.59)	0.93 (0.56-1.55)	0.92 (0.54-1.55)
rs78378222	Yes	0.77 (0.31-1.92)	0.82 (0.32-2.08)	0.84 (0.32-2.19)
rs7538876	Yes	1.06 (0.82-1.38)	1.03 (0.78-1.34)	1.03 (0.78-1.36)
rs801114	Yes	0.94 (0.72-1.22)	1.01 (0.77-1.33)	0.99 (0.75-1.32)
rs214782	Yes	0.91 (0.67-1.23)	0.93 (0.68-1.26)	0.95 (0.69-1.31)
rs13014235	Yes	1.05 (0.81-1.35)	1.04 (0.80-1.35)	1.05 (0.80-1.38)
rs57244888	Yes	0.96 (0.59-1.57)	1.02 (0.62-1.69)	1.05 (0.63-1.76)
rs401681	Yes	1.00 (0.78-1.29)	0.93 (0.72-1.21)	0.92 (0.70-1.21)
rs12203592	Yes	1.50 (0.97-2.33)	1.50 (0.94-2.32)	2.11 (1.25-3.58)**
rs12202284	Yes	0.79 (0.53-1.17)	0.73 (0.49-1.10)	0.55 (0.35-0.88)*
rs12210050	Yes	0.88 (0.61-1.26)	0.86 (0.60-1.24)	
rs157935	Yes	0.97 (0.73-1.28)	0.95 (0.71-1.27)	0.95 (0.71-1.29)
rs28727938	Yes	0.87 (0.49-1.53)	0.74 (0.41-1.32)	0.70 (0.38-1.27)
rs7006527	Yes	0.76 (0.53-1.09)	0.72 (0.50-1.05)	0.72 (0.49-1.06)
rs2151280	Yes	1.15 (0.90-1.47)	1.15 (0.89-1.49)	1.18 (0.91-1.53)
rs59586681	Yes	1.07 (0.82-1.38)	1.10 (0.84-1.44)	1.07 (0.81-1.42)

[1] Compared to nodular BCCs only; all mixed-type BCCs with a superficial component were excluded.

[2] Odds ratios with 95% confidence intervals between parentheses.

[3] Included one SNP at a time, adjusted for age at first BCC, sex and first 4 principal components.

[4] Full model, adjusted for age at first BCC, sex and first 4 principal components. No selection procedures used.

* P-value < 0.05; ** P-value < 0.01; *** P-value < 0.001.

BCC, basal cell carcinoma.

Model fit full multivariable model

Cox and Snell R^2:	6.9%
Nagelkerke R^2:	10.4%
Hosmer and Lemeshow Test:	p-value 0.802

Chapter 5

Competing risk of death in Kaplan-Meier curves when analyzing subsequent keratinocyte cancer

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To the Editor

We have read with great interest the article by Wehner et al[1] about the timing of subsequent new keratinocyte carcinomas in patients who present with basal cell carcinoma (BCC) or cutaneous squamous cell carcinoma (SCC).[1] The authors estimated the probability of developing a subsequent KC by calculating 1 minus the Kaplan-Meier (KM) survival probability. The use of the KM method for other end points than overall mortality can lead to a violation of a key assumption, which is the independent censoring assumption. In a KM curve with subsequent KC as the event of interest, patients who die are censored. The independent censoring assumption means that we assume that patients who are censored at time *t* have the same risk of developing the event of interest as those patients who are still in follow-up at time *t*. It is impossible to develop a KC after death, and not adjusting for this will lead to an overestimation of the probability of developing a new KC.

One possibility to take the competing risk of death into account, is to compute a cumulative incidence curve (CIC). Other methods are also available and described elsewhere.[2-4] A CIC is calculated by the sum of the multiplication of the overall survival probability with the hazard of a subsequent KC at each time point.

To show the difference between both methods (KM and CIC), we used data from the Rotterdam Study.[5] We calculated 1 minus the KM survival probability and the CIC of the second metachronous KC (BCC or SCC, including keratoacanthoma but no in situ SCC) between January 1, 1990, and December 31, 2013, among 1644 patients with a first KC. After 10 years of follow-up the probability of a subsequent KC was 40% using the KM method (Figure 1). This probability was an overestimation—the actual probability was 34% using the CIC. Twenty years after diagnosis, the difference was even larger (74% for KM vs 52% for CIC) because the problem of competing risk due to death became larger.

In conclusion, the problem of competing risk can occur for all end points other than overall mortality when using the KM method (eg, melanoma-specific death—patients cannot first die due to other causes and then due to melanoma). It especially occurs in older populations (ie, higher probability of other competing events such as death) and when the follow-up time is long.

We would like to ask if Wehner et al[1] could re-analyze their data using the CIC method.

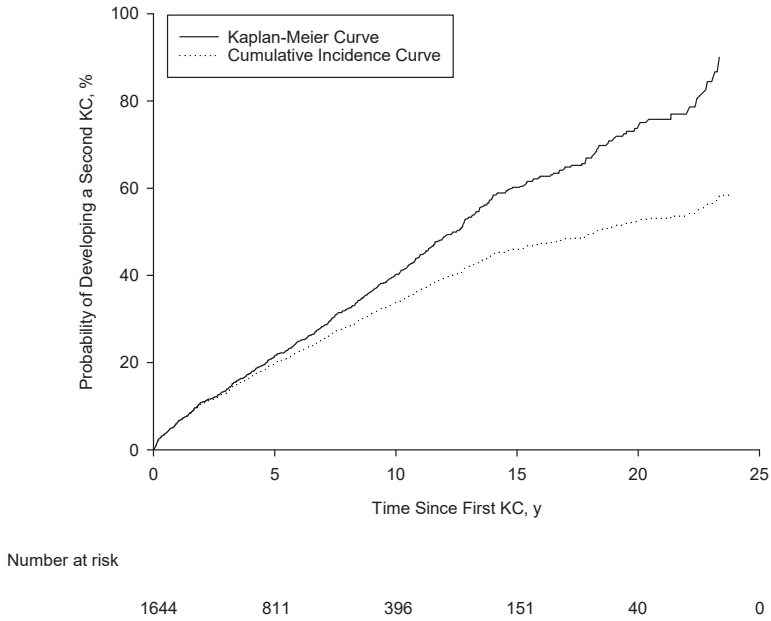

Figure 1. Kaplan-Meier Curve vs Cumulative Incidence Curve of the Probability of Developing a Second Keratinocyte Cancer (KC)

The solid line represents the biased Kaplan-Meier estimate of the probability of a subsequent KC due to the competing risk of death. The dotted line represents the correct probability of a subsequent KC using a cumulative incidence curve, taking the competing risk of death into account.

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Chapter 6

Predicting the risk of a second basal cell carcinoma

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ABSTRACT

A third of basal cell carcinoma (BCC) patients will develop subsequent BCCs. We aimed to develop a simple model to predict the absolute risk of a second BCC. We observed 14,628 participants of Northern European ancestry from a prospective population-based cohort study. BCCs were identified using a linkage with the Dutch Pathology Registry (Pathological Anatomy National Automated Archive). Predictors for a second BCC included 13 phenotypic, lifestyle, and tumor-specific characteristics. The prediction model was based on the Fine and Gray regression model to account for the competing risk of death from other causes. Among 1,077 participants with at least one BCC, 293 developed a second BCC at a median of 3 years. Several well-known risk factors for a first BCC were not prognostic for a second BCC, whereas having more than one initial BCC was the strongest predictor. Discriminative ability at 3 years was reasonable (bootstrap validated c-index= 0.65). Three groups were created, with 7, 12, and 28% risk of a second BCC within 3 years. We conclude that a combination of readily available clinical characteristics can reasonably identify patients at high risk of a second BCC. External validation and extension with stronger predictors is desirable to further improve risk prediction.

INTRODUCTION

Patients with previously treated basal cell carcinoma (BCC) have a high risk of subsequent BCCs (Epstein, 1973). A recent meta-analysis showed that 29% of the patients with a first BCC will develop at least one more BCC (Flohil et al., 2013b). The increasing incidence of BCC, with ~ 5% annually, suggests that primary prevention campaigns have not been very effective so far (Lomas et al., 2012). Secondary prevention (i.e., detecting new BCCs at an early stage among patients with a prior BCC) is important to reduce the high disease burden (i.e., morbidity and costs) associated with this very common cancer (Housman et al., 2003; Flohil et al., 2013a; Hollestein et al., 2014).

The most well-known risk factor for a BCC is UVR, in particular acute and intermittent exposure (Kricker et al., 1995; Armstrong and Kricker, 2001). Recently, Weinstock coined the term "actinic neoplasia syndrome" to underline the fact that patients with a keratinocyte carcinoma (BCC or squamous cell carcinoma) frequently develop another keratinocyte carcinoma and various other signs of cutaneous photodamage (e.g., solar keratosis and actinic keratosis) due to the field dysplasia (Weinstock et al., 2009). However, BCC is a complex disease and not only UVR-related factors are important in its carcinogenesis.

In contrast to risk factors for a first BCC, prognostic factors for a second BCC are less well documented. Male sex, higher age at initial BCC, and a history of BCC have been found associated with metachronous BCCs (Karagas et al., 1992; Richmond-Sinclair et al., 2010; Flohil et al., 2011). The value of other phenotypic (e.g., skin type) and environmental (e.g., UVR) characteristics in predicting a new BCC is under debate (Robinson, 1987; Karagas et al., 1992; Lovatt et al., 2005; Kiiski et al., 2010; Richmond-Sinclair et al., 2010). However, no prediction models have been developed yet that allow for individualized risk stratification.

The objective of this study is to develop a prognostic model for predicting the occurrence of a second BCC. We hereto analyzed a prospective population-based cohort (Rotterdam Study; RS) including over a 1,000 BCC patients.

MATERIALS AND METHODS

Study population

The RS is a prospective population-based cohort study of people aged 45 years or older (Hofman et al., 2013). From July 1989 to September 1993, the first cohort of 7,983 recruited persons (RS-I, 78% of the invitees) aged 55 years or older was realized. In 2000– 2001, another 3,011 participants (RS-II, 67% of the invitees) who had become 55 years of age or older, or applied to this age minimum and had moved into the

district, were added to the cohort. The last addition of 3,932 Ommoord inhabitants (RS-III, 65% of the invitees) aged 45–54 years took place during 2006–2008. These three cohorts together comprise 14,926 participants. Data were acquired by interviews at home and by thorough examinations in a specially built research facility in their district. These examinations were repeated every 3–4 years. The RS has been approved by the Medical Ethics Committee of the Erasmus MC and by the Ministry of Health, Welfare and Sport of The Netherlands, implementing the Wet Bevolkingsonderzoek: ERGO (Population Studies Act: RS). All participants provided written informed consent to participate in the study and to obtain information from their treating physicians. The RS was conducted according to the Declaration of Helsinki Principles.

Case definition

The RS participants were linked to the Dutch nationwide network and registry of histopathology and cytopathology (PALGA) to retrieve their medical history of histopathologically confirmed BCCs. PALGA was founded in 1971 and achieved complete national coverage in 1991 (i.e., since 1991 all Dutch histopathology laboratories are linked to this databank; Casparie et al., 2007). Every pathology excerpt located on PALGA's central databank contains encrypted patient data and a PALGA diagnosis line derived from the Systematized Nomenclature of Medicine. In collaboration with a dermatopathologist, the following information from the excerpts was retrieved: date of diagnosis, anatomical location, body side, type of procedure (i.e., biopsy or excision), radicality, and diagnosis (including tumor subtype). To obtain all pathology reports concerning BCC, we used the PALGA diagnosis lines attached to all subtypes of BCC (i.e., M80903, M80913, M80923, M80933, M80943, M80963, M80973, and M80983).

The linkage was done using encrypted patient data both available in the RS and PALGA. This encrypted data consisted of the patient's date of birth, gender, and first four to eight letters of the (maiden) family name. The combination of these identifiers produced a linkage key. This key showed 98% sensitivity and 98% positive predictive value in earlier record linkage research (Van den Brandt et al., 1990).

Of the 14,926 RS participants, 298 did not sign informed consent for a linkage and could not be linked to PALGA. Every BCC excerpt between 1 July 1989 and 31 December 2013 was retrieved from the network of PALGA. Participants who had developed a BCC before entering the RS were excluded from the analyses.

All excerpts mentioned a date of diagnosis, and, the majority of excerpts included a precise anatomical location and information about the type of procedure and the radicality of the excision, which made it possible to distinguish between different BCCs over time. If information about location was not available, we assumed that a biopsy

followed by an excision within a logical time frame (<3 months) concerned the same BCC.

The next tumor following a radical excision was always scored as a new BCC. If an excision was irradical, the next reported tumor on the same or adjacent location was regarded as the same tumor. Metachronous BCCs occurring within 6 months of the first BCC were counted as additional tumors at the date of the initial diagnosis, as those BCCs were most likely present at this earlier date. If a BCC consisted of different histopathological subtypes, a superiority rule was used, namely infiltrative greater than micronodular greater than nodular greater than superficial. Unclear excerpts were discussed with an experienced dermatologist, and, if available, missing information was obtained from medical records.

Candidate predictors

Three phenotypic factors were selected—namely, age at first BCC (years), sex, and pigment status (Robinson, 1987; Karagas et al., 1992). The latter was a combination of eye color and hair color when young (e.g., a participant with blue eyes and red hair was scored as light). Hair color for RS-III was determined during the second examination round.

Three questions related to UVR exposure were selected and concerned the tendency to develop sunburn, a history of outdoor work for at least 4 hours per day during at least 25 years, and sun protective behavior measured by wearing sunglasses or a hat (Karagas et al., 1992). A history of outdoor work was not included in the questionnaire for the RS-II cohort. All UVR-related questions for RS-III were determined during the second examination round. In addition, smoking, alcohol consumption (glasses per week), coffee consumption (cups per day), and BMI (kg/m^2) were selected as other lifestyle factors (Freedman et al., 2003; Gerstenblith et al., 2012; Miura et al., 2014). Alcohol and coffee consumption for RS-II were determined during the third examination round.

Finally, three variables concerning BCC characteristics were included: localization of the first BCC, superficial histopathological subtype of the first BCC, and the number of BCCs at first date of diagnosis (Karagas et al., 1992; Lovatt et al., 2005).

Model development

All included participants had at least a first BCC and therefore have a date of first BCC diagnosis that served as starting point of the follow-up. Participants were followed from this point forward until they developed a second BCC, died, or reached the end of the linkage period (31 December 2013) without developing a subsequent BCC. Mortality dates were obtained from the municipal register. The localization and histopathological subtype of the first BCC of participants who had more than one BCC at the first date of diagnosis were randomly selected before the analyses.

Missing predictor values were imputed 50 times using multivariate imputation by chained equations (Van Buuren, 2012). The imputation model included all candidate predictors, the outcome (i.e., second BCC or censored), the follow-up time, the side of the first BCC, the level of education, and the RS cohort number.

As a large proportion (28%) of the elderly participants with a first BCC died before they could have developed a second BCC, the analyses were adjusted for competing risk of death from other causes (Wolbers et al., 2009). We used the Fine and Gray semiparametric proportional hazards model to estimate univariable and multivariable regression coefficients (Fine and Gray, 1999). The subdistribution hazard of the event of interest (i.e., second BCC) is the absolute risk of a second BCC. We explored the association of the continuous predictors with the risk of metachronous BCCs by plotting several transformations (e.g., linear, natural logarithm, or square).

We entered all (possibly transformed) candidate predictors in a multivariable model, independent of their p-values in the univariable models. To reduce the multivariable model with backward stepwise selection, we used Wald tests based on Rubin's rules for combining estimated regression coefficients and variances from the 50 different completed data sets (Vergouwe et al., 2010). To reduce selection bias, we used a liberal P-valueo0.20 to include predictors (Steyerberg et al., 2000; Steyerberg, 2009). No significant interactions were observed among the included predictors. The regression coefficients in the final model were multiplied with a shrinkage factor, which was estimated with bootstrapping (Steyerberg, 2009). Shrinkage was applied to prevent that predictions for new patients were too extreme (i.e., low predictions being too low and high predictions being too high).

We performed a sensitivity analysis by including only participants with complete data in the multivariable modeling.

Model performance

We focused on discrimination as a key aspect of model performance. The discriminative ability of the model was evaluated using the c-index. In the available survival data, the c-index represents the probability that, for a randomly chosen pair of patients, the patient who experiences a second BCC earlier in time has a higher predicted risk. A c-index of 0.5 is equivalent to a coin toss, whereas 1.0 implies perfect predictability. We corrected the c-indices for optimism using a bootstrap procedure (500 replications; Steyerberg, 2009).

Clinical application

For illustrative purposes, we divided patients in three risk groups (low, intermediate, and high) using the 25th and 75th percentiles of the risk score distribution as cut points. Next, a score chart was developed to facilitate clinical application of the final

prediction model. Scores were based on the shrunken regression coefficients, which were multiplied by 6.7 and then rounded to an integer. A constant was subtracted or added to rescale the scores conveniently.

IBM SPSS Statistics for Windows version 21.0 (Chicago, IL) was used for data management and R version 3.1.1 for more advanced statistical analysis (R Core Team, 2013), using the cmprsk and riskRegression libraries.

RESULTS

Study population

After the linkage between Pathological Anatomy National Automated Archive (PALGA) and the RS, 1,528 patients with at least one BCC were identified. Of those, 451 were excluded because they developed at least one BCC before entry of the RS. Overall, 1,077 patients were included, of whom 293 developed a second BCC during a median follow-up of 3.0 years, 479 did not develop a new BCC before the end of follow-up (median 3.8 years), and 305 died before they reached the end of follow-up (median 4.6 years; Table 1). The median age at first BCC in the overall group was 74.5 years, whereas in the group of participants who died it was 80.0 years. In all groups, there were more females than males.

Age at first BCC diagnosis

When using ordinary Cox models, there appeared a nonlinear relationship between age at first BCC diagnosis and the hazard of developing a second BCC (Figure 1a) and a linear relationship between age and the hazard of dying (Figure 1b). The subdistribution hazard of a second BCC—using the Fine and Gray model—also had a nonlinear relationship with age (Figure 1c). Compared with the cause-specific hazard, the subdistribution hazard of developing a second BCC is lower for older age, because it takes into account the fact that people may die and therefore are no longer at risk of a second BCC. The nonlinear relation between age at first BCC diagnosis and developing a second BCC could best be approximated by adding a squared term for age to the model.

Predictors for a second BCC

Of the 13 potential predictors, a lower age at first BCC (hazard ratio (HR): 1.6, 95% confidence interval (CI): 1.3–2.0 for 13 years younger) and two tumor-specific factors (i.e., superficial subtype of the first BCC and more than one BCC at first date of diagnosis) were significantly associated with an increased risk of a second BCC in the univariable analyses (Table 2). Furthermore, several other characteristics showed

Table 1. Characteristics of 1,077 patients from the Rotterdam study with at least a first BCC diagnosis

Patient and tumor characteristics	Category	Overall	New BCC	Death without new BCC	Alive without new BCC
Number of patients		1,077 (100%)	293 (100%)	305 (100%)	479 (100%)
Follow-up time (years)	Median (IQR)	3.8 (1.7-7.2)	3.0 (1.3-5.6)	4.6 (2.0-7.7)	3.8 (1.8-7.3)
Age at first BCC (years)	Median (IQR)	74.5 (67.6-80.7)	73.1 (67.3-77.6)	80.0 (74.6-85.6)	71.8 (65.4-78.7)
Sex	Male	484 (45%)	143 (49%)	137 (45%)	204 (43%)
BMI (kg/m^2)	Median (IQR)	26.0 (23.8-28.6)	25.9 (23.8-28.1)	26.5 (24.3-29.0)	25.9 (23.7-28.4)
	Missing	77 (7%)	15 (5%)	33 (11%)	29 (6%)
Pigment status	Dark	174 (16%)	42 (14%)	41 (13%)	91 (19%)
	Intermediate	514 (48%)	138 (47%)	135 (44%)	241 (50%)
	Light	240 (22%)	75 (26%)	74 (24%)	91 (19%)
	Missing	149 (14%)	38 (13%)	55 (18%)	56 (12%)
Easily sunburned	Yes	365 (34%)	111 (38%)	92 (30%)	162 (34%)
	Missing	68 (6%)	20 (7%)	20 (7%)	28 (6%)
Outdoor work	Yes	142 (13%)	40 (14%)	50 (16%)	52 (11%)
	Missing	294 (27%)	90 (31%)	43 (14%)	161 (34%)
Sun protection	No or almost never	404 (38%)	108 (37%)	133 (44%)	163 (34%)
	Missing	63 (6%)	19 (6%)	19 (6%)	25 (5%)
Smoking	Current or ever	705 (65%)	188 (64%)	198 (65%)	319 (67%)
	Missing	18 (2%)	9 (3%)	3 (1%)	6 (1%)
Alcohol consumption (glasses/week)	Median (IQR)	3.8 (0.4-11.2)	3.5 (0.5-11.2)	3.1 (0.2-11.1)	4.3 (0.3-11.2)
	Missing	241 (22%)	52 (18%)	86 (28%)	103 (22%)
Coffee consumption (cups/day)	Median (IQR)	3.3 (2.0-5.0)	3.3 (2.0-4.0)	4.0 (3.0-5.0)	3.3 (2.0-5.0)
	Missing	241 (22%)	52 (18%)	86 (28%)	103 (22%)
Localization of first BCC	Head	663 (62%)	175 (60%)	207 (68%)	281 (59%)
	Extremities	137 (13%)	35 (12%)	32 (10%)	70 (15%)
	Trunk	265 (25%)	81 (28%)	59 (19%)	125 (26%)
	Missing	12 (1%)	2 (1%)	7 (2%)	3 (1%)
Superficial first BCC	Yes	199 (18%)	64 (22%)	39 (13%)	96 (20%)
	Missing	76 (7%)	22 (8%)	34 (11%)	20 (4%)
>1 BCC at first diagnosis date	Yes	132 (12%)	69 (24%)	26 (9%)	37 (8%)

Abbreviations: BCC, basal cell carcinoma; BMI, body mass index; IQR, interquartile range.

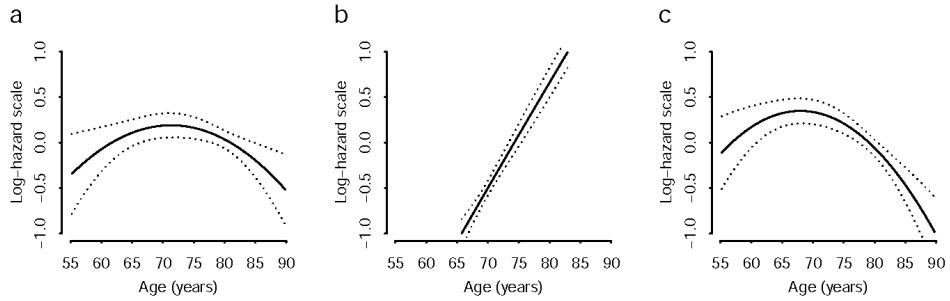


Figure 1. Relationships between age at first BCC diagnosis and risk of a second BCC or death (**a**) *Cause-specific hazard of second BCC.* Nonlinear relation between age at first BCC (x axis) and the logarithmic transformation of the cause-specific hazard of developing a second BCC (y axis) using a Cox model. The dotted lines represent the 95% confidence intervals. (**b**) *Cause-specific hazard of death.* Linear relation between age at first BCC (x axis) and the logarithmic transformation of the cause-specific hazard of dying (y axis) using a Cox model. The dotted lines represent the 95% confidence intervals. (**c**) *Subdistribution hazard of second BCC.* Non-linear relation between age at first BCC (x axis) and the logarithmic transformation of the subdistribution hazard of developing a second BCC (y axis) using a Fine and Gray model. The dotted lines represent the 95% confidence intervals. BCC, basal cell carcinoma.

borderline significant associations with an increased risk of a new BCC, namely male sex, easily sunburned, and truncal localization of the first BCC. In contrast, an increase in coffee consumption of 3 cups per day (HR: 0.8, 95% CI: 0.6–1.0) was borderline significantly associated with a decreased risk of a second BCC.

After backward selection, five predictors remained in the reduced multivariable model: age at first BCC, sex, coffee consumption, superficial subtype of the first BCC, and more than one BCC at first date of diagnosis (Table 2). None of the UVR-related predictors were associated with a second BCC. Being "easily sunburned" also lost its significance after adjustment for all other predictors. The strongest predictor was having more than one BCC at first date of diagnosis (adjusted HR: 2.5, 95% CI: 1.9–3.3). Coffee consumption remained significantly associated with a decreased risk of a second BCC (adjusted HR: 0.7, 95% CI: 0.6–0.9). A complete case analysis on 567 participants resulted in the same reduced multivariable model and comparable HRs (data not shown).

The apparent concordance index (c-index) of the multivariable model was 0.66 (95% CI: 0.58–0.73) at 1 year, 0.67 (95% CI: 0.62–0.72) at 3 years, and 0.65 (95% CI: 0.61–0.69) at 5 years after first BCC diagnosis. After correction for optimism, the c-index of the model was 0.64 at 1 year, 0.65 at 3 years, and 0.63 at 5 years after first BCC diagnosis. When using the score chart for predictions, the apparent c-indices were nearly identical to those of the original model (0.65 at 1 year, 0.67 at 3 years, and 0.65 at 5 years after first BCC diagnosis).

Table 2. Associations between predictors and occurrence of a second BCC (n=293) using the Fine and Gray model for competing risks

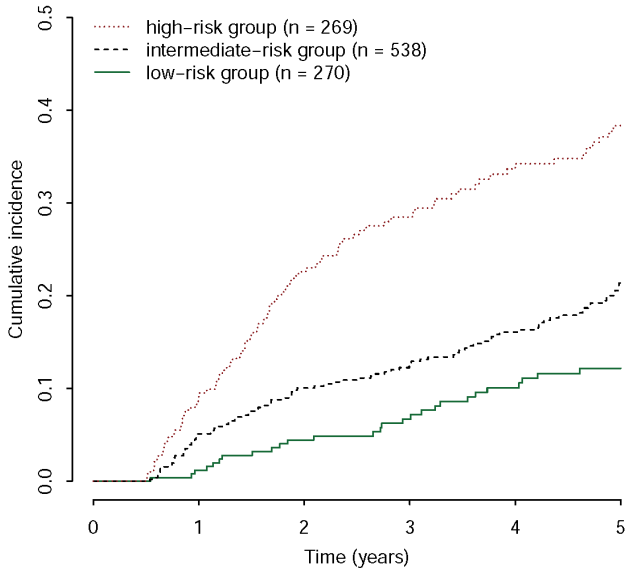
Patient and tumor characteristics	Coding	Univariable models		Multivariable model[1]
Age at first BCC (years)	68 versus 81[2]	1.6 (1.3-2.0)	***	1.6 (1.3-2.0)
Sex	Male	1.3 (1.0-1.6)	*	1.2 (0.9-1.5)
BMI (kg/m2)	24 versus 29[2]	1.1 (0.9-1.3)		-
Pigment status	Dark	Reference		-
	Intermediate	1.2 (0.8-1.6)		-
	Light	1.4 (0.9-2.0)		-
Easily sunburned	Yes	1.3 (1.0-1.6)		-
Outdoor work	Yes	1.1 (0.8-1.5)		-
Sun protection	No or almost never	0.9 (0.7-1.2)		-
Smoking	Ever	1.1 (0.8-1.3)		-
Alcohol consumption (glasses/ week)[3]	10 versus 0[2]	1.1 (0.8-1.6)		-
Coffee consumption (cups/day)	5 versus 2[2]	0.8 (0.6-1.0)	*	0.7 (0.6-0.9)
Localization of first BCC	Head	Reference		-
	Extremities	1.1 (0.8-1.5)		-
	Trunk	1.3 (1.0-1.7)	*	-
Superficial first BCC	Yes	1.5 (1.1-2.0)	**	1.3 (0.9-1.7)
>1 BCC at first diagnosis date	Yes	2.6 (2.0-3.4)	***	2.5 (1.9-3.3)

Abbreviations: BCC, basal cell carcinoma; BMI, body mass index.
The baseline cumulative subdistribution hazard is 0.035 at 1 year, 0.106 at 3 years, and 0.170 at 5 years.
* P-value <0.05, **P-value <0.01, and ***P-value <0.001.
[1] After backward selection.
[2] Interquartile range.
[3] Truncated at 10 glasses per week.

Clinical application

The observed cumulative incidence curve of the high-risk group showed a distinct pattern compared with the observed cumulative incidence curves of the other risk groups (Figure 2). Table 3 shows the score chart that was based on the shrunken regression coefficients of the final prediction model; the estimated shrinkage factor was 0.88. Using the score chart, the physician can easily calculate the predicted risk of a second BCC for a patient currently having a first BCC. The patient obtains a score for each predictor, and these are added up to form a total score. The corresponding predicted risks of a second BCC (within 1, 3, and 5 years) can be found in Table 3 as well. For example, a 63-year-old (two points, when age is rounded to 65 years) man (one point) who drinks no coffee (two points) presenting with one (zero points)

superficial (one point) BCC has a total score of 6, which corresponds to a 3-year risk of 21%.



Numbers at risk				
High−risk	269	223	144	92
Intermediate−risk	538	466	348	248
Low−risk	270	222	142	80

Figure 2. Observed cumulative incidence curves of the three risk groups
Observed cumulative incidence (y axis) curves of the three risk groups (low, intermediate, and high-risk) with follow-up time (x axis) using the 25th and 75th percentiles of the risk score distribution as cut points. Below the figure are the numbers at risk at start of follow-up and at 1, 3, and 5 years of follow-up for each risk group.

Table 3. On the left, score chart for predicting an individual's risk of a second BCC at the time of a first BCC. On the right, total scores and corresponding absolute risks of a second BCC at the time of a first BCC

Predictor	Value	Score
Age at first BCC[1] (years)	≤ 55	0
	60	1
	65	2
	70	2
	75	1
	80	0
	85	-3
	≥ 90	-5
Sex	Female	0
	Male	1
Daily intake of cups of coffee	0	2
	1	1
	2	1
	3	0
	4	-1
	5	-1
	≥ 6	-2
Superficial subtype of first BCC	No	0
	Yes	1
> 1 BCC at first date of diagnosis	No	0
	Yes	5
Total score		…

Total score	1-year risk	3-year risk	5-year risk
≤ -5	1%	4%	6%
-4	2%	5%	8%
-3	2%	6%	9%
-2	2%	6%	10%
-1	2%	7%	11%
0	3%	9%	13%
1	3%	10%	16%
2	4%	11%	18%
3	5%	13%	20%
4	5%	15%	23%
5	6%	18%	28%
6	8%	21%	32%
≥ 7	10%	27%	40%

Abbreviation: BCC, basal cell carcinoma.
The predicted risk (%) of a second BCC within 1 year after the primary BCC was determined by: $P = [1-(\exp(-\exp(B) \times 0.035))] \times 100\%$, where
$B = 0.285 \times \text{age} - 0.002 \times \text{age}^2 + 0.152$ (if male sex) $- 0.093 \times$ coffee cups per day $+ 0.209$ (if superficial subtype) $+ 0.796$ (if more than one BCC).

DISCUSSION

This prospective population-based cohort study shows that the absolute risk of a second BCC could be predicted with reasonable accuracy using simple phenotypic, lifestyle and tumor-specific characteristics. The strongest predictor of a second BCC in time was having more than one BCC at initial BCC diagnosis. Participants were 2.5-fold more likely to develop a new BCC compared with individuals who only had one BCC at the initial date of diagnosis. From the concept of field cancerization, this observation is

expected. It is consistent with the results from the Skin Cancer Prevention Study Group and a retrospective Spanish study demonstrating that the total number of prior BCCs was strongly associated with the risk of metachronous BCCs (Karagas et al., 1992; Graells, 2004).

A superficial subtype of the first BCC gave a participant a significantly higher (+30%) risk to develop a second BCC, which is in accordance with data from a British retrospective cohort study (Lovatt et al., 2005). In previous studies, the histopathological subtype of a BCC has also been associated with tumor localization, as most of the truncal BCCs are superficial, and most of the head and neck BCCs are nodular (Bastiaens et al., 1998; Scrivener et al., 2002). We noted a similar pattern, suggesting a good validity of these predictors.

A nonlinear (parabolic) relationship between age at first BCC diagnosis and the risk of a second BCC was detected. As expected, the risk of a second BCC increased with age, but this risk decreased after approximately 68 years of age. Several other cohort studies have shown a similar risk increase with age but not a risk decrease as patients get even older. Reasons could be that they analyzed a younger cohort and/or changed age into a categorical variable so that a possible nonlinear relationship was hidden (Karagas et al., 1992; Richmond-Sinclair et al., 2010; Flohil et al., 2011).

After adjusting for other factors in the multivariable model, male gender was a modest prognostic factor for a second BCC. Other cohorts demonstrated weak to strong relations between male sex and risk of a subsequent BCC, but they did not adjust for tumor characteristics, such as histological subtype and/or localization, that differ across gender (Karagas et al., 1992; Richmond-Sinclair et al., 2010; Flohil et al., 2011).

Remarkably, coffee consumption reduced the risk of a second BCC (adjusted HR per increase in three cups per day: 0.7, 95% CI: 0.6–0.9). Although caffeinated and decaffeinated coffee consumers could not be differentiated in the overall population, ~ 90% of the coffee consumers in RS-I, which accounts for most of the included participants, used caffeinated coffee. Several observational studies investigated the association between coffee intake and BCC development. Recently, a large prospective follow-up study from Australia showed protective effects of coffee consumption (Miura et al., 2014), whereas two European case–control studies did not find a significant association with BCC development (Corona et al., 2001; Milan et al., 2003). Animal studies have shown that oral and topical administration of caffeine inhibit UVB-induced carcinogenesis and selectively increase apoptosis in squamous cell carcinomas (Huang et al., 1997; Lu et al., 2002). In vitro research on human keratinocytes has demonstrated that this inhibitory effect of caffeine may be due to the induction of apoptosis in UVB-damaged keratinocytes (Heffernan et al., 2009; Han et al., 2011). However, people consuming more coffee may also differ from those drinking less coffee for which the analyses were unable to adjust for (i.e., residual confounding). A recent review argues

that coffee intake reflects an, often unmeasured, healthy life style and is indirectly associated with multiple health outcomes (Mirza et al., 2014).

It is interesting that no significant influence was found for pigment status and UVR-related characteristics (easily sunburned, outdoor work, and sun protection) on the development of a second BCC. The lack of this association was consistent with earlier studies (Lovatt et al., 2005; Richmond-Sinclair et al., 2010). A reason for this apparently paradoxical observation could be the so-called index event bias (Dahabreh and Kent, 2011). UVR is a strong risk factor for a first BCC, and participants who have been exposed to high levels of UVR could have a relatively favorable risk factor profile with respect to the other known and unknown risk factors for a first BCC. This relatively favorable risk profile could, with respect to the other risk factors in the statistical analysis, show a seemingly nonsignificant or an even protective relation with the development of a new BCC within this group with high UVR exposure compared with the group without high levels of UVR exposure.

The prediction model and simple score chart allow for identification of high-risk patients for more intensive follow-up, while excluding the low-risk patients from subsequent follow-up visits. This will lower the strain that the group of BCC patients is putting on the limited (specialized) health care. In addition, an earlier detection of BCCs most likely leads to smaller tumor sizes, which in turn will reduce treatment-related morbidity and costs (Mudigonda et al., 2010). Our 1-year (0.64), 3-year (0.65), and 5-year (0.63) discriminative ability is far from perfect, which suggests that other (unknown) predictors also have a role in the development of a second BCC. Combining genetic and non-genetic predictors into one model might increase the c-index.

Limitations

Cohort members may have developed BCCs prior to the complete national coverage of the pathology database (PALGA) in 1991, leading to misclassification bias, which reduces the generalizability. However, between 1971 and 1991 partial coverage was achieved and the mean age of the included participants in 1991 was 61 years, which is seven years younger compared with the mean BCC age of diagnosis (Arits et al., 2011), suggesting that the impact of this bias is at most modest. In addition, approximately 30% of the participants with a first BCC developed at least a second BCC, which is in line with another Dutch PALGA study and a recent meta-analysis (Flohil et al., 2011; Flohil et al., 2013b), suggesting excellent internal validity of the study design.

Because we obtained our BCC cases through a linkage with PALGA, we have missed BCC diagnoses that were not made based on histopathology. However, a recent study showed that only a small percentage (ca. 7%) of patients with metachronous BCCs had subsequent non-histologically confirmed BCCs (Flohil et al., 2013c). In addition, the evidence-based guideline regarding BCC from the Dutch Society for Dermatology

and Venereology (NVDV) states that all biopsied/excised BCCs should be sent for a histopathological diagnosis (http://www.nvdv.nl/wp-content/uploads/2014/08/Richtlijn-Basaalcelcarcinoom-2014.pdf).

The UVR-related items in the questionnaires for this study may not have been optimal but probably picked up major differences in UVR exposure between participants. Although lifestyle characteristics may change over a lifetime, we only measured UVR-related variables, smoking, alcohol consumption, coffee consumption, and BMI at baseline for most participants. However, we do not believe that non-UVR-related behavior changes after a first BCC diagnosis, as most patients do not associate predictors such as smoking, alcohol consumption, and coffee consumption with BCC development. UVR-related behavior may change, but most of the UV damage has already been done years before diagnosis. We did not have UVR exposure information during childhood and adolescence, which is important in the etiopathogenesis of BCC, but because of the potential recall bias this information is often inaccurate (Glanz et al., 2010).

We have tried to find an external cohort for validation of our prediction model (Leiden Skin Cancer Study, Nurses' Health Study and Framingham Heart Study). Unfortunately, multiple BCC data and detailed information on our predictors are scarce.

Conclusion

The risk factor profile for a second BCC differs from that of a first BCC. The strongest predictor is the presentation of multiple BCCs at index date. Other factors associated with a second BCC are age at first BCC, male gender, coffee consumption, and superficial subtype of the first BCC. These simple variables provide a tool to assist physicians to identify high-risk patients, to give a tailored follow-up, and to give information on the risk of subsequent BCCs. External validation and improvement of the discriminative ability are needed.

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Chapter 7

Occurrence of metachronous basal cell carcinomas: a prognostic model

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ABSTRACT

Background: A third of patients with a first basal cell carcinoma (BCC) will develop subsequent (metachronous) BCCs.

Objectives: To study the prognostic effect of the number of previous BCC diagnosis dates a patient has experienced to derive a prediction model to assess the risk of metachronous BCCs that may inform individualized decision making on surveillance.

Methods: We considered participants of north-western European ancestry from a prospective population-based cohort study (Rotterdam Study). After linkage with the Dutch Pathology Registry, 1077 patients with a first BCC were included. Candidate predictors for metachronous BCCs included patient, lifestyle and tumour characteristics. The prognostic model was developed with Fine and Gray regression analysis to account for competing risk of death. We used bootstrapping to correct for within-patient correlation and statistical optimism in predictive performance.

Results: Second to fifth BCCs occurred in 293, 122, 58 and 36 patients, with median follow-up times of 3.0, 2.1, 1.7 and 1.8 years after the previous BCC, respectively. The risk of a new BCC was higher for patients with more metachronous BCCs. Having more than one BCC at diagnosis was another strong predictor of metachronous BCCs. Discriminative ability of the model was reasonable with an optimism-corrected c-index of 0.70 at 3 years.

Conclusions: The number of previous BCC diagnosis dates was a strong prognostic factor and should be considered when predicting the risk of metachronous BCCs. When the number of previous BCC diagnosis dates is combined with other readily available characteristics into a prognostic model, patients at high risk of a new BCC can be identified.

INTRODUCTION

Basal cell carcinoma (BCC) places a large burden on healthcare systems, resulting from the high incidence of new tumours over time (metachronous BCCs), which need treatment and follow-up.[1–4] The incidence of BCC is increasing, which is reflected in the significant increase in disability-adjusted life years and costs in different countries in the last decades.[2,5,6] Patients tend to develop subsequent skin cancers of the same type, illustrating the concept of field cancerization.[4,7,8] Most metachronous BCCs occur within the first 3 years after diagnosis, but the risk remains elevated over time.[4,9,10] A meta-analysis selected nine studies and found a pooled mean 5-year cumulative risk of a metachronous BCC of 36%, which was comparable with the most recent observational study published.[3,4]

We recently developed a prognostic model to discriminate between patients with a low risk and a high risk of a second BCC. Having more than one BCC at initial diagnosis was the strongest risk factor, followed by age, superficial first BCC and male sex.[11] These predictors have been found associated with metachronous BCCs in other observational research.[4,7,10,12–14] Previous studies have typically focused on the first metachronous BCC, whereas patients frequently develop more metachronous BCCs.[4,10] A prognostic model for metachronous BCCs could identify high-risk patients who need active surveillance, improving secondary prevention.

The question arises of whether the predictors of a second BCC will be predictive for the risk of metachronous BCCs. At least the number of previous BCC diagnosis dates a patient has experienced might be an important addition to the prognostic model. The purpose of our study was to determine the frequency and timing of metachronous BCCs with cumulative incidence curves and to develop a prognostic model to predict the absolute risk of metachronous BCCs. We analysed a prospective population-based cohort (Rotterdam Study)including > 1000 patients with BCC. In the model development, we considered death as a competing risk and adjusted for the multiple events per patient. Reporting was according to the TRIPOD Statement.[15,16]

MATERIALS AND METHODS

Study population

The Rotterdam Study is a prospective population-based follow-up study in a well-defined district of Rotterdam in the Netherlands and comprises 14 926 participants aged 45 years or older.[17] The cohort started in July 1989 and predominantly consists of people of north-western European ancestry. All the participants were interviewed and examined at baseline and these examinations were repeated about every 4 years. The

Rotterdam Study has been approved by the Medical Ethics Committee of the Erasmus MC and by the Ministry of Health, Welfare and Sport of the Netherlands, implementing the Wet Bevolkingsonderzoek: ERGO (Population Studies Act: Rotterdam Study). All participants provided written informed consent to participate in the study and for us to obtain information from their treating physicians.

Case definition

Identification of BCC cases has been described previously.[11] In short, the Rotterdam Study participants were linked to the nationwide network and registry of histo- and cytopathology in the Netherlands (PALGA) to obtain their medical history of histopathologically confirmed BCCs until 1 January 2014. PALGA was founded in 1971 and achieved complete national coverage in 1991.[18] The pathology excerpts we received contained information on date of diagnosis, anatomical location, body side, type of procedure (biopsy or excision), radicality (whether or not the BCC was completely excised with no tumour cells in the studied transection margins according to the pathologist) and diagnosis [including subtype(s)]. We used this information to distinguish metachronous BCCs from recurrent BCCs (i.e., irradically treated). Furthermore, if a new BCC was diagnosed within 6 months of an earlier BCC, it was considered to be present at the previous diagnosis date. This rule was applied during the entire follow-up period.

Of the 14 926 Rotterdam Study participants, 298 did not sign informed consent for a linkage and could not be linked to PALGA. To maintain the prospective design, participants who had a BCC before Rotterdam Study entry were excluded from the analyses.

All included patients had a first BCC with a date of diagnosis that served as starting point of the follow-up. Participants were followed from this point forward until they died, or reached the end of the linkage period (31 December 2013). We considered a maximum of four metachronous BCCs per patient, to have strata with at least 50 patients at time of prediction. Mortality dates were obtained from the municipal registry. Figure 1 shows the data structure of the BCC patients.

Candidate predictors

The same candidate predictors were considered as in our prognostic model for a second BCC: age at BCC diagnosis, sex, pigment status, tendency to develop sunburn, history of outdoor work, sun-protective behaviour, BCC localization, superficial subtype and having more than one BCC at date of diagnosis.[11] We added one new categorical predictor: the number of previous BCC diagnosis dates (0, 1, 2 or 3). More specifically, this predictor represents the number of previous diagnosis dates on which one or more BCCs were diagnosed. Pigment status was a combination of eye colour and hair

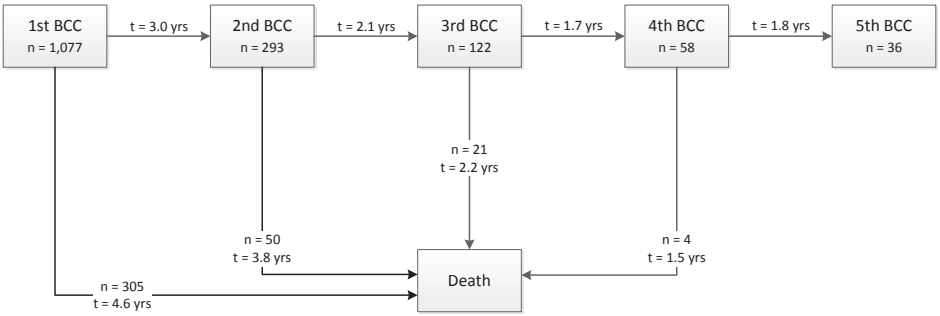

Figure 1. Structure of the metachronous basal cell carcinoma (BCC) dataset with numbers of patients (n) and median follow-up times in years (t)
Censoring occurred for 479, 121, 43 and 18 patients after having a first, second, third and fourth BCC diagnosis date, respectively.

colour when young. Mixed-type BCCs with a superficial component were coded as superficial. When participants had more than one BCC at a certain date of diagnosis, the localization and histopathological subtype at this date were randomly selected before the analyses. The latter was the case for 12– 25% of the patients, depending on the number of previous BCC diagnosis dates.

Model development

Missing predictor values were imputed 20 times using multivariate imputation by chained equations.[19] The imputation model included all candidate predictors, the outcome (i.e., new BCC, death or censored) and the follow-up time.

As a large proportion of the patients died during follow-up (24.5%), time to event analyses were adjusted for the competing event 'death'.[20] We used the Fine and Gray semiparametric proportional hazards model to estimate univariable and multivariable regression coefficients.[21] The subdistribution hazard of a new BCC corresponded to the absolute risk of a new BCC.

The proportionality of subdistribution hazards was tested for all predictors.[22] The assumption was not met for age, but adding an interaction term between age and time did not show sufficient relevance according to the likelihood ratio test to extend the model beyond the main effect of age. We explored the association of the continuous predictors with the risk of metachronous BCCs by plotting several transformations (linear, natural logarithm and square). Only the predictor age showed a nonlinear relationship with the outcome, which could be approximated by adding a quadratic term. To allow for event-specific effects of the predictors, we tested the interactions between the number of previous BCC diagnosis dates and each of the other predictors with likelihood ratio tests. None of these was of sufficient relevance to change the model specification.

The Fine and Gray model does not account for within-patient correlation resulting from multiple events per patient. Consequently, variance estimates of the regression coefficients will be too low and confidence intervals (CIs) will be too narrow. To adjust for the within-patient correlation, we assessed SEs with bootstrapping.[23] For each imputed dataset, we drew 1000 bootstrap samples. Backward stepwise selection was based on a liberal P-value (< 0.20), to reduce selection bias.[24] The regression coefficients in the final model were multiplied with a heuristic shrinkage factor.[25] Shrinkage was applied to prevent predictions for new patients being too extreme (i.e., low predictions being too low and high predictions being too high).

Model performance

We focused on discrimination as a key aspect of model performance. The discriminative ability of the model was evaluated using the concordance index (c-index) adapted for competing risks data.[26] In our survival data, the c-index represents the probability that, for a randomly chosen pair of patients, the patient who experiences a new BCC earlier in time has a higher predicted risk. A c-index of 0.5 is equivalent to a coin toss, whereas 1.0 implies perfect discrimination. We corrected the c-indices for optimism using a bootstrap procedure with 500 replications, including the backward selection procedure.[25]

Clinical application

We developed a score chart to facilitate clinical application of the final prognostic model. Scores were based on the shrunken regression coefficients, which were multiplied by 7.1 and then rounded to an integer. A constant was subtracted or added to rescale the scores conveniently. IBM SPSS Statistics for Windows version 21.0 (IBM, Armonk, NY, U.S.A.) was used for data management and R version 3.2.0 for more advanced statistical analysis,[27] using the 'cmprsk' and 'riskRegression' libraries.

RESULTS

Study population

After linkage, 1528 patients with at least one BCC were identified. Of those, 451 were excluded because they developed at least one BCC before entry into the Rotterdam Study. Included patients (n = 1077) had a median age of 75 years and 45% were male. Among them 293, 122, 58 and 36 developed a second, third, fourth and fifth BCC, respectively. The median follow-up until the next BCC was 3.0, 2.1, 1.7 and 1.8 years, respectively (Figure 1). In total, 380 patients died during a median follow-up for survivors of 5.0 years. The cumulative incidence of a metachronous BCC at 3 years

was 15%, 34%, 45% and 67% for the second, third, fourth and fifth BCC, respectively (Figure 2).

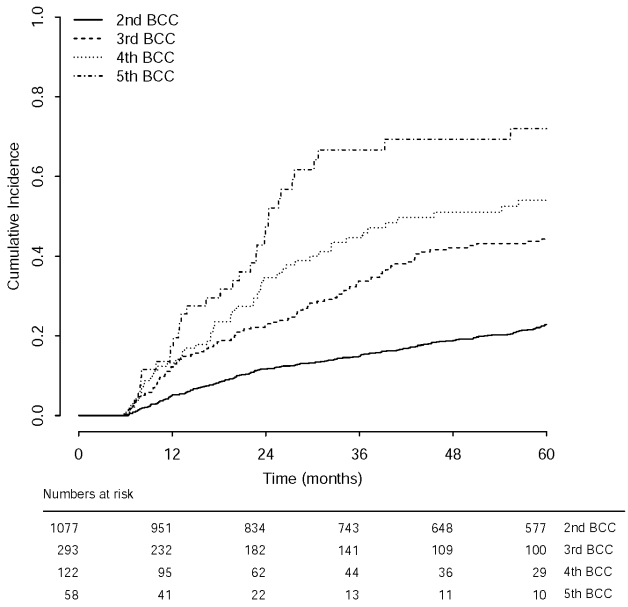

Figure 2. Cumulative incidence functions per basal cell carcinoma (BCC) diagnosis date sequence number
Below the figure are the number of patients at risk at the specific time points.

Predictors for metachronous basal cell carcinomas

The frequency of known predictors for a first BCC – such as light pigment status, easily sunburned and no sun protection-increased when patients experienced more metachronous BCCs (Table 1). More than one BCC at diagnosis was a strong predictor in the univariable analyses together with the number of previous BCC diagnosis dates a patient had experienced (Table 2). The effect of the localization of the BCC was similar for trunk and extremities.

Nine of the 14 candidate predictors remained in the multivariable model: age at BCC diagnosis, sex, pigment status, easily sunburned, coffee consumption, more than one BCC at diagnosis, superficial subtype of BCC, localization of BCC and the number of previous BCC diagnosis dates (Table 2). The apparent overall c-index of the multivariable model was 0.68 (95% CI 0.63–0.72) at 1 year; 0.71 (95% CI 0.69–0.74) at 3 years; and 0.69 (95% CI 0.67–0.72) at 5 years after any BCC diagnosis. Optimism-corrected c-indices were 0.67 (95% CI 0.62–0.71), 0.70 (95% CI 0.68–0.73) and 0.68 (95% CI 0.66–0.71), respectively.

Table 1. Distribution of candidate predictor values at first and metachronous basal cell carcinomas (BCCs)

		At 1st BCC N = 1077		At 2nd BCC N = 293		At 3rd BCC N = 122		At 4th BCC N = 58	
Age at BCC diagnosis	Years	75	(68-81)	77	(71-83)	79	(73-84)	77	(73-85)
Gender	Male	484	(45%)	143	(49%)	68	(56%)	31	(53%)
Pigment status	Total[a]	928		255		105		52	
	Dark	174	(19%)	42	(17%)	13	(13%)	6	(11%)
	Intermediate	514	(55%)	138	(54%)	56	(53%)	27	(52%)
	Light	240	(26%)	75	(29%)	36	(34%)	19	(37%)
Easily sunburned	Total[a]	1009		273		111		54	
	Yes	365	(36%)	111	(41%)	55	(50%)	30	(56%)
Sun protection	Total[a]	1014		274		111		54	
	Yes	404	(40%)	108	(39%)	35	(31%)	17	(31%)
Outdoor work	Total[a]	783		203		86		44	
	Yes	142	(18%)	40	(20%)	19	(22%)	9	(20%)
BMI	Total[a]	1000		278		118		56	
	Kg per m^2	26.0	(23.8-28.6)	25.9	(23.8-28.1)	25.8	(24.0-27.8)	26.6	(24.4-29.0)
Smoking	Total[a]	1059		284		117		56	
	Ever	705	(67%)	188	(66%)	80	(68%)	36	(64%)
Alcohol consumption	Total[a]	836		241		101		48	
	Glasses per week	3.8	(0.4-10.0)	3.5	(0.5-10.0)	4.0	(0.6-10.0)	2.5	(0.5-9.9)
Coffee consumption	Total[a]	836		241		101		48	
	Cups per day	3.3	(2.0-5.0)	3.3	(2.0-4.0)	3.3	(2.0-4.0)	4.0	(2.9-4.0)
More than 1 BCC at diagnosis	Yes	132	(12%)	40	(14%)	30	(25%)	12	(21%)
Superficial BCC	Total[a]	1001		287		119		56	
	Yes	199	(20%)	77	(27%)	37	(31%)	18	(32%)
Localization of BCC	Total[a]	1065		289		122		57	
	Head	663	(62%)	165	(57%)	63	(52%)	25	(44%)
	Trunk	265	(25%)	84	(29%)	36	(29%)	24	(42%)
	Extremities	137	(13%)	40	(14%)	23	(19%)	8	(14%)

Data are n (%) unless otherwise indicated. IQR, interquartile range; BMI, body mass index. [a] For predictors with missing values, the number of observed values is given.

Table 2. Uni- and multivariable associations between predictors and occurrence of metachronous basal cell carcinomas (BCCs) using the Fine and Gray model for competing risks

Patient, lifestyle, and tumour characteristics	Coding	Univariable models HR (95% CI)[a]	Multivariable model HR (95% CI)[a]
Age at BCC diagnosis, years	69 versus 82[b]	1.3 (1.1-1.6)	1.4 (1.2-1.7)
Gender	Male	1.3 (1.0-1.6)	1.2 (1.0-1.4)
Pigment status	Dark	1.0	1.0
	Intermediate	1.3 (1.0-1.8)	1.2 (0.9-1.5)
	Light	1.6 (1.1-2.2)	1.3 (1.0-1.7)
Easily sunburned	Yes	1.4 (1.1-1.7)	1.2 (1.0-1.4)
Sun protection	Yes	0.8 (0.7-1.0)	—
Outdoor work	Yes	1.1 (0.9-1.4)	—
BMI, kg per m^2	24 versus 29[b]	1.0 (0.9-1.1)	—
Smoking	Ever	1.1 (0.8-1.3)	—
Alcohol consumption, glasses per week	10 versus 0[b]	1.1 (0.8-1.4)	—
Coffee consumption, cups per day	5 versus 2[b]	0.8 (0.7-0.9)	0.8 (0.7-0.9)
> 1 BCC at diagnosis	Yes	2.2 (1.8-2.6)	1.9 (1.5-2.4)
Superficial BCC	Yes	1.5 (1.3-1.9)	1.2 (0.9-1.5)
Localization of BCC	Head	1.0	1.0
	Trunk	1.5 (1.2-1.9)	1.2 (1.0-1.5)
	Extremities	1.4 (1.1-1.9)	1.3 (1.0-1.7)
Number of previous BCC diagnosis dates	0	1.0	1.0
	1	2.1 (1.7-2.6)	2.1 (1.7-2.6)
	2	2.8 (2.1-3.8)	2.6 (1.9-3.5)
	3	4.8 (3.4-6.9)	3.9 (2.5-6.2)

HR, hazard ratio; CI, confidence interval. [a] Based on 1000 bootstrap samples; [b] interquartile range.

Clinical application

A score chart was based on the regression coefficients of the final prognostic model (with shrinkage by a factor 0.89). The patient obtained a score for each predictor, and these were added to form a total score (Table 3). The corresponding predicted risks of a metachronous BCC are shown for 1, 3 and 5 years [Figure. 3; Table S1 (see Supporting Information)]. For example, a 75-year-old (4 points) male patient (1 point) with light pigment status (2 points), who sunburns easily (1 point), who drinks no coffee (4 points) and who presents for the first time (0 points) with one (0 points) superficial (1 point) BCC located at the head (0 points) has a total score of 13, which corresponds to a 3-year risk of 26% of experiencing a metachronous BCC. If this same male patient had presented with exactly the same BCC but already experienced three previous

BCCs, his score would be 22, corresponding to a 3-year risk of 59% of experiencing a metachronous BCC.

Table 3. Score chart for predicting metachronous basal cell carcinomas (BCCs)

Predictor	Value	Score
Age at BCC diagnosis, years	≤ 55	4
	60-70	5
	75	4
	80	3
	85	2
	≥ 90	0
Gender	Female	0
	Male	1
Pigment status	Dark	0
	Intermediate	1
	Light	2
Easily sunburned	No	0
	Yes	1
Daily intake of cups of coffee	0	4
	1-2	3
	3-4	2
	5-6	1
	≥ 7	0
More than 1 BCC at diagnosis	No	0
	Yes	4
Superficial BCC	No	0
	Yes	1
Localization of BCC	Head	0
	Trunk	1
	Extremities	2
Number of previous BCC diagnosis dates	0	0
	1	5
	2	6
	3	9
Total score		…

DISCUSSION

We studied the occurrence of metachronous BCCs and developed a prognostic model to predict the absolute risk of metachronous BCCs. The number of previous BCC

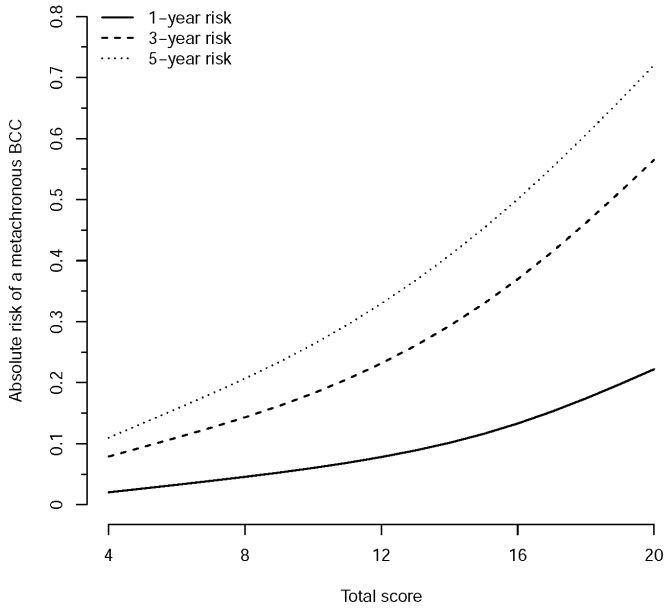

Figure 3. Total score obtained from the score chart (x-axis) and corresponding absolute risks of a metachronous basal cell carcinoma (BCC; y-axis) within 1, 3 and 5 years after the current BCC diagnosis date

diagnosis dates a patient experienced was the strongest prognostic factor. The risks of a third, fourth and fifth BCC increased compared with the risk of a second BCC. The number of previous BCC diagnosis dates was combined with easily obtainable patient, lifestyle and tumour-specific characteristics into a prognostic model.

Most predictors that were included in the current model and in our previous model for predicting a second BCC had similar effect sizes.[11] Three additional predictors with moderate effects were included in the model for metachronous BCC: pigment status, easily sunburned and localization of BCC.

The risk of a metachronous BCC was high in the first 2 years after a diagnosis, as illustrated by the cumulative incidence curves. Up to 50% of patients with a fourth BCC will develop a fifth within 24 months of follow-up. Indeed, previous studies have also shown that most metachronous BCCs occur within the first 2–3 years after diagnosis, but the risk remains elevated over time.[4,9,10] The model may guide in assessing individualized surveillance: who should be monitored and when, and extends our previous prognostic model, where we ended the follow-up after the first metachronous BCC (i.e., second BCC).[11]

Patients who experienced more previous BCCs were far more likely to develop a new BCC than patients who were diagnosed with their first BCC (Figure. 2). The number of previous BCC diagnosis dates and the number of BCC diagnosis dates at diagnosis

could be proxies of field cancerization, a well-known concept in patients with recurrent and multiple primary oral squamous cell cancers.[28] Like the oral cavity, the skin is one of the predominant sites of oncogenesis because it comes into direct contact with many carcinogens, in particular ultraviolet radiation (UVR). Throughout life, large fields of UVR-exposed skin accumulate genetically altered cells and become preneoplastic, which is analogous to, for example, head and neck squamous cell carcinoma of the digestive system.[29] Normal-looking human skin and epithelial tissue surrounding BCCs often contains precancerous changes, such as TP53 mutations.[30–32] Thus, physicians should always perform a full-body skin examination at first BCC diagnosis, because of the high likelihood of synchronous BCCs, and follow those who have a history of synchronous and/or metachronous BCCs. Our results are in line with other prospective U.S.-based follow-up studies, where the number of previous BCC diagnosis dates was the strongest predictor for metachronous BCCs in multivariable adjusted analyses.[7,33] A U.K. case–control study showed that the presence of more than one BCC at first presentation was significantly associated with a decreased time to the next BCC.[34]

Two other tumour-specific characteristics – histopathological subtype and localization – were also predictive of metachronous BCCs. The findings are comparable with U.K. case–control and cohort studies.[12,35] The results could indicate that patients with a superficial and/or BCC on the trunk or extremities comprise a subgroup in which different mechanisms are at work (e.g., different UVR exposure patterns and/or genetic susceptibility). However, in a Dutch retrospective cohort study both subtype and localization were not associated with a second BCC after multivariable adjustment.[36] Previous studies found that superficial BCCs are predominantly located on the trunk, whereas nodular BCCs are found more often in the head and neck area.[37–39] We noted a similar distribution of the different subtypes and localizations in our study.

The risk of metachronous BCCs increased with age but decreased again after approximately 68 years of age. This is similar to our previous finding, when we analysed the follow-up until the first metachronous BCC (i.e., second BCC).[11] Other prospective cohort studies have found a risk increase with age –but categorized age, which could have hidden a nonlinear relation.[7,10,13,33] Moreover, these studies also did not take the potential competing risk of death into account, which could lead to an unrealistic estimation of absolute risks.[20,40]

Our patients were relatively old (median age 75 years) as a result of linking the clinical data of the patients to the epidemiological data of the Rotterdam cohort containing mainly elderly people. Nevertheless, our model can be applied to patients from about 50 years of age, as the minimum age of our patients was 48 years.

Coffee consumption significantly reduced the risk of metachronous BCCs, for each increase of three cups of coffee per day the risk decreased by 20%. As discussed in our previous study, this could be a true biological effect or the result of selection

bias.[11] The findings on coffee consumption are diverse. A large prospective follow-up study from Australia showed protective effects of coffee consumption,[41] whereas two somewhat older European case–control studies did not find a significant association with BCC development.[42,43] A recent meta-analysis suggested a protective effect of coffee consumption, although this effect disappeared when the study that contributed the most heterogeneity between studies was left out of the analysis.[44] Despite these varying findings, the strong predictive effect of coffee consumption on developing a second or later BCC and the ease of asking patients about their coffee use allowed us to include this predictor in our model.

The apparent c-indices of our model varied between 0.68 and 0.71. As we had no external cohort, the c-index measured the internal validity of our model. But even after correcting for the possibly resulting optimism, the c-indices still varied between 0.67 and 0.70, which indicated a reasonable discriminative ability. As there are not yet any tools for predicting the occurrence of metachronous BCCs, we think our model is a good first step. We propose that the simple score chart can help physicians identify high-risk patients to prevent serious morbidity and high healthcare costs.[5,6] A cost-effectiveness analysis may identify a threshold value that justifies active surveillance and a reasonable frequency of follow-up.

The existing follow-up protocols differ per country and even per hospital within a country. Nevertheless, our model may well assist clinicians to better identify patients at high risk of metachronous BCC and to determine surveillance frequencies accordingly. For example, BCC patients in the Netherlands usually are not under follow-up, because 'only' 30% will get a next BCC and BCC is rarely lethal. Moreover, following up every patient would be unrealistic because of insufficient capacity and high costs. However, when clinicians can use our model to estimate an individual patient's absolute risk, they can decide to see high-risk patients regularly.

This study has some limitations. Some cohort members may have developed BCCs prior to the complete national coverage of PALGA; BCC diagnoses that were not based on pathology were missed (most likely only a small percentage);[45] and the UVR-related items in the questionnaires may not have been optimal. These issues were also encountered when we developed a model for a second BCC, but were shown to have little or no effect on the internal validity of the study design.[11] We did not have the availability of an external validation cohort and had to rely on bootstrap validation to support our claims of predictive performance in new patients. Future studies should validate the proposed score and try to improve the discriminative ability.[46]

To accommodate both competing risks and repeated events, we based our prognostic model on the Fine and Gray model. We corrected the variances of the predictor estimates post hoc to account for the within-patient correlation between metachronous BCCs. Other advanced techniques might have been used, such as the multistate model or the

joint frailty model.[47,48] Unfortunately, absolute risk calculation for repeated events is not yet readily possible with these techniques in currently available statistical software.

In conclusion, the absolute risk of a metachronous BCC can be predicted with reasonable accuracy using a prognostic model that consists of a combination of patient, lifestyle and tumour-specific characteristics. The number of previous BCC diagnosis dates a patient has experienced is the strongest prognostic factor with higher risk of a metachronous BCC, when patients have experienced more previous metachronous BCCs. When proven to be valid at external validation, the model can assist clinicians in identifying high-risk patients and in tailoring surveillance frequencies for individual patients.

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SUPPORTING INFORMATION

Table S1. Formula to calculate the individual absolute risks of metachronous basal cell carcinomas.

Table S1. Formula to calculate the individual absolute risks of metachronous BCCs

The predicted risk (%) of a new BCC within *t* years after the current BCC can be calculated as:

$$Pr = \left[1 - \left(\exp\left(-\exp(lp) \times CSH_0(t)\right)\right] \times 100\%\right., \text{ where}$$

$$\begin{aligned} lp = {} & 0.145 \times (Age - 75) - 0.001 \times (Age^2 - 5746) + 0.141\,(\text{if male gender}) \\ & + 0.134 \times (\text{if intermediate pigment status}) \\ & + 0.221 \times (\text{if light pigment status}) \\ & + 0.166 \times (\text{if easily sunburned}) - 0.073 \times (\text{coffee cups per day} - 3.6) \\ & + 0.570 \times (\text{if more than one BCC}^a) + 0.146 \times (\text{if superficial subtype}) \\ & + 0.257 \times (\text{if localized on extremities}) \\ & + 0.166 \times (\text{if localized on trunk}) \\ & + 0.660 \times (\text{if 1 previous BCC diagnosis date}) + 0.849 \\ & \times (\text{if 2 previous BCC diagnosis dates}) + 1.220 \times (\text{if 3 previous BCC diagnosis dates}) \end{aligned}$$

$$CSH_0(1) = 0.033;\ CSH_0(3) = 0.109;\ CSH_0(5) = 0.163$$

[a] If more than 1 BCC is currently diagnosed, the observed value corresponding to the highest regression coefficient should be used for superficial and localization. BCC, basal cell carcinoma; $CSH_0(t)$, cumulative subdistribution baseline hazard at time point t in years; exp, exponent; lp, linear predictor; Pr, probability.

Chapter 8

Common variants affecting susceptibility to develop multiple basal cell carcinomas

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To the editor

Among the millions of people who develop a first basal cell carcinoma (BCC) annually, ~30% will get subsequent BCCs (Flohil et al., 2013). The majority of BCCs occur on the head and neck, where tumor growth and surgery can lead to functional and cosmetic morbidity. Because of the high incidence, risk of multiple tumors, and morbidity, the disease burden and healthcare costs are considerable (Housman et al., 2003; Hollestein et al., 2014).

Several candidate gene approaches (CGAs) suggest that polymorphisms in the genes encoding cytochrome P450, glutathione S-transferase, and HLA are implicated in the development of multiple BCCs (mBCC; Cerimele et al., 1988; Rompel et al., 1995; Lear et al., 1996; Ramachandran et al., 2000). Most of these studies have a small sample size and include only a few variants per gene. To date, there are no studies investigating whether more recently identified BCC loci also confer susceptibility to mBCC. We investigated whether single-nucleotide polymorphisms (SNPs) previously associated with BCC increase the risk of mBCC using a CGA. In addition, we conducted a genome-wide association study (GWAS) to identify previously unreported loci associated with the risk of mBCC.

A detailed description of all the methods is presented in the Supplementary Material online. We used participants from the Rotterdam Study (RS), which is a population-based follow-up study that consists of three cohorts (RS-I, II, and III; Hofman et al., 2013). The Medical Ethics Committee of the Erasmus Medical Center and the review board of the Dutch Ministry of Health, Welfare and Sport have ratified the RS. All participants who gave informed consent were linked with a nationwide registry of histopathology in The Netherlands (PALGA, up to 31 December 2013) to identify histopathologically confirmed BCCs, squamous cell carcinomas (SCCs), and melanomas (Casparie et al., 2007).

DNA from whole blood was extracted and genotyped following standard protocols (Hofman et al., 2013). Quality control procedures were applied to the genotyped SNP data. The GWA data sets were imputed to the 1,000 Genomes data set using MACH-minimac v1.0.18 (Howie et al., 2012). In total, 30,072,738 markers were genotyped and/or imputed. We excluded markers with a minor allele frequency <3% and an imputation quality <0.3. After quality control, 7,260,691 markers were available for analysis.

From the 9,810 RS participants with genotype and phenotype information, 1,219 individuals with BCC were identified, of whom 472 had mBCC (38.7%). Participants with mBCC had a significantly higher proportion of SCCs and/or melanomas compared with those with single BCC (sBCC; Supplementary Table S1 online).

First, 19 candidate SNPs and 17 loci from well-powered GWASs/CGA of BCC were selected (Supplementary Table S2 and Supplementary Material online). We then

conducted SNP- and gene-based logistic regression analyses in two data sets, comparing BCC to no BCC (i.e., validation set) and mBCC to sBCC to investigate whether these BCC loci also increase the risk of mBCC. The analyses were adjusted for age at study entry or age at first BCC, sex, and four principal components (PCs). As these SNPs and loci were previously significantly associated with BCC, we only adjusted for multiple testing in the mBCC versus sBCC data set using the Bonferroni correction. All analyses were performed in PLINK v1.07 (Purcell et al., 2007).

In the CGA on BCC against no BCC, 12/19 (63%) candidate SNPs and 5/17 (29%) loci were replicated, demonstrating a good external validity of the study (Table 1 and Supplementary Table S3A online). Interestingly, the CGA comparing sBCC to mBCC did not yield any significant associations between these BCC-related SNPs/loci and risk of mBCC (Table 1 and Supplementary Table S3B online).

Table 1. SNP-based and gene-based association analyses

		BCC vs. no BCC			mBCC vs. sBCC		
SNP	**Locus**	**SNP-based p-value**	**Gene-based p-value**	**SNP-based OR**	**SNP-based p-value**	**Gene-based p-value**	**SNP-based OR**
rs1126809	TYR	**0.0027**	0.14	1.16	0.99	0.16	1.0010
rs4911414	20q11.22	0.067	0.96	1.086	0.49	0.77	0.94
rs1015362	20q11.22	0.83	0.96	1.010	0.15	0.77	0.87
rs7538876	PADI6	**0.00062**	0.075	1.16	0.063	0.11	1.17
rs801114	1q42.13	**0.0070**	**0.049**	1.13	0.63	0.75	0.96
rs11170164	KRT5	0.17	**0.0020**	1.12	0.13	0.24	1.27
rs2151280	CDKN2B-AS1	**0.0043**	0.13	0.88	0.97	0.64	1.00
rs157935	LINC-PINT	**0.00077**	0.10	0.85	0.18	0.86	0.88
rs16891982	SLC45A2	**0.0081**	**0.038**	0.65	0.82	0.47	0.93
rs401681	CLPTM1L	**0.00053**	**0.022**	0.86	0.36	0.83	0.92
rs12210050	EXOC2	**0.030**	0.35	1.15	0.69	0.13	0.95
rs7335046	UBAC2	0.19	0.35	1.087	0.10	0.016	1.22
rs1805007	MC1R	0.069	0.60	1.16	0.43	0.25	0.88
rs78378222	TP53	**0.037**	0.17	1.34	0.13	0.39	1.49
rs12203592	IRF4	**7.7E-05**	0.063	1.31	0.35	0.30	0.89
rs12202284	EXOC2	0.12	0.35	1.096	0.88	0.13	1.017
rs8015138	14q22.1	0.79	0.088	0.99	0.91	0.72	0.99
rs214782	TGM3	**1.2E-05**	**0.0070**	1.27	0.056	0.19	1.22
rs7006527	RGS22	**0.0018**	0.17	0.82	0.49	0.85	1.088

Abbreviations: BCC, basal cell carcinoma; mBCC, multiple basal cell carcinomas; OR, odds ratio; sBCC, single basal cell carcinoma; SNP, single-nucleotide polymorphism. Numbers in bold display significant differences (P-value <0.05). The gene-based P-value for mBCC versus sBCC should be corrected for multiple testing.

Finally, we conducted a pilot GWAS using logistic regression (additive model) to test for associations between markers and mBCC, adjusting for age at diagnosis, sex, and four PCs (Supplementary Material online). A meta-analysis of the GWAS results per cohort was performed. Despite the low overall power to detect genome-wide significant hits (Supplementary Figure S1 online), we identified genome-wide suggestive associations in chromosomes 2, 3, 18, and 22 (P-values $<5 \times 10^{-6}$, Supplementary Figure S2 online and Table 2). The most significant SNP was rs78857623 (P-value $=1.2 \times 10^{-7}$, Table 2), which mapped to an intron in FHIT, and it was in linkage disequilibrium ($r^2=0.65$) with another significantly associated intronic FHIT SNP (rs78316259; P-value$=4.6 \times 10^{-6}$). FHIT is a tumor suppressor gene that encodes a diadenosine polyphosphate hydrolase involved in purine metabolism (Barnes et al., 1996). Aberrant FHIT transcripts as well as germline mutations in this gene have been found in different cancers including BCC (Ohta et al., 1996; Goldberg et al., 2006; Ding et al., 2008).

Given the high proportion of SCCs and melanomas in mBCC cases, a sensitivity analysis investigating the influence of other cutaneous cancers was performed (Supplementary Table S1 online). We observed changes in the P-values of the associations due to a 13% decrease in sample size, but all top SNPs remained significant with a P-value $<5 \times 10^{-5}$ (data not shown), showing that our findings were driven by BCC cases.

In contrast to other BCC GWASs, we performed a GWAS on histopathologically confirmed mBCC. By combining national pathology data with genomewide SNP data from a population-based study, we accurately distinguished between sBCC and mBCC. It is a pilot GWAS because the sample size is small and replication data are not easily available. All existing cohorts, which have performed genetic epidemiology on skin cancer, do not have data on mBCC. Like in other GWASs, the significant associations are only statistical and therefore any inference about the functional impact of the variants to the risk of mBCC needs to be investigated with other approaches. Despite these limitations, our data set contains the largest collection of cases with mBCC to date and may serve as a valuable reference for future studies.

Because of the high risk of subsequent BCCs in individuals with a first BCC, we expected that loci predisposing to sBCCs would also contribute to the risk of mBCC. However, the CGA analysis did not support this hypothesis, suggesting that other biological factors, including genes, may confer an increased risk to mBCC. The earlier onset of first BCCs in patients with mBCC may indeed suggest that these patients have a stronger genetic burden compared with those with sBCC (68.7 vs. 72.4 years; P-value <0.05, Supplementary Table S1 online). A heritability analysis comparing mBCC against sBCC in well-powered samples will help validate this hypothesis. It could also be argued that other yet-to-be-identified loci conferring susceptibility to BCC may also increase risk of mBCC, which will require larger consortia on the genetics of BCC.

Table 2. SNPs with highly significant associations from GWAS on mBCC

Chr.	Marker	Frequency[1]	P-value	Direction[2]	HetISq[3]	HetPVal[4]	Gene	Functional consequence marker
3	rs78857623	0.048	1.2E-07	---	0.4	0.37	FHIT	intronic
2	2:168946822:D	0.084	3.9E-07	---	0	0.53	STK39	intronic
18	rs4371253	0.18	2.1E-06	+++	0	0.76	SLC14A2	intronic
22	rs4824031	0.34	2.2E-06	---	0	0.88		intergenic
9	rs17717641	0.94	3.4E-06	---	0	0.57		intergenic
16	rs41305755	0.20	3.6E-06	---	56.1	0.10	ABAT	intronic
16	rs45545237	0.80	3.6E-06	+++	56.1	0.10	ABAT	intronic
6	rs79899616	0.96	3.6E-06	+++	0	0.57		intergenic
22	rs8138971	0.38	3.6E-06	---	0	0.92		intergenic
18	rs4890560	0.19	3.7E-06	+++	0	0.87	SLC14A2	intronic
18	rs4890291	0.19	3.8E-06	+++	0	0.87	SLC14A2	intronic
11	rs80160790	0.031	4.0E-06	--?	0	0.58	GRIA4	intronic, upstream variant 2KB & 5'UTR variant
22	rs9616609	0.36	4.1E-06	---	0	0.80		intergenic
18	rs8093237	0.18	4.2E-06	+++	0	0.75	SLC14A2	intronic
18	rs11875624	0.18	4.5E-06	+++	0	0.74	SLC14A2	intronic
18	18:43175190:D	0.18	4.5E-06	+++	0	0.74		deletion
3	rs78316259	0.057	4.6E-06	---	0	0.89	FHIT	intronic
9	rs1412279	0.46	5.2E-06	---	0	0.47		intergenic
9	9:78186385:D	0.065	5.2E-06	+++	0	0.69		deletion
9	rs75858454	0.065	5.2E-06	+++	0	0.63		intergenic
9	rs74403342	0.94	5.4E-06	---	0	0.68		intergenic

Abbreviations: Chr., chromosome; GWAS, genome-wide association study; mBCC, multiple basal cell carcinomas; SNP, single-nucleotide polymorphism; UTR, untranslated region.

[1] Frequency: weighted average of frequency for allele 1 across all cohorts.

[2] Direction: summary of effect direction for each study, with one '+' or '–' per cohort ('?' is unknown).

[3] HetISq: I^2 statistic which measures heterogeneity on scale of 0–100%.

[4] HetPVal: P-value for heterogeneity statistic.

In conclusion, genetic loci previously associated with BCC do not increase the risk of mBCC. A pilot GWAS on mBCC identified to our knowledge previously unreported susceptibility variants, but these findings need to be replicated in other mBCC cohorts.

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SUPPLEMENTARY MATERIAL

Materials and methods

Study population

The Rotterdam Study (RS) is a prospective population-based follow-up study of the determinants and prognosis of chronic diseases, including skin diseases and cancer, in the elderly. Only participants living in Rotterdam, The Netherlands, are included. The RS consists of a major cohort (RS-I) and two extensions (RS-II and RS-III). RS-I started in 1990 and initially included 7,983 participants living in the Ommoord district in Rotterdam. RS-II started in 2000 and now includes 3,011 participants. RS-III is a further extension of the cohort, started in 2006, and now includes 3,932 participants. By the end of 2008, the RS comprised 14,926 subjects aged 45 years or over. The overall response rate for all three cycles at baseline was 72.0%. The cohort consists predominantly (90%) of participants of North-European ancestry. A detailed description of the design of the RS is presented in Hofman et al. (2013). The Medical Ethics Committee of the Erasmus Medical Center and the review board of the Dutch Ministry of Health, Welfare and Sport have ratified the RS. Our study was conducted according to Declaration of Helsinki Principles. From each participant, written informed consent was obtained.

Ascertainment of (multiple) basal cell carcinoma cases

All the participants of the RS who gave informed consent (n = 14,628) were linked with the nationwide network and registry of histopathology and cytopathology in The Netherlands (PALGA; up to 31st December 2013) to identify histopathologically confirmed BCCs, squamous cell carcinomas and melanomas. PALGA was founded in 1971 and achieved complete national coverage in 1991. Every obtained pathology excerpt contains encrypted patient data, a report identifier, the conclusion of the pathologist and a PALGA diagnosis line derived from the Systematized Nomenclature of Medicine (Casparie et al., 2007). The majority of excerpts extracted from PALGA include an anatomical location, which makes it possible to distinguish between participants with single or subsequent tumors. If location was not available, we assumed that a biopsy followed by an excision for the same type of skin cancer, concerned the same tumor. The next BCC following a radical excision was always counted as a new neoplasm. If an excision was incomplete, the next reported tumor on the same/adjacent location was regarded as recurrent and not a new BCC. If the diagnosis or the number of unique BCCs remained unclear, the medical files were searched by hand and a consensus decision was made based on these data.

DNA collection, genotyping, imputation and quality control

DNA from whole blood was extracted following standard protocols (Hofman et al., 2013). The Illumina Infinium II HumanHap550 BeadChips were used to genotype the RS-I (n = 6,291) and RS-II (n = 2,157) cohorts while Illumina Human610-Quad BeadChips were used to genotype the RS-III cohort (n = 3,048). Quality control criteria included the removal of SNPs with Hardy-Weinberg equilibrium deviations ($p<10-6$), genotyping call rate < 97%, gender mismatch and a high mean autosomal heterozygosity. We also excluded duplicates or first-degree relatives using identity-by-descent (IBD) estimates and outliers (three standard deviations away from the population mean) using multi-dimensional scaling (MDS) analysis with four principal components. After excluding related participants, 9,810 participants remained in our dataset.

To increase the coverage of the genome, we imputed the RS-I, RS-II and RS-III cohorts separately, using 1000Genomes (GIANT Phase I version 3) as the reference panel and using a two-step procedure imputation algorithm implemented in the program MACH-Minimac v1.0.18 using default parameters (Howie et al., 2012). In total 30,072,738 markers were genotyped and/or imputed. We filtered out markers with a MAF < 3% and an imputation quality score (r^2_pihat) < 0.3. This resulted in 7,260,691 markers that passed quality control and were used in the meta-analysis.

Selection of SNPs and candidate loci associated with BCC

We selected English publications indexed in PubMed until December 2013 that reported associations between common SNP variants (MAF > 1%) and BCC or keratinocyte carcinoma. To reduce the burden of multiple testing for our CGA we limited our CGA to variants identified in high-powered GWASs and CGAs of BCC in humans. For a GWAS we considered SNPs with a p-value < $10-^{6}$ to choose candidate genes.

To select the candidate gene/locus of an associated SNP located within a gene/locus, we retrieved the coordinates of the RefSeq longest transcript from the UCSC Genome Browser (http://www.http://genome.ucsc.edu/; GRCh37/hg19 assembly) and added 15Kb downstream and upstream of the locus region (Kent et al., 2002). The 30Kb region was selected to only include regulatory regions nearby the locus to which the variants have been previously annotated. For intergenic SNPs located more than 15Kb from a gene, we added 30Kb (15Kb downstream and 15Kb upstream) to the position of the candidate SNP. Further, we used the genomic coordinates of the selected loci and extracted all SNPs available from the RS genome-wide SNP data for the gene/locus association analyses. The list of SNPs and loci associated with the SNPs from the studies we included for our analysis, are shown in Supplementary Table S2 online.

Statistics and analyses

Candidate SNP and gene association analyses on (m)BCC

To test for associations between (m)BCC and common SNPs and candidate genes, best-guessed genotypes of the three RS cohorts were estimated from the imputed data using the Genome wide Complex Trait Analysis (GCTA) software with default parameters (Yang et al., 2011).

We first carried-out a SNP-based association analysis on SNPs that were previously associated with BCC using two case-control designs, namely: all prevalent cases of BCC against no BCC (BCC/controls), and all prevalent cases with mBCC against all prevalent cases with sBCC. We used the first design as a validation dataset and the second design to address the research questions. For the SNP association we carried-out a logistic regression analysis, adjusting the model for age at study entry/age at first BCC, sex and four principal components. Since the SNPs were already replicated in previous GWAS we only adjusted for multiple testing in the mBCC/sBCC design using the Bonferroni correction. These analyses were carried-out in PLINK v1.07 (Purcell et al., 2007).

To screen for additional variants in the genomic region (15Kb up- and downstream) to which the candidate SNPs are mapped to, a gene-based logistic regression analysis using a set-based test implemented in PLINK v1.07 was performed with the following parameters: $r^2 = 0.5$, p-value = 0.05, maximum number of SNPs = 15 and permutations = 1000. This test calculates whether the mean statistics of SNP associations within a gene are larger than those calculated under the null hypothesis of no association, taking into account the linkage disequilibrium (LD) between SNPs within a set/gene and adjusting for multiple testing per gene using permutations. For these analyses we adjusted for the same covariates as in the SNP-based associations. P-values were further adjusted for the number of evaluated genes in the mBCC/sBCC dataset.

Genome-wide association analysis

To discover to our knowledge previously unreported loci associated with an increased risk for mBCC, a pilot GWAS per RS cohort with participants with mBCC as cases and subjects with sBCC as controls was performed. We used logistic regression with an additive model to test for associations between SNPs and the phenotype, adjusting for age at diagnosis, sex and four principal components. The significance of the association was tested using the likelihood ratio test with one degree of freedom. The GWAS analyses were implemented in the ProbABEL package, which incorporates dose imputed data within a logistic regression framework (Aulchenko et al., 2010). Subsequently, because the standard error of the effects may not be accurate due to the small sample size, we used the p-values from the likelihood-ratio tests as summary statistics to meta-analyze.

The meta-analysis of the three RS cohorts was carried out using the METAL software, allowing for genomic control correction and heterogeneity (Willer et al., 2010).

Supplementary table S1. Study population characteristics

Characteristics	**sBCC**[1]	**mBCC**[2]
Number of participants	747	472
Mean age (range) at study entry, years	66.4 (46-99)	65.8 (48-92)
Mean age (range) at first BCC diagnosis, years	**72.4 (36-99)**	**68.7 (30-96)**
Sex (%)		
Female	429 (57.4)	233 (49.4)
Male	**318 (42.6)**	**239 (50.6)**
Other cutaneous cancers (%)		
SCC[3]	62 (8.3)	76 (16.1)
Melanoma	6 (0.8)	13 (2.8)
SCC & Melanoma	2 (0.3)	4 (0.8)
All combined	**70 (9.4)**	**93 (19.7)**

[1] Single basal cell carcinoma
[2] Multiple basal cell carcinoma
[3] Squamous cell carcinoma
Numbers in bold display significant differences (p-value < 0.05) according to the independent-samples t-test or Chi-squared test

Supplementary table S2. Selected candidate SNPs and loci

Study type	SNP[1]	Position[2]	Chr[3]	Mutation	Locus	Genomic coordinates[4]	Other genes within region
CGA[5]	rs1126809[7]	89017961	11	missense	TYR	88896039- 89043927	
CGA	rs4911414[7]	32729444	20	intergenic	20q11.22	32714444-32879444	ASIP, AHCY (partial)
CGA	rs1015362[7]	32738612	20	intergenic	20q11.22	32714444-32879444	ASIP, AHCY (partial)
GWAS[6]	rs7538876[8]	17722363	1	intronic	PADI6	17683690-17743195	RCC2 (partial)
GWAS	rs801114[8]	228997835	1	intergenic	1q42.13	228982835-229147835	
GWAS	rs11170164[9]	52913668	12	missense	KRT5	52893358-52929243	
GWAS	rs2151280[9]	22034719	9	intronic	CDKN2B-AS1	21979789-22136093	CDKN2B, CDKN2A (partial)
GWAS	rs157935[9]	130585553	7	intronic	LINC-PINT	130550750-130809675	
GWAS	rs16891982[9]	33951693	5	3'UTR	SLC45A2	33930971-33999780	AMACR (partial), RXFP3 (partial)
GWAS	rs401681[9]	1322087	5	intronic	CLPTM1L	1302999-1360002	
GWAS	rs12210050[9]	475489	6	intergenic	EXOC2	470137-708141	HUS1B
GWAS	rs7335046[10]	100041738	13	intergenic	UBAC2	99837678-100053753	GPR18, GPR183, UBAC2-AS1, FKSG29
GWAS	rs1805007[10]	89986117	16	missense	MC1R	89969286-90002385	TUBB3 (partial)
GWAS	rs78378222[11]	7571752	17	3'UTR	TP53	7556719- 7605868	WRAP53 (partial)
GWAS	rs12203592[12]	396321	6	intronic	IRF4	376738- 426443	
GWAS	rs12202284[12]	471136	6	intergenic	EXOC2	470137-708141	HUS1B
GWAS	rs8015138[12]	52310104	14	intergenic	14q22.1	52295104-52325104	
GWAS	rs214782[13]	2281970	20	intronic	TGM3	2261612-2336725	
GWAS	rs7006527[13]	101024505	8	intronic	RGS22	100958165-101133344	

[1] Single nucleotide polymorphism
[2] Base pair positions were based on GRCh37/hg19 assembly
[3] Chromosome
[4] Genomic coordinates were based on GRCh37/hg19 assembly
[5] Candidate gene approach
[6] Genome-wide association study
[7] Gudbjartsson *et al.* (2008); [8] Stacey *et al.* (2008); [9] Stacey *et al.* (2009); [10] Nan *et al.* (2011); [11] Stacey *et al.* (2011); [12] Zhang *et al.* (2013); [13] Stacey *et al.* (2014)

Supplementary table S3a. SNP-based and gene-based associations for BCC[1]

Set[2]	Total no. SNPs/set	No. significant SNPs[3]	No. significant independent SNPs[4]	P-value[5]	SNPs/insertions/deletions[6]
TYR	552	110	6	0.14	rs28521275|rs12363772|rs147546939|rs140959774|11:88897675:D|rs150457098
20q11.22	378	5	4	0.96	rs819135|rs11907546|rs6087563|20:32783615:I
PADI6	339	89	7	0.075	rs2800696|rs144412112|rs12127366|rs72637458|rs3094884|rs10788668|rs76455718
1q42.13	816	60	15	**0.049**	rs10916373|rs116307216|rs61824935|rs142159847|rs114257119|1:229010041:D|rs710805|rs6680713|rs710813|rs6685751|rs11582420|rs78351254|1:229101395:D|rs190427483|rs188101343
KRT5	230	1	1	**0.0020**	rs142879390
CDKN2B-AS1	574	94	15	0.13	rs72652409|rs10757265|9:21980969:D|9:22062751:I|rs597816|rs72655437|rs10757281|rs74992648|rs141348408|9:22036252:D|rs3731212|rs72655422|rs72654280|rs10965228|rs2811711
LINC-PINT	927	46	15	0.10	rs157934|rs17737947|7:130567125:I|7:130670612:D|rs2895195|rs73159889|rs205755|rs28564708|7:130675843:D|rs56219258|rs150200398|rs113171594|rs7781295|rs55968167|rs34211697
SLC45A2	191	24	4	**0.038**	5:33956141:D|rs35406|5:33944592:D|rs173662
CLPTM1L	236	94	15	**0.022**	rs55901723|rs37002|rs37004|rs428499|rs190785038|rs182017427|rs76879431|rs27066|rs182898174|rs186023279|rs144439878|rs140648021|rs62329688|rs186156459|rs71575565
EXOC2	1310	74	15	0.35	rs111739182|rs150161396|rs186862400|rs151237719|rs115663764|rs9504290|rs1213054|rs1747586|rs139546405|rs181005467|rs115686195|rs181391484|6:505139:I|rs12210050|rs2985278
UBAC2	967	19	13	0.35	rs192022243|rs138435721|rs188034987|rs116740777|rs142525959|rs61970285|13:99959963:I|rs145902748|rs148859462|rs1160294|rs138258516|rs17472050|rs139945409
MC1R	180	6	5	0.60	rs76302987|rs3212379|16:89974729:D|rs3212349|rs140585935
TP53	232	27	12	0.17	rs1641548|rs2908807|rs9894946|rs76032029|17:7600126:D|rs17883670|17:7579643:D|rs12938947|rs78378222|rs74351250|rs1642785|rs72829452
IRF4	227	8	4	0.063	rs12203592|rs9405192|rs190846031|6:384956:I
14q22.1	256	4	3	0.088	rs2748146|rs2884137|rs2993998
TGM3	284	131	13	**0.0070**	rs214748|rs188612684|rs151233784|rs192816818|rs192147751|rs6048207|rs2014017|20:2336433:I|rs6082627|rs214816|rs214827|rs6082867|rs6515233

Supplementary table S3a. SNP-based and gene-based associations for BCC[1] (*continued*)

Set[2]	Total no. SNPs/set	No. significant SNPs[3]	No. significant independent SNPs[4]	P-value[5]	SNPs/insertions/deletions[6]
RGS22	618	52	15	0.17	8:101111131:D|rs17391663|rs13340637|rs76209450|rs6994747|rs187405154|rs2885 5477|8:101081191:I|rs140377173|rs28457202|rs146325009|rs141450089|rs1153461-50|rs181290644|rs150640895

[1] BCC: 1,219 cases (≥1 BCC) were compared to 8,591 controls (no BCC)

[2] Locus region, see Supplementary Table S2 for exact genomic coordinates

[3] Total number of SNPs with p-value < 0.05

[4] Total number of significant SNPs (p-value < 0.05) also passing LD-criterion ($r^2 < 0.5$)

[5] Numbers in bold display significant associations

[6] List of significant SNPs/deletions/insertions also passing the LD criterion

Supplementary table S3b. SNP-based and gene-based associations for mBCC[1]

Set[2]	Total no. SNPs/set	No. significant SNPs[3]	No. significant independent SNPs[4]	P-value[5]	SNPs/insertions/deletions[6]
TYR	552	9	1	0.16	rs35963892
20q11.22	378	4	4	0.77	rs146491521|rs139914983|rs13042965|rs7262908
PADI6	339	62	5	0.11	rs183603588|rs3094884|rs7191|rs76550244|rs7556072
1q42.13	816	4	4	0.75	rs146666914|rs115475209|rs77541106|rs187651720
KRT5	230	52	3	0.24	rs11170161|12:52898781:D|rs11549951
CDKN2B-AS1	574	1	1	0.64	rs61743293
LINC-PINT	927	31	14	0.86	rs117798053|rs205730|rs11767836|rs6959008|rs11760691|rs181060962|rs11980581|rs116519273|7:130755763:D|7:130657365:I|rs62473520|7:130652737:D|rs6953084|rs17165262
SLC45A2	191	3	2	0.47	rs77175283|rs141549575
CLPTM1L	236	7	3	0.83	rs12332579|rs189368366|rs144439878
EXOC2	1310	36	13	0.13	rs145280257|rs58022076|rs17756753|rs6933777|rs113608863|rs72835985|rs3823124|rs186862400|6:675542:D|rs3799307|rs143388344|rs150891820|rs17236435
UBAC2	967	148	9	0.016	rs2390237|rs2296046|13:100022283:D|13:99934484:I|13:99933087:D|rs79145601|rs9513608|rs61968345|rs188179841
MC1R	180	32	8	0.25	rs72813442|rs76302987|rs12920483|rs4785736|rs111398992|rs188455950|rs182927424|rs186542844
TP53	232	2	2	0.39	rs17883670|MERGED_DEL_2_87391
IRF4	227	10	8	0.30	rs79324228|rs11756234|6:425200:D|rs9378774|rs3823305|rs6935510|6:393837:D|rs72833949
14q22.1	256	1	1	0.72	rs140107698
TGM3	284	26	8	0.19	rs6515233|rs6082627|rs112763701|rs2422689|rs2076408|rs214755|rs45440896|rs214802
RGS22	618	5	5	0.85	rs191382884|rs62532694|8:101065714:D|rs140899826|8:100989341:I

[1] mBCC: 472 cases (>1 BCC) were compared to 747 controls (1 BCC)

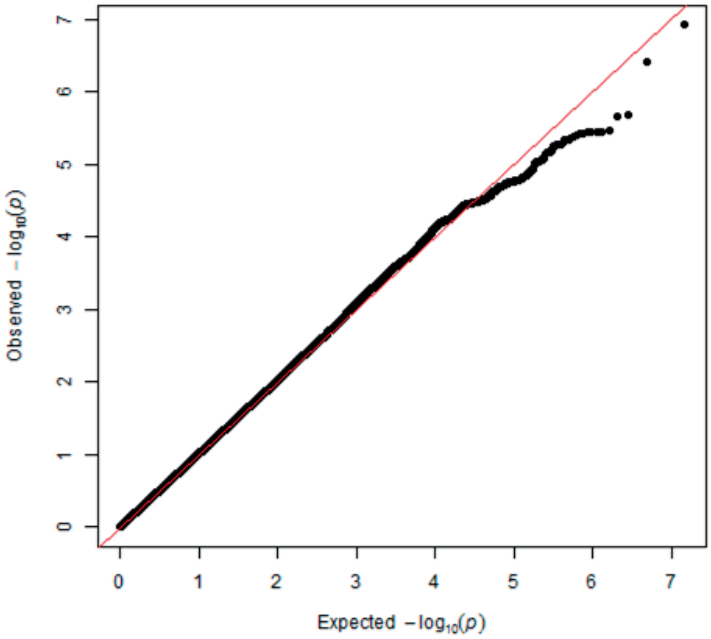
[2] Locus region, see Supplementary Table S2 for exact genomic coordinates

[3] Total number of SNPs with p-value < 0.05

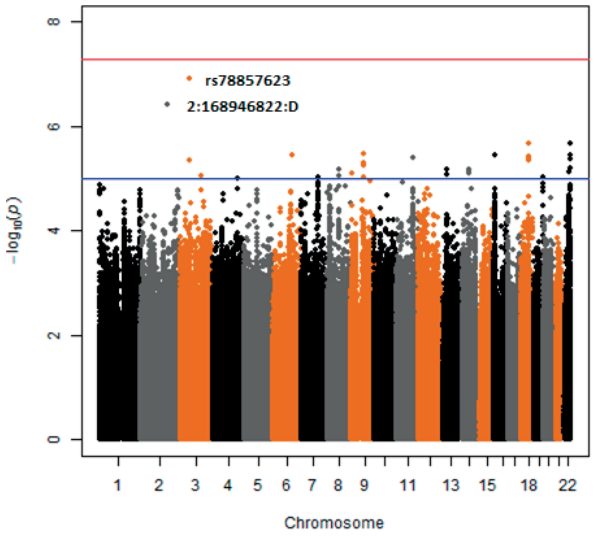
[4] Total number of significant SNPs (p-value < 0.05) also passing LD-criterion ($r^2 < 0.5$)

[5] There are no significant associations, because all p-values need to be corrected for multiple testing

[6] List of significant SNPs/deletions/insertions also passing the LD criterion



Supplementary Figure S1. Q-Q-plot of the meta-analysis of the Rotterdam Study on mBCC
The $-\log_{10}$ of observed p-values of the associations between mBCC and SNPs (Y-axis) are plotted against the expected p-values under the assumption of no association (X-axis).



Supplementary Figure S2. Manhattan plot of the meta-analysis of the Rotterdam Study on mBCC
The $-\log_{10}$ of observed p-values of the associations between mBCC and SNPs (Y-axis) for all SNPs (dots) are represented per chromosome (X-axis). The horizontal lines indicate the significant threshold of p-value = 5×10-6 (blue) and p-value = 5×10^{-8} (red).

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Chapter 9

Genome-wide association studies of multiple keratinocyte cancers

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ABSTRACT

There is strong evidence for a role of environmental risk factors involved in susceptibility to develop multiple keratinocyte cancers (mKCs), but whether genes are also involved in mKCs susceptibility has not been thoroughly investigated. We investigated whether single nucleotide polymorphisms (SNPs) are associated with susceptibility for mKCs. A genome-wide association study (GWAS) of 1,666 cases with mKCs and 1,950 cases with single KC (sKCs; controls) from Harvard cohorts (the Nurses' Health Study [NHS], NHS II, and the Health Professionals Follow-Up Study) and the Framingham Heart Study was carried-out using over 8 million SNPs (stage-1). We sought to replicate the most significant statistical associations (p-value $\leq 5.5 \times 10^{-6}$) in an independent cohort of 574 mKCs and 872 sKCs from the Rotterdam Study. In the discovery stage, 40 SNPs with suggestive associations (p-value $\leq 5.5 \times 10^{-6}$) were identified, with eight independent SNPs tagging all 40 SNPs. The most significant SNP was located at chromosome 9 (rs7468390; p-value = 3.92×10^{-7}). In stage-2, none of these SNPs replicated and only two of them were associated with mKCs in the same direction in the combined meta-analysis. We tested the associations for 19 previously reported basal cell carcinoma-related SNPs (candidate gene association analysis), and found that rs1805007 (*MC1R* locus) was significantly associated with risk of mKCs (p-value = 2.80×10^{-4}). Although the suggestive SNPs with susceptibility for mKCs were not replicated, we found that previously identified BCC variants may also be associated with mKC, which the most significant association (rs1805007) located at the *MC1R* gene.

INTRODUCTION

Basal cell carcinoma (BCC) and squamous cell carcinoma (SCC) of the skin are known together as keratinocyte carcinomas (KC), since they both originate from keratinocytes of the epidermal layer of the skin, and share similar risk factors, treatments and prognosis [1]. KC is the most common cancer in adults of northern-European descent and is becoming a major health burden due to the high prevalence and increasing incidence in Western countries [2, 3]. A systematic review showed that patients with a primary BCC or SCC are likely to develop subsequent KCs with proportions as high as 44% in USA and 32% in The Netherlands [4]. However, it was recently shown that patients with only single KCs have a lower risk for subsequent KCs when compared with patients with a history of two or more KCs suggesting a differential risk profile of patients with single KCs than patients with a history of prior multiple KCs [3].

Environmental, tumour, and individual risk factors, including ultraviolet radiation (UVR), pale hair and skin, and male gender have been associated with an increased risk for multiple KCs (mKCs) [5-7]. There is also suggestive evidence for a genetic predisposition to mKCs, since genetic mutations in *PTCH1* [1, 8] and *PTCH2* [9] cause multiple BCCs [10] in individuals with nevoid BCC syndrome (NBCCS), a Mendelian disease. In addition, over 19 loci have been associated with sporadic BCC [11-13] and two with SCC. However, these previous studies included all prevalent non-melanoma skin cases and therefore it is not clear whether mKCs patients share the same genetic susceptibility variants as these with single KC.

In a recent study we found that common variants associated with BCC did not predict susceptibility for mBCC [14]. Other studies assessing genetic susceptibility in patients with mKCs are scarce. Here, we carried out a meta-analysis of GWAS on mKCs to investigate genetic susceptibility for mKCs comparing 1,241 mKCs to 2,822 single KCs (sKCs). We used patients with single KC as controls to increase the chance of identifying variants associated with susceptibility for having multiple KCs.

MATERIALS AND METHODS

Study population

The nurses' health study (NHS), NHS II and the health professionals follow-up study (HPFS)-harvard cohorts.

Study participants were included from three ongoing longitudinal cohorts: NHS, NHS II and HPFS. The NHS was established in 1976 when 121,701 married, female registered nurses aged 30-55 in the US were enrolled using a mailed questionnaire inquiring about

their medical history and lifestyle practices. Between 1989 and 1990, blood samples were collected from 32,826 cohort members. NHS II began in 1989 when 116,430 female nurses aged 25-42 completed a mailed questionnaire. Between 1996 and 1998, blood samples were collected from 29,616 cohort members. The HPFS consisted of 51,529 male health professionals who completed their baseline questionnaire in 1986. Between 1993 and 1994, blood samples were collected from 18,159 cohort members. Information on lifestyle factors and medical history was collected biennially by mailed questionnaire. The follow-up rate exceeds 90% in each cohort. The study protocol was approved by the Institutional Review Board of Brigham and Women's Hospital and the Harvard School of Public Health.

We combined data from several case-control studies nested within the cohorts for type 2 diabetes (NHS and HPFS), coronary heart disease (NHS and HPFS), breast cancer (NHS and NHS II), colon cancer (NHS and HPFS), kidney stone (NHS, NHS II and HPFS), advanced prostate cancer (HPFS), endometrial cancer (NHS), gout (NHS and HPFS), glaucoma (NHS and HPFS), mammographic density (NHS), and pancreatic cancer (NHS and HPFS). The description of the studies is presented elsewhere [13].

mKCs case ascertainment

Participants reported diagnoses of cancers biennially. Medical records were reviewed to confirm the diagnoses. Medical records were not obtained for self-reported cases of BCC, but previous studies showed high validity of BCC self-reports [15, 16]. Information on the cumulative number of KCs was collected in 2004 (NHS), 2005 (NHS II) and 2008 (HPFS); details are presented elsewhere [5, 7]. A validation study among 200 cases who reported 5-10 and ≥11 KC showed a confirmation rate of 92% [7]. All the participants included in the analysis were Caucasians who reported at least one pathologically confirmed diagnosis of SCC or self-reported BCC in the cohort follow-up. For this study, cases were defined as individuals with more than one KC (mKCs) and controls were defined as those with single KC (sKCs).

The framingham heart study

The Framingham Heart Study (FHS) is a community-based prospective study that began in 1948 to characterize cardiovascular disease and its risk factors. The Original Cohort was composed of 5,209 Framingham residents primarily of white European-ancestry. In 1971, 5,124 offspring of the Original Cohort and their spouses were recruited into the Offspring Cohort. In 2002, 4095 children of the offspring cohort were invited to the Third Generation Cohort. The study design and participant descriptions of the three cohorts have been published elsewhere [17-19].

mKCs case ascertainment

Participants have undergone routine research examinations every two to six years. Cancer cases were identified at the research examinations or by medical history updates for participants who did not attend an examination. Two independent reviewers examined the medical records of all cancer cases and used the World Health Organization ICD-O coding and in 2010 ICD-10 coding to classify all primary tumours. All skin cancer cases were verified with pathology reports. FHS participants with GWAS genotype information and with pathologically confirmed skin cancer (until December 31 2013, melanoma excluded) were included in the current study. Participants with more than one KC were defined as cases and these with single KCs were defined as controls.

The rotterdam study (RS)

The RS is a prospective population-based follow-up study of the determinants and prognosis of chronic diseases, including skin cancer, in the elderly [20]. The RS consists of a major cohort (RS-I) and two extensions (RS-II and RS-III). RS-I started in 1990 and included 7,983 participants living in the Ommoord district (Rotterdam, the Netherlands). RS-II began in 2000 and now includes 3,011 participants. RS-III was started in 2006 and now includes 3,932 participants. By the end of 2008, the RS comprised 14,926 subjects aged 45 years or over. The RS consists predominantly (90%) of participants of North-European ancestry. A detailed description of the design of the RS is presented elsewhere [20].The Medical Ethics Committee of the Erasmus Medical Center and the review board of the Dutch Ministry of Health, Welfare and Sports have ratified the RS. Written informed consent was obtained from each participant.

mKCs case ascertainment

To identify histopathologically confirmed BCCs, SCCs and melanomas, RS participants were linked with the nationwide registry of histo- and cytopathology in the Netherlands (PALGA; up to 31st December 2013) [21]. The case definition for KC has been described previously [14]. In the majority of reports extracted from PALGA it was possible to distinguish between participants with single or subsequent tumours. If the diagnosis or the number of unique KC remained unclear, the medical files were searched by hand and a consensus decision was made. The number of KC was recorded separately for BCC and SCC. Individuals with either a single BCC or SCC were considered as controls while multiple BCC and/or SCC were taken as mKCs cases.

Genotyping and imputation

Details of DNA collection, genotyping and quality control for the Harvard cohorts [13, 22], the FHS [23] and the RS [20] cohorts has been detailed elsewhere. A summary of

the genotyping quality control for the all cohorts is presented in the supplementary file S1 Appendix.

Harvard GWAS

Genotyping was performed on three platforms: Affymetrix (n = 1230: 539 controls, 691 cases), Illumina HumanHap (n = 845: 363 controls, 482 cases), and Illumina Omni Express (n = 645: 287 controls, 358 cases). The genotypes per platform were merged from the different cohorts (NHS, NH II and HPFS) [13] and thus, had men and women. Based on combined GWAS genotypes on each genotyping platform and the 1000 Genomes Project ALL Phase I Integrated Release Version 3 Haplotypes (2010-11 data freeze, 2012-03-14 haplotypes) as reference panel, we imputed the genotypes of markers in the 1000 Genomes Project using MACHv.1.0.18.c [24]. Only SNPs with imputation Rsq > 0.95 and minor allele frequency (MAF) >1% were included in meta-analysis.

FHS GWAS

Genotyping was conducted using the Affymetrix 500K mapping array and the Affymetrix 50K gene-focused molecular imprinted polymer array. We imputed using 1000Genomes Phase I Version 3 as the reference panel using MACH-Minimac [24]. SNPs with MAF ≤1% and imputation quality value <0.3 were excluded.

RS GWAS

Details of genotyping approach is presented elsewhere [20]. Briefly, cohorts RS-I and RS-II were genotyped with the Infinium II HumanHap550K Genotyping BeadChip version 3 (Illumina, San Diego, California USA) and the cohort RS-II was genotyped using the Illumina Human 610 Quad Arrays. We imputed the RS-I, RS-II and RS-III cohorts separately, using 1000Genomes (GIANT Phase I version 3) as the reference panel and using MACH-Minimac with default parameters [24]. Next, markers with a MAF ≤1% and an imputation quality score (Rsq) <0.3 were removed.

Statistical analysis

Stage-1; discovery phase

The discovery samples (stage-1) consisted of the Harvard cohorts (NHS, NHS II, and HPFS) and the FHS cohort. The association analyses between the SNPs and mKCs were performed using an additive logistic regression model on subjects with more than one KC as cases and subjects with only one KC as controls. As the Harvard cohorts were genotyped on three different platforms [13], GWAS analyses were conducted for each platform, adjusting for age at first diagnosis of SCC/BCC, sex and four principal

components of genetic variance (PCAs) using ProbABEL [25]. The association for each SNP from three platforms for the Harvard cohorts was combined in an inverse-variance-weighted meta-analysis using METAL [26].

The FHS GWAS was carried out using an additive generalized estimation equation (GEE) model [27] that takes into account the pedigree structure of the FHS study. The model was adjusted for age at first diagnosis, sex and four PCAs. These analysis were performed using the R package [27].

The quality control of the GWAS summary statistics from Harvard cohorts and the FHS GWAS summary statistics was performed using the EasyQC software [28]. After quality control there were 9,001,799 markers from Harvard cohorts and 8,246,930 markers from FHS. The cleaned files of both datasets (Harvard cohorts and FHS) were meta-analysed using the inverse variance approach implemented in METAL[26]. SNP heterogeneity was tested using I^2 and Cochran's Q, both of which are implemented in METAL. The inflation factor lambda (genomic control) was close to 1.0 (ć= 1.08) and therefore no further adjustments for genomic control were done The SNPs that showed significant associations with mKCs (p-value ≤5.5x10-6) were selected for stage-2 phase.

Stage-2 phase; replication and joint meta-analysis

The stage-2 analysis of the top SNPs identified in the discovery phase was carried out in the RS cohort. A logistic regression with an additive model to test for associations between SNPs and mKCs was implemented adjusting for age at diagnosis, sex and four PCs. The significance of the association was tested using the likelihood ratio test (LRT) with one degree of freedom. To correct for multiple testing, we calculated the pair-wise linkage disequilibrium (LD; r^2) between the top SNPs using SNAP [29] and the p-values were adjusted by dividing the nominal p-value by the number of independent tests (SNPs were considered independent with $r^2 \leq 0.6$).

We also carried out a GWAS on the RS cohort as described previously [14] and used thep-values from the LRT [25] of the three cohorts for a meta-analysis. The quality control of the GWAS summary statistics per cohort was done with EasyQC [28]. After QC there were 7,898,815 markers. The cleaned files of the RS were then meta-analyzed with the FHS and Harvard cohorts using the weighted Z-score method, implemented in METAL [26]. SNP heterogeneity was tested using I^2 and Cochran's Q methods. The top SNPs were annotated to genes using Ensembl (http://browser.1000genomes.org/index.html).

RESULTS

The age and sex distribution of cases (mKCs) and controls (sKCs) for the stage-1 (discovery) and stage-2 cohorts are presented in Table 1. The discovery cohorts consisted of 1,666 subjects with mKCs and 1,950 subjects with sKCs. The ascertainment of KC was done primarily using self-reports in the Harvard cohorts. There were differences in the proportion of males in the cohorts, since the NHS and NHS II are women's cohorts and HPFS is men's cohort. We also observed that cases were older than controls.

Table 1. Demographic characteristics of the population-based cohorts

Cohorts	KC ascertainment	Cases (mKC)	Controls (sKC)	Sex (%male cases)	Sex (%male controls)	Median age[a] cases (IQR)	Median age[a] controls (IQR)
Stage 1							
Harvard[b]	Self-report	1,531	1,189	38.3	28.5	66 (59-73)	64 (58-71)
NHS	Self-report	920	817	0	0	64 (57-70)	66 (59-72)
NHS II	Self-report	23	33	0	0	45 (40-52)	50 (46-54)
HPFS	Self-report	588	339	100	100	67 (60-73)	69 (62-73)
FHS	Pathology records	135	761	60	50	66 (58-78)	66 (54-77)
Stage 2							
RS combined	Pathology records	574	872	40	50	73 (66-81)	69 (72-77)
RS1	Pathology records	345	542	43	52	78 (72-84)	74 (68-90)
RS2	Pathology records	142	178	53	52	68 (62-72)	70 (66- 76)
RS3	Pathology records	88	152	39	36	57 (51-64)	60 (53-65)

KC: keratinocyte carcinoma; mKC: multiple KC; sKC: single KC; IQR: inter-quantile range; NHS: Nurses' Health Study; HPFS: Health Professionals Follow-up Study; RS: Rotterdam Study

[a] Median age at first diagnosis

[b] Combined; dataset from the combined NHS, NHS II and HPFS cohorts. GWAS analysis for the Harvard cohorts were performed per GWAS platforms (see Materials and Methods) not per cohort.

In the discovery stage, suggestive genome-wide associations (p-value $\leq 5.5 \times 10^{-6}$) were identified for 40 SNPs (Figure 1 and S1 Table). Due to the strong LD ($r^2 > 0.6$) among these 40 SNPs (eight SNPs tagged 32 of the top SNPs), only eight of them were considered independent signals (S1 Table). The most significant hit was an intergenic SNP on the short arm of chromosome 9 (rs7468390, p-value = 3.92×10^{-7}), with an OR (95% CI) of 0.73 (0.64-0.82) for the C allele (Table 2). This is a common SNP in strong LD with 13 other SNPs with suggestive associations (S1 Table and S1 Figure). The region of LD of rs7468390 spans approximately 13 kb. Of the 40 SNPs with

suggestive associations, 29 were intergenic, three mapped to non-coding RNA, four within regulatory regions and four to the *NCKAP5* gene (S1 Table).

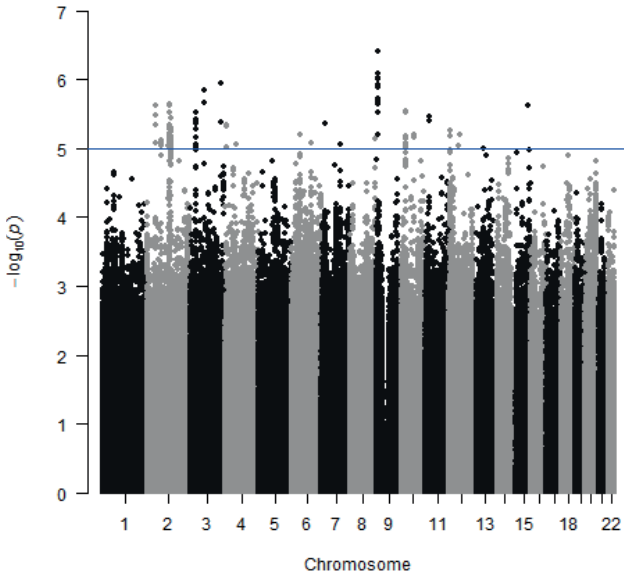

Figure 1. Manhattan plot of the GWAS associations for mKCs in the discovery sample (FHS and Harvard cohorts)

The observed -log10 p-values (Y-axis) of the association between the SNPs and susceptibility for mKC are shown. All SNP are represented by dots and displayed per chromosome (X-axis).

For the stage-2, we tested for associations between the 40 top SNPs from the stage-1 and mKC in an independent sample of 574 mKCs and 872 sKCs from the RS using an adjusted p-value of 0.006 (corresponding to a p-value of 0.05 divided by eight independent SNPs/tests). None of the SNPs replicated at this threshold (S2 Table). A combined analysis of both the stage-1 and stage-2 datasets showed suggestive associations for the 40 significant SNPs, but none reached genome-wide significance, and there was significant heterogeneity in the estimates, most likely due to the different direction of the effects in the RS [30] (Table 2 and S2 Table).

Other than the above-described 40 SNPs, we found suggestive statistical signals in the combined meta-analysis (p-values $\leq 5.5 \times 10^{-6}$, S3 Table) for other SNPs. The most significant SNP was rs4761496 that mapped to an intergenic region of chromosome 12 (p-value = 4.5×10^{-7}). Other SNPs with suggestive associations mapped to protein coding genes including *CSMD1* (rs11777268, rs116045237, rs17393453) and *PRF1* (rs35401316), both of which have an indirect involvement with SCC [31, 32].

Since up to 80% of KCs are BCCs, we also looked at whether SNPs previously associated with susceptibility for BCC conferred susceptibility to mKCs. Six of the

Table 2. Top SNPs identified in the discovery samples (Harvard cohorts and FHS) and p-values of stage 2 in the RS and joint-meta-analysis stages (Harvard cohorts, FHS and RS all combined)

			Stage1 (discovery phase)			Stage 2 (replication)		Combined analysis		
SNP id	**A1[a]/A2[b]**	**Freq[c]**	**OR[d] (95% CI)**	**P-value**	**Direction[e]**	**Z-score[f]**	**P-value**	**Z-score[g]**	**P-value**	**Direction[h]**
rs7468390	C/G	0.64	0.73 (0.64-0.82)	3.92×10^{-7}	--	1.459	0.145	-3.300	9.69×10^{-4}	--+
3:171255288:ID	D/I	0.98	3.10 (1.97-4.88)	1.11×10^{-6}	++	-0.898	0.369	3.551	3.84×10^{-4}	**++-**
rs58848026	T/C	0.85	0.71 (0.62-0.82)	2.43×10^{-6}	--	0.629	0.529	-3.664	2.48×10^{-4}	**--+**
rs4749296	T/G	0.36	0.78 (0.70-0.87)	2.83×10^{-6}	--	-0.947	0.344	-4.358	1.31×10^{-5}	---
rs6803721	T/C	0.34	0.77 (0.69-0.86)	3.03×10^{-6}	--	1.538	0.124	-2.964	3.04×10^{-3}	--+
rs4923076	A/T	0.52	1.28 (1.15-1.41)	3.46×10^{-6}	++	-0.431	0.666	3.602	3.15×10^{-4}	++-
rs10167336	T/C	0.51	1.27 (1.15-1.41)	3.70×10^{-6}	++	0.286	0.775	3.894	9.88×10^{-5}	+++
rs7799651	A/A	0.45	0.73 (0.64-0.83)	4.37×10^{-6}	--	2.043	0.041	-2.553	0.011	--+

SNP: single nucleotide polymorphism; FHS: Framingham Heart Study; RS: Rotterdam Study; Freq: frequency; OR: odds ratio; CI: confidence interval

[a] A1: reference allele

[b] A2: other allele

[c] Frequency of A1

[d] ORs of A1. ORs and 95% CIs were calculated from the weighted average of the effect size (regression coefficients and standard error) from the inverse-variance meta-analysis

[e] Direction of the effect of A1 with +/- indicating a higher/lower disease risk for Harvard and FHS cohorts, respectively

[f] Z-scores from the replication

[g] Z-scores from the meta-analysis

[h] Direction of the effect of the A1 with +/- indicating a higher/lower disease risk for Harvard cohorts, FHS and RS, respectively.

19 SNPs tested were significantly associated with susceptibility for mKCs (Table 3). However, only rs1805007, which that mapped to *MC1R* was significant (p-value = $2.8x10^{-4}$) after Bonferroni correction (adjusted p-value ≤0.0026, 0.05/19). This gene is a well-known susceptibility locus for melanoma and KC. Other candidate loci identified in a recent GWAS for mBCC [14] were investigated but none were significantly associated with mKCs after Bonferroni correction (data not shown).

Table 3. Association analysis of BCC-loci and mKC susceptibility from the combined analyses (Harvard cohorts, FHS and RS)

SNP id	Gene	Alleles[a]	Freq[b]	Z-score[c]	P-value	Direction[d]	I^{2e}	ChiSq[f]
rs1126809	*TYR*	A/G	0.270	2.523	0.012	+++	0	1.97 (0.37)
rs4911414	20q11.22	T/G	0.345	0.892	0.373	+-+	0	1.65 (0.44)
rs1015362	20q11.22	T/C	0.276	-0.652	0.514	+--	52.9	4.25 (0.12)
rs7538876	*PADI6*	A/G	0.382	-0.049	0.961	-++	57.2	4.67 (0.10)
rs801114	1q42.13	T/G	0.65	0.109	0.913	++-	0	1.55 (0.46)
rs11170164	*KRT5*	T/C	0.081	0.639	0.523	-++	0	1.40 (0.50)
rs2151280	*CDKN2B-AS1*	A/G	0.470	-1.94	0.052	---	0	1.02 (0.60)
rs157935	*LINC-PINT*	T/G	0.704	0.991	0.322	+++	0	0.10 (0.95)
rs16891982	*SLC45A2*	C/G	0.051	-1.427	0.154	---	0	0.79 (0.67)
rs401681	*CLPTM1L*	T/C	0.427	-2.748	$6.00x10^{-3}$	---	53.6	4.31 (0.12)
rs12210050	*EXOC2*	T/C	0.167	2.04	0.041	+++	0	1.20 (0.55)
rs7335046	*UBAC2*	C/G	0.871	1.569	0.117	+++	0	0.45 (0.80)
rs1805007	MC1R	T/C	0.083	3.633	**$2.80x10^{-4}$**	**+++**	0	1.63 (0.44)
rs78378222	*TP53*	T/G	0.985	-1.689	0.091	-??	0	0.00 (1.00)
rs12203592	*IRF4*	T/C	0.171	2.37	0.018	+?+	0	0.19 (0.67)
rs12202284	*EXOC2*	A/C	0.214	2.224	0.026	+?+	0	0.00 (0.97)
rs8015138	*GNG2*	A/C	0.486	-1.3	0.194	-++	69.7	6.61 (0.04)
rs214782	*TGM3*	A/G	0.815	-1.953	0.051	---	0	0.81 (0.67)
rs7006527	*RGS22*	A/C	0.851	0.735	0.462	+++	0	0.08 (0.96)

BCC: basal cell carcinoma; mKC: multiple keratinocyte carcinoma; FHS: Framingham Heart Study; RS: Rotterdam Study; SNP: single nucleotide polymorphism; Freq: frequency; ChiSq: chi-squared

[a] Alleles for the SNP (left reference, right other)

[b] Frequency of the first allele selected by the METAL software as the reference allele

[c] Z-scores from the meta-analysis

[d] Direction of the effect of the first allele, with +/- indicating a higher/lower disease risk for Harvard, FHS and RS cohorts, respectively

[e] I^2 statistic of the amount of heterogeneity

[f] Cochran's Q-test statistics for heterogeneity with degrees of freedom equal to number of studies -1

Significant p-value after Bonferroni correction (adjusted p-value≤ 0.0026) is highlighted in bold

DISCUSSION

In this two-stage GWAS of mKCs, we did not identify genome-wide significant associations between SNPs and mKCs. Several SNPs with suggestive associations mapped to genes involved in cancer pathology, but the findings need to be confirmed in larger samples. A candidate SNP-based analysis of previous BCC/SCC variants showed significant associations between mKCs and only one SNP (rs1805007) at *MC1R*, known to be associated with BCC, SCC and melanoma was significant. This suggests that genetic susceptibility for mKCs may partly overlap with that for BCC, which is expected given that up to 80% of KC are BCCs.

The lack of replication of the suggestive associations in might be due to several factors. First, phenotypic heterogeneity due to a differential ascertainment of KCs (pathology-confirmed versus self-reports) in the cohorts could have led to some phenotypic heterogeneity, although common variants for KCs have been replicated in the NHS, NHS II and HPFS cohorts [13] as well as in RS [14]. In addition, the ratio BCC/SCC may be different for the American and the European populations. In the RS, BCCs accounted for 82% of all mKCs. For the USA cohorts, BCC/SCC ratios were not available, but a higher proportion of SCCs after prior KCs in the USA were shown previously [4]. In addition, this study was underpowered to detect variants with small to moderate effects (S2 Figure). Indeed, in the joint meta-analysis it was shown that the eight variants with the most significant associations in the discovery samples had the opposite direction in the RS, which led to significant heterogeneity (Table 2). This may have caused a drop in the significance of the associations in the meta-analysis [30]. Interestingly, we found other SNPs hits in the joint analysis that had the same direction in the three cohorts, although the sample size was not large enough to reach genome-wide significance (lowest p-value was $4.5x10^{-7}$). Most likely, the lack of replication is a combination of both phenotype heterogeneity and low power to detect variants with moderate to low effects in the RS. Last but not least, one may argue that due to differences in the imputation quality thresholds between the Harvard cohorts and the RS and FHS, we may have missed GWAS hits. However, we did not expect a dramatic drop in power due to this reason because the Harvard cohorts, where a very stringent threshold was used to include SNPs for final meta-analysis (Rsq ≥0.95) provided most of the markers (9,001,799 SNPS).

In the candidate SNP analysis nested within the GWAS, we found that*MC1R*, a gene previously associated with BCC was also associated with an increased risk for mKCs. This contrasts with a recent study from the RS where no association between known BCC-SNPs and susceptibility for mBCC was found [14]. Since the BCC cases were included in the replication dataset of this study, this shows that the previous findings were most likely due to a lack of power of the RS. Although only one of the 19 BCC-

related SNPs was significant after Bonferoni correction, we found nominal associations for six of the previously identified BCC SNPs, suggesting that larger sample sizes will be necessary to validate these associations.

As shown previously [33], most variants identified through GWAS are expected to have low to moderate risks effects and therefore large consortia of participants with phenotype and GWA-SNP data are needed. While this is feasible for traits such weight or blood pressure for disease-related phenotypes this can be challenging. As mentioned above, all previous GWAS studies of BCC or non-melanoma skin cancer published so far did not separate cases with mKCs from those with sKC, and thus our series of mKCs cases could be considered as a rare phenotype. With our findings one may argue that there are no common variants with strong effects contributing to the genetic susceptibility for mKCs, although we only tested eight million common SNPs (frequencies higher than 2%). Whether the differential risk between patients with mKC and sKCs is due to genes or mostly due to environmental factors, or an interaction of genes and environmental factors remains to be elucidated. We did not test for SNP and environmental interactions that may be relevant in explaining susceptibility to mKCs, because we did not have all environmental risk factors assessed in all cohorts and the sample size was already small to detect SNP main effects. Heritability studies could help to determine to what extent genetic risk factors explain susceptibility for mKC. We found an heritability of 8% using GWAS data from the RS(data not shown), but the power was low to have a significant estimate. Determining the heritability for mKC as well as to identify individual susceptibility loci will require larger consortia of well characterized cases and controls. In addition, rare variants were not evaluated. Although such variants may not be clinically relevant to predict disease risk, they may reveal new pathways predisposing to mKCs and new targets for drug discovery, as in the example of vismodegib, a drug used to treat patients with NBCCS and sporadic, metastatic BCC [34].

Conclusion

We found suggestive associations of common variants that were not replicated. To identify new loci and to confirm the suggestive associations found in this study, larger mKCs cohorts will be required.

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SUPPLEMENTARY MATERIAL

Materials and Methods

Quality control of the NHSI-II-HPFS GWAS

SNPs with deviations from Hardy-Weinberg equilibrium (HWE, $p<10^{-07}$), call rates <95%, or MAF <1% were excluded. Samples with genotype call rates ≤95%, gender mismatch, non-European ancestry or outliers from the population sample were removed.

Quality control of the FHS GWAS

Quality control was performed by excluding samples with high heterozygosity (mean±3×s.d.), gender mismatch or sample call rates of <95%. SNPs with the following criteria were included for the GWAS: Hardy-Weinberg equilibrium test with a p-value $>10^{-06}$, MAF ≥1% and SNP call rate of ≥98%.

Quality control of RS GWAS

The quality control included the removal of SNPs with Hardy-Weinberg equilibrium deviations ($p<5x10^{-06}$), genotyping call rate <97%, gender mismatch and a high heterozygosity. Duplicates or first-degree relatives using identity-by-descent (IBD) estimates and outliers (three standard deviations away from the population mean) using multi-dimensional scaling (MDS) analysis with four principal components (PCs) were excluded.

Supplementary table 1. Summary statistics of the most significant statistical associations between SNPs and mKCs in the discovery sample

Genomic regions[a]	MarkerName	RS_ID	Alleles	Freq[b]	Effect	StdErr	Odds ratio (CI)[c]	P-value	Dir[d]	I^{2e}	Q Statistic[f]	Associated genes
2:129763945-129770253	2:129763945	rs11689142	a/g	0.85	-0.3272	0.0715	0.72 (0.63-0.83)	4.68E-06	--	0	0.13 (0.72)	intergenic_variant
2:129763945-129770253	2:129764366	rs11690587	t/c	0.85	-0.3269	0.0715	0.72 (0.63-0.83)	4.77E-06	--	0	0.13 (0.72)	intergenic_variant
2:129763945-129770253	2:129764693	rs13407770	t/c	0.15	0.3266	0.0715	1.39 (1.20-1.59)	4.86E-06	++	0	0.13 (0.72)	intergenic_variant
2:129763945-129770253	2:129765122	rs748597	t/c	0.85	-0.3261	0.0714	0.72 (0.63-0.83)	4.90E-06	--	0	0.13 (0.72)	intergenic_variant
2:129763945-129770253	2:129765144	rs748598	t/c	0.15	0.326	0.0714	1.39 (1.20-1.59)	4.92E-06	++	0	0.13 (0.72)	intergenic_variant
2:129763945-129770253	2:129766797	rs10170738	a/g	0.16	0.3265	0.0716	1.39 (1.20-1.59)	5.19E-06	++	0	0.12 (0.73)	intergenic_variant
2:129763945-129770253	2:129768845	rs72851082	a/g	0.15	0.3392	0.0719	1.40 (1.22-1.62)	2.35E-06	++	0	0.22 (0.64)	intergenic_variant
2:129763945-129770253	2:129768875	rs78282480	a/g	0.85	-0.3393	0.0719	0.71 (0.62-0.82)	2.33E-06	--	0	0.22 (0.64)	intergenic_variant
2:129763945-129770253	2:129769495:ID	2:129769495:ID	d/i	0.85	-0.3368	0.0721	0.71 (0.62-0.82)	3.03E-06	--	0	0.25 (0.62)	intergenic_variant
2:129763945-129770253	**2:129770253**	**rs58848026**	**t/c**	**0.85**	**-0.339**	**0.0719**	**0.71 (0.62-0.82)**	**2.43E-06**	**--**	**0**	**0.23 (0.63)**	**intergenic_variant**
2:133963827-133972362	2:133963827	rs11695854	a/g	0.50	-0.2394	0.0525	0.79 (0.71-0.87)	5.03E-06	--	34.4	1.53 (0.22)	*NCKAP5* (intron)
2:133963827-133972362	2:133972362	rs956560	t/c	0.50	0.2381	0.0523	1.27 (1.15-1.41)	5.40E-06	++	38.2	1.62 (0.20)	*NCKAP5* (intron)
2:133963827-133972362	2:133973007	rs6706037	a/t	0.50	0.2368	0.0521	1.27 (1.14-1.40)	5.48E-06	++	40.1	1.67 (0.20)	*NCKAP5* (intron)
2:133963827-133972362	**2:133973773**	**rs10167336**	**t/c**	**0.51**	**0.2415**	**0.0522**	**1.27 (1.15-1.41)**	**3.70E-06**	**++**	**52.8**	**2.12 (0.15)**	***NCKAP5* (intron)**
3:35190794-35190794	**3:35190794**	**rs6803721**	**t/c**	**0.34**	**-0.2602**	**0.0557**	**0.77 (0.69-0.86)**	**3.03E-06**	**--**	**0**	**0.56 (0.46)**	**intergenic_variant**
3:35190794-35190794	3:35192891	rs7619909	a/g	0.34	-0.2554	0.0561	0.77 (0.69-0.86)	5.35E-06	--	0	0.59 (0.44)	intergenic_variant
3:35190794-35190794	3:35195059	rs6786729	t/c	0.66	0.2585	0.0559	1.29 (1.16-1.44)	3.81E-06	++	0	0.54 (0.46)	intergenic_variant
3:35190794-35190794	3:35197212	rs6808770	t/c	0.34	-0.2572	0.056	0.77 (0.69-0.86)	4.32E-06	--	0	0.79 (0.38)	intergenic_variant
3:35190794-35190794	3:35200632	rs4678501	a/g	0.33	-0.2601	0.0563	0.77 (0.69-0.86)	3.92E-06	--	0	0.51 (0.48)	intergenic_variant
3:171255288	**3:171255288:ID**	**3:171255288:ID**	**d/i**	**0.98**	**1.1308**	**0.2322**	**3.10 (1.97-4.88)**	**1.11E-06**	**++**	**0**	**0.06 (0.81)**	**intergenic_variant**
7:19988731	**7:19988731**	**rs7799651**	**a/g**	**0.45**	**-0.3146**	**0.0685**	**0.73 (0.64-0.83)**	**4.37E-06**	**--**	**60.5**	**2.53 (0.11)**	***LOC101927668 (intron)***
9:13517915-13530711	**9:13517965**	**rs7468390**	**c/g**	**0.64**	**-0.3184**	**0.0628**	**0.73 (0.64-0.82)**	**3.92E-07**	**--**	**0**	**0.61 (0.44)**	**intergenic_variant**
9:13517915-13530711	9:13518209	rs12000970	t/c	0.69	-0.3267	0.0686	0.72 (0.63-0.83)	1.95E-06	--	0	0.77 (0.38)	intergenic_variant
9:13517915-13530711	9:13520083	rs1543712	a/g	0.40	0.2774	0.0587	1.32 (1.18-1.48)	2.32E-06	++	0	0.93 (0.33)	intergenic_variant
9:13517915-13530711	9:13520190	rs1543713	a/g	0.40	0.278	0.0586	1.32 (1.18-1.48)	2.14E-06	++	0	0.92 (0.34)	intergenic_variant

Supplementary table 1. Summary statistics of the most significant statistical associations between SNPs and mKCs in the discovery sample (*continued*)

Genomic regions[a]	MarkerName	RS_ID	Alleles	Freq[b]	Effect	StdErr	Odds ratio (CI)[c]	P-value	Dir[d]	I^{2e}	Q Statistic[f]	Associated genes
9:13517915-13530711	9:13520224	rs1543714	a/g	0.25	0.3324	0.0711	1.39 (1.21-1.60)	2.98E-06	++	0	0.24 (0.62)	ENSR00001469254**
9:13517915-13530711	9:13520569	rs10961102	c/g	0.41	0.2766	0.058	1.32 (1.18-1.48)	1.86E-06	++	0	0.68 (0.41)	intergenic_variant
9:13517915-13530711	9:13520598	rs10961103	t/c	0.41	0.2757	0.0579	1.32 (1.18-1.48)	1.95E-06	++	0	0.67 (0.41)	intergenic_variant
9:13517915-13530711	9:13522809	rs4741308	a/c	0.58	-0.2839	0.0587	0.75 (0.67-0.84)	1.31E-06	--	0	0.37 (0.54)	intergenic_variant
9:13517915-13530711	9:13523961	rs7864569	a/t	0.58	-0.2867	0.059	0.75 (0.67-0.84)	1.20E-06	--	0	0.51 (0.47)	intergenic_variant
9:13517915-13530711	9:13528855	rs2382398	a/g	0.58	-0.2826	0.0574	0.75 (0.67-0.84)	8.42E-07	--	0	0.42 (0.52)	ENSR00001300196
9:13517915-13530711	9:13528905	rs1333988	a/g	0.58	-0.2826	0.0574	0.75 (0.67-0.84)	8.42E-07	--	0	0.41 (0.52)	intergenic_variant
9:13517915-13530711	9:13530105	rs7870726	t/c	0.41	0.2687	0.0553	1.31 (1.17-1.46)	1.17E-06	++	0	0.46 (0.50)	intergenic_variant
9:13517915-13530711	9:13530295	rs933035	t/g	0.56	-0.2789	0.0569	0.76 (0.68-0.85)	9.66E-07	--	0	0.42 (0.52)	intergenic_variant
9:13517915-13530711	9:13530711	rs933034	a/g	0.41	0.2701	0.0552	1.31 (1.18-1.46)	9.97E-07	++	0	0.45 (0.50)	intergenic_variant
10:28296558-28300409	10:28296558	rs7076786	a/g	0.65	0.2532	0.0542	1.29 (1.16-1.43)	2.98E-06	++	15.9	1.19 (0.28)	RP11-218D6.4
10:28296558-28300409	**10:28300409**	**rs4749296**	**t/g**	**0.36**	**-0.2481**	**0.053**	0.78 (0.70-0.87)	**2.83E-06**	**--**	**1.6**	**1.02 (0.31)**	**RP11-218D6.4**
11:23246771-23248177	**11:23246771**	**rs4923076**	**a/t**	**0.52**	**0.2437**	**0.0525**	**1.28 (1.15-1.41)**	**3.46E-06**	**++**	**0**	**0.32 (0.57)**	**intergenic_variant**
11:23246771-23248177	11:23248157	rs1351640	t/c	0.52	0.2423	0.0526	1.27 (1.15-1.41)	4.03E-06	++	0	0.27 (0.60)	ENSR00001052093
11:23246771-23248177	11:23248177	rs1351639	a/t	0.48	-0.2425	0.0526	0.78 (0.71-0.87)	3.95E-06	--	0	0.28 (0.60)	ENSR00001052093

[a] Genomic regions of SNPs in strong LD with the most significant SNP (bold)

[b] Frequency of the first allele used by METAL as reference allele

[c] Odds ratio and confidence intervals were calculated using the weighted regression coefficients of the meta-analysis using the inverse-variance approach

[d] Direction of the effect (first) allele with +/- indicating a higher/lower disease risk for Harvard and FHS cohorts, respectively

[e] I^2 statistics to test for heterogeneity

[f] Cochran's Q test statistics for heterogeneity with degrees of freedom equal to number of studies -1 (p-value in brackets)

** regulatory variant

Supplementary table 2. Summary statistics of the most significant statistical associations from the discovery phase in the replication and joint meta-analysis phase

	Discovery datasets (Harvard cohorts, FHS)					Replication dataset (Rotterdam Study)						Joint meta-analysis of Harvard cohorts, FHS and RS						
SNP name	**Freq[a]**	**Effect**	**StdErr**	**Dir[b]**	**Pvalue**	**Freq[a]**	**Weight**	**Z-score[c]**	**P-value**	**I[2d]**	**HetChiSq[e]**	**Freq[a]**	**Weight**	**Z-score[c]**	**P-value**	**Dir[f]**	**I[2d]**	**HetChiSq[e]**
rs11689142	0.85	-0.33	0.07	--	5E-06	0.85	1447	0.666	0.505	0	0.20 (0.91)	0.85	5063	-3.523	4.26E-04	--+	78.1	9.12 (0.01)
rs11690587	0.85	-0.33	0.07	--	5E-06	0.85	1447	0.666	0.506	0	0.20 (0.91)	0.85	5063	-3.521	4.30E-04	--+	78	9.10 (0.01)
rs13407770	0.15	0.33	0.07	++	5E-06	0.15	1447	-0.667	0.505	0	0.20 (0.90)	0.15	5063	3.519	4.33E-04	++-	78	9.10 (0.01)
rs748597	0.85	-0.33	0.07	--	5E-06	0.85	1447	0.667	0.505	0	0.20 (0.90)	0.85	5063	-3.515	4.39E-04	--+	78	9.10 (0.01)
rs748598	0.15	0.33	0.07	++	5E-06	0.15	1447	-0.667	0.505	0	0.20 (0.90)	0.15	5063	3.515	4.40E-04	++-	78	9.09 (0.01)
rs10170738	0.16	0.33	0.07	++	5E-06	0.16	1447	-0.755	0.450	0	0.22 (0.90)	0.16	5063	3.459	5.43E-04	++-	79	9.51 (0.01)
rs72851082	0.15	0.34	0.07	++	2E-06	0.15	1447	-0.637	0.524	0	0.20 (0.91)	0.15	5063	3.668	2.45E-04	++-	78.9	9.47 (0.01)
rs78282480	0.85	-0.34	0.07	--	2E-06	0.85	1447	0.637	0.524	0	0.20 (0.91)	0.85	5063	-3.669	2.43E-04	--+	78.9	9.48 (0.01)
2:129769495:ID	0.85	-0.34	0.07	--	3E-06	0.84	1447	0.639	0.523	0	0.20 (0.91)	0.85	5063	-3.624	2.91E-04	--+	78.6	9.33 (0.01)
rs58848026	**0.85**	**-0.34**	**0.07**	**--**	**2E-06**	**0.85**	**1447**	**0.629**	**0.529**	**0**	**0.20 (0.91)**	**0.85**	**5063**	**-3.664**	**2.48E-04**	**--+**	**78.7**	**9.41 (0.01)**
rs11695854	0.50	-0.24	0.05	--	5E-06	0.48	1447	-0.083	0.934	0	1.71 (0.43)	0.50	5063	-3.707	2.09E-04	---	76.8	8.62 (0.01)
rs956560	0.50	0.24	0.05	++	5E-06	0.52	1447	0.136	0.892	0	1.68 (0.43)	0.50	5063	3.746	1.79E-04	+++	75.9	8.30 (0.02)
rs6706037	0.50	0.24	0.05	++	5E-06	0.52	1447	0.115	0.909	0	1.78 (0.41)	0.50	5063	3.742	1.83E-04	+++	76.1	8.37 (0.02)
rs10167336	**0.51**	**0.24**	**0.05**	**++**	**4E-06**	**0.53**	**1447**	**0.286**	**0.775**	**0**	**0.89 (0.64)**	**0.51**	**5063**	**3.894**	**9.88E-05**	**+++**	**76.3**	**8.45 (0.01)**
rs6803721	**0.34**	**-0.26**	**0.06**	**--**	**3E-06**	**0.36**	**1447**	**1.538**	**0.124**	**54**	**4.35 (0.11)**	**0.34**	**5063**	**-2.964**	**3.04E-03**	**--+**	**87.3**	**15.78 (0.00)**
rs7619909	0.34	-0.26	0.06	--	5E-06	0.35	1447	1.515	0.130	53.7	4.32 (0.12)	0.34	5063	-2.877	4.01E-03	--+	86.9	15.23 (0.00)
rs6786729	0.66	0.26	0.06	++	4E-06	0.64	1447	-1.498	0.134	51.4	4.11 (0.13)	0.66	5063	2.948	3.20E-03	++-	87	15.34 (0.00)
rs6808770	0.34	-0.26	0.06	--	4E-06	0.36	1447	1.54	0.124	55	4.44 (0.11)	0.34	5063	-2.886	3.90E-03	--+	87.3	15.80 (0.00)
rs4678501	0.33	-0.26	0.06	--	4E-06	0.35	1447	1.67	0.095	51.7	4.14 (0.13)	0.34	5063	-2.854	4.31E-03	--+	87.8	16.34 (0.00)
3:171255288:ID	**0.98**	**1.13**	**0.23**	**++**	**1E-06**	**0.98**	**887**	**-0.898**	**0.369**	**0**	**0.00 (1.00)**	**0.98**	**5063**	**3.551**	**3.84E-04**	**++-**	**83.3**	**11.99 (0.00)**
rs7799651	**0.45**	**-0.31**	**0.07**	**--**	**4E-06**	**0.44**	**1447**	**2.043**	**0.041**	**0**	**0.25 (0.88)**	**0.45**	**5062**	**-2.553**	**0.011**	**--+**	**90.6**	**21.26 (0.02)**

Supplementary table 2. Summary statistics of the most significant statistical associations from the discovery phase in the replication and joint meta-analysis phase (*continued*)

	Discovery datasets (Harvard cohorts, FHS)					Replication dataset (Rotterdam Study)						Joint meta-analysis of Harvard cohorts, FHS and RS						
SNP name	**Freq[a]**	**Effect**	**StdErr**	**Dir[b]**	**Pvalue**	**Freq[a]**	**Weight**	**Z-score[c]**	**P-value**	**I[2d]**	**HetChiSq[e]**	**Freq[a]**	**Weight**	**Z-score[c]**	**P-value**	**Dir[f]**	**I[2d]**	**HetChiSq[e]**
rs7468390	**0.64**	**-0.32**	**0.06**	**--**	**4E-07**	**0.64**	**1447**	**1.459**	**0.145**	**0**	**0.99 (0.61)**	**0.64**	**5063**	**-3.3**	**9.69E-04**	**--+**	**88.7**	**17.64 (0.00)**
rs12000970	0.69	-0.33	0.07	--	2E-06	0.69	1447	1.942	**0.052**	0	0.15 (0.93)	0.69	5063	-2.753	5.90E-03	--+	89.8	19.58 (0.06)
rs1543712	0.40	0.28	0.06	++	2E-06	0.39	1447	-1.024	0.306	0	0.70 (0.70)	0.40	5063	3.189	1.43E-03	++-	85.8	14.13 (0.00)
rs1543713	0.40	0.28	0.06	++	2E-06	0.39	1447	-1.06	0.289	0	0.79 (0.67)	0.40	5063	3.189	1.43E-03	++-	86.1	14.42 (0.00)
rs1543714	0.25	0.33	0.07	++	3E-06	0.25	1447	-1.632	0.103	0	0.08 (0.96)	0.25	5063	2.901	3.72E-03	++-	87.7	16.28 (0.00)
rs10961102	0.41	0.28	0.06	++	2E-06	0.40	1447	-1.137	0.256	0	0.73 (0.69)	0.41	5063	3.184	1.45E-03	++-	86.3	14.60 (0.00)
rs10961103	0.41	0.28	0.06	++	2E-06	0.40	1447	-1.139	0.255	0	0.84 (0.66)	0.41	5063	3.178	1.48E-03	++-	86.3	14.55 (0.00)
rs4741308	0.58	-0.28	0.06	--	1E-06	0.58	1447	1.019	0.308	0	0.73 (0.69)	0.58	5063	-3.340	8.37E-04	--+	85.4	13.71 (0.00)
rs7864569	0.58	-0.29	0.06	--	1E-06	0.59	1447	0.797	0.426	0	0.70 (0.71)	0.58	5063	-3.442	5.78E-04	--+	84.5	12.89 (0.00)
rs2382398	0.58	-0.28	0.06	--	8E-07	0.58	1447	0.688	0.492	0	0.67 (0.72)	0.58	5063	-3.570	3.57E-04	--+	83.9	12.44 (0.00)
rs1333988	0.58	-0.28	0.06	--	8E-07	0.58	1447	0.687	0.492	0	0.67 (0.72)	0.58	5063	-3.571	3.55E-04	--+	83.9	12.44 (0.00)
rs7870726	0.41	0.27	0.06	++	1E-06	0.40	1447	-0.535	0.593	0	0.89 (0.64)	0.41	5063	3.578	3.46E-04	++-	82.8	11.61 (0.00)
rs933035	0.56	-0.28	0.06	--	1E-06	0.57	1447	0.757	0.449	0	0.46 (0.79)	0.56	5063	-3.506	4.54E-04	--+	84.3	12.75 (0.00)
rs933034	0.41	0.27	0.06	++	1E-06	0.40	1447	-0.537	0.591	0	0.89 (0.64)	0.41	5063	3.600	3.18E-04	++-	82.9	11.70 (0.00)
rs7076786	0.65	0.25	0.05	++	3E-06	0.63	1447	1.012	0.312	0	1.57 (0.46)	0.65	5063	4.349	1.37E-05	+++	60.4	5.05 (0.08)
rs4749296	**0.36**	**-0.25**	**0.05**	**--**	**3E-06**	**0.38**	**1447**	**-0.947**	**0.344**	**0**	**1.95 (0.38)**	**0.36**	**5063**	**-4.358**	**1.31E-05**	**---**	**58.1**	**4.77 (0.09)**
rs4923076	**0.52**	**0.24**	**0.05**	**++**	**3E-06**	**0.52**	**1447**	**-0.431**	**0.666**	**0**	**0.86 (0.65)**	**0.52**	**5063**	**3.602**	**3.15E-04**	**++-**	**78.1**	**9.11 (0.01)**
rs1351640	0.52	0.24	0.05	++	4E-06	0.52	1447	-0.391	0.696	0	0.86 (0.65)	0.52	5063	3.600	3.18E-04	++-	77.1	8.73 (0.01)
rs1351639	0.48	-0.24	0.05	--	4E-06	0.48	1447	0.39	0.697	0	0.87 (0.65)	0.48	5063	-3.603	3.15E-04	--+	77.2	8.75 (0.01)

[a] Frequency of the first allele used by METAL as reference allele

[b] Direction of the effect (first) allele with +/- indicating a higher/lower disease risk for Harvard and FHS cohorts, respectively

[c] Z-scores from the meta-analysis (see results)

[d] I^2 Statistics to test for heterogeneity

[e] Cochran's Q test statistics for heterogeneity with degrees of freedom equal to number of studies -1 (p-value in brackets)

[f] Direction of the effect of the first allele, with +/- indicating a higher/lower disease risk for Harvard, FHS, RS cohorts, respectively

Supplementary table 3. Summary statistics of the most significant statistical associations for other SNPs and mKCs in the joint meta-analysis phase

RS_id	Alleles[a]	Z-score[b]	P-value	Direction[c]	I^{2d}	HetChiSq[e]	Associated genes
rs4761496	t/c	-5.046	**4.51E-07**	---	39.7	3.32 (0.19)	intergenic_variant
rs75790006	t/g	4.836	**1.33E-06**	+++	0	0.50 (0.78)	*RP11-395F4.1 (intron)*
3:80136427	a/g	-4.825	1.40E-06	?-?	0	0.00 (1.00)	intergenic_variant
15:89432448	a/g	4.751	2.02E-06	++?	0	0.65 (0.42)	*HAPLN3 (intron)*
3:79848880	a/c	-4.739	2.15E-06	?-?	0	0.00 (1.00)	intergenic_variant
rs17393453	a/t	-4.734	**2.20E-06**	---	0	1.60 (0.45)	*CSMD1 (intron)*
rs59112743	a/g	-4.699	**2.61E-06**	---	0	0.12 (0.94)	intergenic_variant
rs2040609	t/c	4.678	**2.89E-06**	+++	0	1.62 (0.45)	intergenic_variant
rs80069861	a/g	4.664	**3.10E-06**	+++	13.6	2.31 (0.31)	intergenic_variant
rs35401316	t/c	-4.646	**3.38E-06**	---	13	2.30 (0.32)	*PRF1 (upstream gene)*
rs11867566	a/g	4.643	**3.43E-06**	+++	0	1.62 (0.44)	intergenic_variant
2:49725708	t/c	-4.617	3.90E-06	--?	52.7	2.12 (0.15)	intergenic_variant
rs9381952	a/g	-4.591	**4.41E-06**	---	0	0.12 (0.94)	intergenic_variant
rs10948606	t/c	-4.589	**4.45E-06**	---	0	0.07 (0.97)	intergenic_variant
4:6338331:ID	d/i	4.585	4.53E-06	+??	0	0.00 (1.00)	*PPP2R2C*
rs116045237	a/t	-4.578	**4.69E-06**	---	0	1.83 (0.40)	*CSMD1 (intron)*
17:59712019:D	d/i	-4.577	**4.71E-06**	---	0	1.35 (0.51)	intergenic_variant
17:59712018:D	d/i	-4.577	**4.72E-06**	---	0	1.34 (0.51)	intergenic_variant
4:6335966	t/c	-4.571	4.86E-06	-??	0	0.00 (1.00)	*PPP2R2C*
2:49762165	a/g	-4.568	4.92E-06	--?	41.5	1.71 (0.19)	intergenic_variant
6:51126577:D	d/i	-4.565	**5.01E-06**	---	0	0.05 (0.98)	intetgenic
rs11777268	t/c	4.555	**5.24E-06**	+++	0	1.71 (0.43)	*CSMD1 (intron)*
rs9370019	a/g	4.546	**5.47E-06**	+++	0	0.08 (0.96)	intergenic_variant

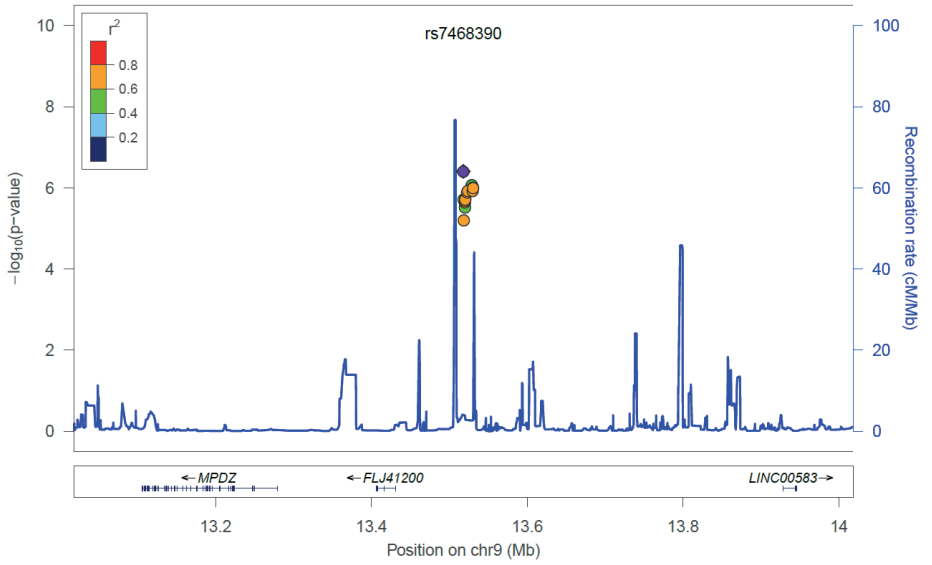
[a] Frequency of the first allele used by METAL as reference allele

[b] Z-scores from the meta-analysis (see results)

[c] Direction of the effect (first) allele with +/-/? indicating a higher/lower/allele not present disease risk for Harvard cohorts, FHS and RS, respectively.

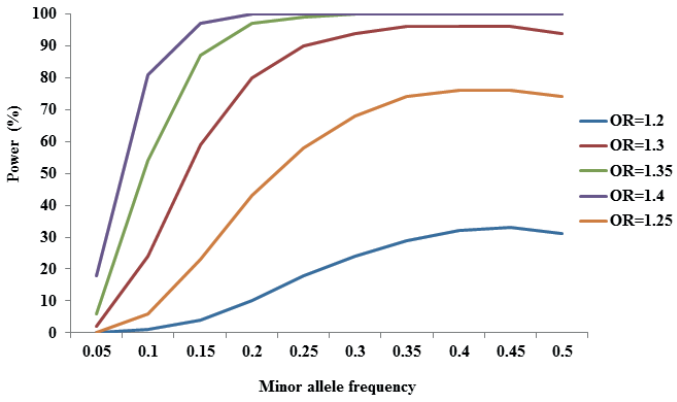
[d] I^2 Statistics to test for heterogeneity

[e] Cochran's Q test statistics for heterogeneity with degrees of freedom equal to number of studies -1 (p-value in brackets)



Supplementary Figure 1. Regional plot of the most significant associations between SNPs in the short arm of chromosome 9 and mKCs

The plots represents the LD patterns of the most significant SNP in the study (rs7468390) and nearby SNPs from this study (+/- 500 kb). Pairwise r^2 is represented in colours. The log p-values of the associations of the rs7468390 SNP and markers from the study is presented in the left Y-axis and the recombination rates is presented in the right Y-axis. The physical position of the markers is presented in Mb. The figure was generated using LocusZoom[35]



Supplementary Figure 2. Power calculation of the study design

The power of the study was calculated using the program CaTS[36] with sample size, p-value ($1x10^{-6}$) and a disease prevalence of 10% as fixed parameters. An 80% power was expected for markers with MAF>25% and Odd ratios of >1.3.

References

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Chapter 10

General discussion

The research presented in this thesis provides insight into the epidemiology of basal cell carcinoma (BCC), in particular into the epidemiology of patients with metachronous BCC (mBCC). An update of the knowledge on the occurrence of mBCC is warranted as the burden of BCC is still increasing (**chapter 2**). In this chapter I will first shortly answer the five research questions posed in the introduction of this thesis. Then I will show the limitations of the included studies. Finally, I will discuss potential implications and future perspectives of my research.

RESEARCH QUESTIONS

1. What is already known about the epidemiology of BCC and where are the gaps?
These questions were answered in **chapter 2** and **3** in a broad non-systemic review of the literature on BCC. Numerous studies on BCC incidence in white-skinned individuals worldwide all point out the high and still increasing incidence. In addition, approximately one third of all individuals with a first BCC will develop at least a second BCC and are at risk of other ultraviolet radiation (UVR) related skin cancers,[1] which is in line with the concept of field cancerization.[2] Nevertheless, the majority of BCC research has been done in patients with one BCC, or without differentiating between single or mBCC or different keratinocyte carcinomas (KC, i.e., BCC and squamous cell carcinoma (SCC)), which is the reason little is known about the true burden of disease (i.e., disability-adjusted life year and health care costs) and both the non-genetic and genetic risk factors (i.e., predictors) of patient with mBCC. The latter makes targeted secondary prevention and tailored follow-up difficult, and screening programs less likely to be cost effective, since it is not known who the high-risk patients exactly are. Thus there is a need for skin cancer consortia and large prospective population-based cohort studies with a long follow-up up in which prediction models for mBCC patients can be developed and validated/replicated.

2. What are the non-genetic and genetic predictors of a superficial first BCC?
These questions were addressed in **chapter 4** using histopathologically confirmed skin cancer data gathered through a linkage between the prospective population-based cohort study named the Rotterdam Study[3] and the Dutch nationwide network and registry of histopathology and cytopathology (PALGA).[4] Based on several previous observational studies eleven non-genetic predictors were included in the binary logistic regression analyses of which three were significantly associated with a superficial first BCC. We found that patients with a superficial first BCC were significantly younger (odds ratio (OR) 0.95, 95% confidence interval (CI) 0.93-0.98), almost two times more often female (OR 1.88, 95% CI 1.16-3.03) and 12-18 times more likely to have their

BCC on the trunk or extremities (truncal OR 12.20, 95% CI 7.08-21.03; extremities OR 17.57, 95% CI 10.06-30.70) than patients with a non-superficial first BCC. We did not find a significant association between a superficial first BCC and having more than 1 BCC at initial diagnosis or having at least another mBCC (*last statement is based on unpublished data*). Based on several previous genome-wide association studies (GWAS) of loci that confer risk of BCC or non-melanoma skin cancer twenty single nucleotide polymorphisms (SNPs) were included in the binary logistic regression analyses of which one SNP (rs12203592), mapped to IRF4, looked promising (OR 1.83, 95% CI 1.13-2.97), but after adjustment for multiple testing, no significant differences in genetic make-up between superficial first BCC and non-superficial first BCC patients were found. Overall, superficial first BCCs could have a different etiology than the other subtypes. Although we did not find a significant association between SNPs previously associated with BCC and risk for superficial first BCC, we cannot rule out that there is no genetic susceptibility for a superficial first BCC, since our cohort of patients was small. Larger genetic studies will be needed to investigate whether this is indeed the case.

3. How to deal with the competing risk of death when analyzing metachronous BCCs?
This question was answered in **chapter 5** with histopathologically confirmed skin cancer data gathered through a linkage between the Rotterdam Study and PALGA. We first pointed out that the competing risk problem could be a real problem in survival analysis of metachronous KC data and then we compared two different methods of estimating the survival probability, namely the Kaplan Meier (KM) curve and the cumulative incidence curve (CIC). After ten years of follow-up the probability of a subsequent KC was 40% using the KM method and only 34% using the CIC method. The KM method gave an overestimation of the real probability by not taking the competing risk of death into account. Twenty years after diagnosis, the difference was even larger (74% for KM vs 52% for CIC) because the problem of competing risk due to death of included patients became larger. Thus, the competing risks problem can occur for all end points other than overall mortality when using the KM method and could be avoided using the CIC method. In chapter 6 and 7 we used this knowledge and showed that the Fine and Gray semiparametric proportional hazards model could be used to deal with competing risk of death and generate valid hazard ratios (HR) for the included predictors.

4. What are the non-genetic predictors, absolute risks and cumulative incidences of metachronous BCCs?
These questions were addressed in **chapter 6** and **7** using histopathologically confirmed skin cancer data gathered through a linkage between the Rotterdam Study and PALGA.

Based on a scarce amount of literature on non-genetic predictors of mBCC several phenotypic, lifestyle, and tumor-specific characteristics were included in the initial prediction models. In **chapter 6** the follow-up was stopped after the second new BCC (i.e., first mBCC) whereas in **chapter 7** the follow-up was extended up to the fifth new BCC (i.e., fourth mBCC).

In **chapter 6** we showed that of the thirteen non-genetic predictors included in our prediction model, only five remained in the multivariable Fine and Gray semiparametric proportional hazards model. These were: age at first BCC, sex, coffee consumption, superficial subtype of the first BCC and more than one BCC at first date of diagnosis. The latter being the strongest predictor of a second BCC (HR 2.5, 95% CI 1.9-3.3). The apparent concordance index (i.e., discriminative ability) of the multivariable model was reasonable, ranging between 0.63-0.65 from 1-5 years after the first BCC diagnosis. A score chart was developed, which makes it easier for a physician to calculate the absolute risk of a second BCC. For example: a 65-year-old (two points) man (one point) who drinks no coffee (two points) presenting with one (zero points) superficial (one point) BCC has a total score of 6, which corresponds to a 3-year risk of 21% of a second BCC. This patient could be regarded as a high-risk patient, however this is a grey area in which dermatologists should come together and define new guidelines on follow-up/ screening.

In **chapter 7** we showed that of the fourteen included non-genetic predictors nine remained in the multivariable Fine and Gray semiparametric proportional hazards model, namely age at BCC diagnosis, sex, pigment status, easily sunburned, coffee consumption, more than one BCC at diagnosis, superficial subtype of BCC, localization of BCC and the number of previous BCC diagnosis dates (newly added variable compared to the other prediction model). The strongest predictors were more than one BCC at diagnosis (HR 1.9, 95% CI 1.5-2.4) and the number of previous BCC diagnosis dates (increasing HR with increasing number of previous diagnosis dates; HR 3 previous dates 3.9, 95% 2.5-6.2), which could be proxies of field cancerization. In contrast to the prognostic model for a second BCC, pigment status, easily sunburned and location of BCC at diagnosis now did remain in the model, but the univariable HRs and 95% CIs were small and quite similar. The discriminative ability of the multivariable model was reasonable, ranging between 0.67-0.70 from 1-5 years after any BCC diagnosis. Again a score chart was developed showing that for example a 65-year-old (five points) man (one point) with a light pigment status (two points) who burns easily (one point), drinks no coffee (four points), presenting with one (zero points) superficial (one point) truncal (one point) BCC on his fourth BCC diagnosis date (nine points) has a total score of 24, which corresponds to a 3-year risk of approximately 34% of a fifth BCC. The cumulative incidence of a mBCC at 3 years was 15%, and 34%, 45% and 67% for the second, third, fourth and fifth BCC, respectively.

In conclusion, a combination of readily available clinical characteristics, especially more than one BCC at diagnosis and number of previous BCC diagnosis dates, can reasonably identify patients at high risk of mBCC. Risk of a mBCC was highest in the first 2-3 years after diagnosis.

5. What are the genetic predictors of multiple/metachronous BCCs?
This question was first answered in **chapter 8** performing a candidate gene approach (CGA) and a pilot GWAS of multiple BCC using data gathered through a linkage between the Rotterdam Study and PALGA. We used the word "multiple" instead of "metachronous" because a small part of the included patients only had multiple BCCs on their first diagnosis date and no further BCCs in time. The CGA comparing single BCC to multiple BCC included nineteen candidate SNPs from GWAS and CGA of BCC or KC and yielded no significant associations between these BCC-related SNPs and the risk of multiple BCC. In addition, the pilot GWAS identified genome-wide suggestive associations in chromosomes 2, 3, 18, and 22 (P-values $<5x10^{-6}$) of which the most significant SNP was rs78857623 (P-value 1.2×10^{-7}) mapped to an intron in the tumor suppressor gene *FHIT*.

In **chapter 9** we presented the results of a combined effort between our dermatology department and two research groups in the USA to identify gene variants for multiple KCs. A CGA and GWAS of multiple KC was performed combining our data with data from three large USA prospective cohort studies, namely the Nurses' Health Study (NHSI and II), Health Professionals Follow-up Study[5] and the Framingham Heart Study.[6] The GWAS on multiple KC identified eight independent SNPs with suggestive associations (p-value $<5.5x10^{-6}$) of which the most significant SNP was located at chromosome 9 (rs7468390; p-value 3.92x10-7). However, in stage two none of these SNPs were replicated in an independent sample of 574 multiple KCs from the Rotterdam Study and only two of them were associated with multiple KCs in the same direction in the combined meta-analysis. The nineteen previously reported candidate BCC SNPs were included in a CGA and we found that rs1805007 (*MC1R* locus) was significantly associated with risk of multiple KCs (p-value$2.80x10^{-4}$).

Overall, it remains likely that there are genetic differences between patients with multiple BCC and single BCC, but we could not confirm this in our genetic analyses. The latter could be explained by our relatively small sample sizes.

LIMITATIONS OF THE STUDIES

The articles on which **chapters 2** and **3** are based, compiled previous literature on BCC epidemiology, which led to some inherent limitations. **Chapter 2** consisted of a

scholarly review, which means that a non-systematic search of the scientific literature took place before the article was written. However, since the purpose of the article was to give a broad epidemiologic overview of BCC, it was practically impossible and not the scope to perform one systematic search. This may have biased the information presented and therefore affected the generalizability of our conclusions. However, we included as many different studies and outcomes as possible (>200 scientific articles). In **chapter 3** we gave our opinion on the scarcity of large population-based cohort studies on mBCC by writing a commentary on an original mBCC article. This means that due to the scope of this commentary only several articles could be included, which therefore could have affected the generalizability of our conclusions.

Chapters 4-9 are all based on data obtained from a linkage between the Rotterdam Study and PALGA. The use of a pathology registry (i.e., PALGA) coupled with a large long ongoing prospective cohort study (i.e., Rotterdam Study) gave us the opportunity to include multiple detailed predictors and distinguish between (metachronous) BCC and SCC, which helped us to discover predictors specific to either KC. This is important because there exist differences in risk factor profiles between BCC and SCC patients (e.g., smoking, UVR exposure patterns). Even though the Rotterdam Study data we used consisted of 14,926 participants, of whom approximately 10% developed at least one BCC, these are still small numbers when performing GWAS, because SNPs often have relatively low allele frequencies and small effect sizes. Therefore we collaborated with three different prospective USA cohort studies (**chapter 9**) to increase the sample size and power for our genetic studies, but it was no longer possible to differentiate between BCC and SCC, since the USA cohorts did not have the same phenotypes available. Likewise we could not (yet) replicate and validate our prediction models (**chapter 6** and **7**) because we could not find external cohorts which had the same detailed data as we had.

Other potential limitations of our studies were:

1) a limited generalizability (i.e., external validity) because the Rotterdam Study population is aged 45 years or older and mainly exists of white-skinned people, whereas current BCC incidence trends show an increasing incidence in young women.[7-9] However, these young women seem to represent a special group and overall BCC is still considered to be a skin disease of the older white-skinned population, suggesting this bias played only a minor role.
2) underestimation of the absolute number of BCCs and potential non-differential misclassification because
   a. we only included histopathologically confirmed BCCs. However, a recent observational study showed that only a small percentage (ca. 7%) of patients with mBCC had subsequent non-histologically confirmed BCCs.[10] In addition,

the evidence based guideline regarding BCC from the Dutch Society for Dermatology and Venereology (NVDV) states that all biopsied/excised BCCs should be sent for a histopathological diagnosis.[11] Besides, including non-histopathological BCCs could have led to a significant misclassification bias because a clinical BCC diagnosis, even made by a dermatologist, has a relatively low diagnostic accuracy.[12,13]

b. of incomplete PALGA coverage. The Rotterdam Study cohort members could have developed BCCs before PALGA had complete nationwide coverage in 1991. However, between 1971 and 1991 partial coverage was achieved and the mean age of the included participants in 1991 was 61 years, which is seven years younger compared with the mean BCC age of diagnosis,[14] suggesting that the impact of this bias is small.

IMPLICATIONS AND FUTURE PERSPECTIVES

In 1973 Epstein already observed that a relatively large part of patients who were previously treated for a BCC developed mBCC.[15] An observation that is in line with the concept of field cancerization[2] and with regard to the skin has been called actinic neoplasia syndrome to emphasize that patients with a first UVR-related cutaneous (pre)malignancy frequently develop other cutaneous (pre)malignancies, in part due to the field dysplasia from which they chronically suffer.[16] These observations were corroborated in a recent meta-analysis, which showed that approximately one third of the BCC patients will at least develop a second BCC and are at risk of SCC (4,3%) and melanoma (0,5%) as well.[1] The risks of developing other UVR-related skin cancers after a first BCC are increased, however, the majority of patients who develop a first BCC will develop BCCs only, something which could be a consequence of particular genetic susceptibilities.[17,18]

Since Epstein's observation no prediction models for mBCC have been developed (until now) and relatively few studies have been conducted to identify patients at risk of mBCC, but plenty of observational studies have pointed out the risk factors of a BCC, and the growing incidence numbers, burden of disease and health care costs.[19-21] It is clear that there is an increased risk of mBCC on a population level, but on an individual level it is still unclear who these patients at risk are. Looking at the increasing incidence and burden it is time we find the missing pieces and translate this into a clinical relevant prediction model for mBCC to help physicians in their therapeutic approach, something which has been done in multiple other research areas as well. A good example is the cardiovascular risk table for Dutch general practitioners which gives a 10-year risk of cardiovascular disease or mortality using age, sex, blood pressure,

smoking and cholesterol, and helps these physicians in determining their treatment plan (e.g., nothing, diet and/or medication).[22] The data presented in this thesis fill some of these knowledge gaps and support the aforementioned findings concerning risks of mBCC and field cancerization. We noticed that approximately 30% of the analyzed Rotterdam Study participants developed mBCC, which is similar to risks found in previous studies. The results presented in **chapters 2-3** and **6-7** demonstrate that proxies of field cancerization, namely more than one BCC at diagnosis and number of previous BCC diagnoses, were the strongest predictors of mBCC (HR varying from 1.9-3.9). However, BCC is a complex disease and the discriminative ability of our models was only reasonable, which most likely could be explained by residual confounding and the fact that we could not include all possible other predictors (because of the sample size) that could add to the total explained variability of mBCC.

In the last decades numerous observational studies (e.g., cohort, case-control, cross-sectional) have been conducted to find BCC risk factors. This has led to the discovery of multiple universally accepted predictors, but also to a lot of questionable (i.e., probably false) risk factors, often with small effect sizes and sometimes opposite effect directions. One could wonder if we should keep looking for (m)BCC predictors or should focus on the strongest and/or most easy to determine risk factors only. I think we should keep looking, as it seems that a significant part of the mBCC risk variation cannot be explained by known predictors, which also shows that risk factors for a first event are not automatically relevant for a second event (pigment status, UVR-related characteristics), and the finding of new predictors (even with small effect sizes) could lead to more focused prevention strategies and new therapeutic options. In the search for mBCC risk factors we should be non-conventional and look into new research areas like the (skin) microbiome as well.[23] In the interest of improving research quality it would be better if we would perform these studies (in particular genetic) in large (inter) national research consortia in order to the increase the validity of study findings and find new risk predictors with small effects sizes and low frequencies as well[24] and stimulate laboratory scientists to study underlying mechanisms in detail. The classical paradigm that most of the explained cancer variability is due to non-genetic/environmental factors (or as more recently stated due to bad luck)[25] seems erroneous and the attributable risk of genetic factors should not be overlooked.[26] Fortunately a paradigm shift took place and the last decade several studies were performed that looked into the genetic epidemiology of BCC. Most of these studies were CGAs or GWASs and the significant genetic predictors found in there usually had small effect sizes (OR <1,5) and only explain a small fraction of the total BCC heritability. Unfortunately, our CGAs and GWASs yielded no relevant significant predictors of multiple BCCs, most likely due to our small sample sizes (**chapter 8-9**). Based on our results one might argue that it is unlikely that there exist common variants (i.e., SNPs) with strong effects contributing

to the susceptibility for multiple BCC. Nevertheless, significant SNPs with weak effects and rare variants might be clinically irrelevant, but could lead to new pathways and therefore new treatment options. The missing heritability could be hidden in SNPs with small effect sizes and low frequencies that were not picked up in the current sample sizes, but another likely explanation is that we should look into for example gene-environment interactions, exome sequencing and epigenetics.[27] Recently, a GWAS found a potential gene-caffeine interaction which could be involved in caffeine-mediated BCC inhibition.[28]

The question remains if we should aim for a perfectly discriminating prediction model, as adding dozens of significant predictors (with small effect sizes) will decrease the applicability of such a model in clinical practice. Even if we would have the ideal (i.e., flawless discrimination, perfectly calibrated and externally validated) prediction model for mBCC, physicians should still be encouraged to think about their follow-up schemes because we, as physicians, would like to give personalized care and pursue shared decision making. However, a recently published cross-sectional study in USA elderly skin cancer patients showed that there were no differences in given treatments (61% surgical) between BCC patients with limited life expectancy and normal life expectancy.[29] A one-size-fits-all approach is not the answer to the growing skin cancer problem in our BCC patients, of whom the majority is over 65 years of age.

Another question concerns the duration and frequency of follow-up once we identified the patients at risk of mBCC. Previous observational studies have shown that most mBCC occur within approximately 3 years after a diagnosis, but that risks remain elevated over time.[30-32] This was in line with our results (**chapter 6-7**) in which we noticed that the median follow-up time until the second BCC was 3.0 years and seemed to shorten until it reached 1.8 years after the fourth BCC. Fortunately, BCCs are rarely lethal[33] and increase slowly in size, with a median growth of approximately 3 millimeters per year as pointed out in a recently published systematic review.[34] This review, which was based on the WHO criteria for screening, also showed that small changes in size can affect treatment options, their effectiveness and associated costs, especially in the H-zone and that current data supports early detection of BCCs on the face. In contrast to this systematic review, the U.S. Preventive Services Task Force concluded in 2016 that "the current evidence is insufficient to assess the balance of benefit and harms of screening for skin cancer in adults with a clinical visual skin examination".[35] Important data in this field comes from the first nationwide skin cancer screening program in Germany,[36] however seven years after the introduction of this program no discernible beneficial effect was found.[37] In addition, an Australian cost-effectiveness analysis of an educational intervention encouraging self-skin examinations for early detection of skin cancers showed that the overall costs and effects outweighed the positive health gains.[38] A way to increase the cost-effectiveness

is to restrict screening to high-risk patients, but the downside of this targeted screening is the so-called 'prevention paradox' in which you do not address the overwhelming majority of low-risk BCC patients that develop mBCC.[39] In the international dermatology society there is currently no consensus on the follow-up scheme of BCC patients, but they almost all promote self-monitoring (Table 1). The national dermatology guidelines differ in the advice on the length and frequency of follow-up, which could depend on a countries' BCC incidence and population composition, and lack prediction models that could define high-risk groups. The Dutch BCC guideline advices not to do regular follow-up (except in well-known high risk groups like immunosuppressed), whereas the German guideline advices, in a population that is similar to that of the Netherlands, half-yearly visits the first 3 years after which lifelong yearly follow-up. The Australian guideline advices lifelong follow-up every 6-12 months, which was expected because they have the highest UVR-related skin cancer incidences rates in the world. Based on these findings I would suggest to follow Dutch BCC patients at risk for a mBCC once per year at least 3-5 years after every new BCC, without forgetting the patients' health status and wishes. Since skin cancer patients already determine a significant amount of the workload of dermatologists, we should consider translocating less complex skin cancer care to general practitioners. Recent studies show that general practitioners are willing to do this but currently often lack the diagnostic capabilities and tools.[40] However, education sessions can improve diagnostic accuracy and surgery skills and diminish unnecessary referrals.[41-43]

The prediction models we created to identify mBCC patients were as far as we know the first prediction models reported for this outcome. However, recently an Australian group developed another prediction model based on prospective population-based cohort data to estimate the future risks of metachronous KC in patients with and without prior KCs.[44] Although this study did not differentiate between BCC and SCC, it did show that the strongest predictors were signs of field cancerization, namely number of prior skin cancers excised and number of skin lesions destroyed. Both our models and the Australian model have room for improvement when looking at the discriminative capacities and have not been externally validated yet. This points to the fact that there are still too few large prospective cohort studies and skin cancer consortia with the same data, which can be used to replicate and validate findings from other BCC studies.

CONCLUSION

The diagnosis, treatment and follow-up of BCC (and other UVR-related cutaneous (pre) malignancies) currently consumes a relatively large part of the dermatological care (in the Netherlands/Western countries) and it is unlikely that this will change in the near

Table 1. Follow-up schemes for BCC patients according to different guidelines

Country	Date	Conclusion[1]	Reference
Netherlands (NVDV)	2015	Primary BCC: no routine follow-up, advice on self-monitoring; except for high-risk patients (like nevoid basal cell carcinoma syndrome, prolonged immunosuppressed and severe actinic damage), whom get at least yearly follow-up	http://www.nvdv.nl/wp-content/uploads/2014/08/20160725-eindversie-richtlijn-BCC-2015.pdf
Germany (ADO, DK, DDG)	2012	Primary BCC: advice on self-monitoring, follow-up every 6 months first 3 years, after which lifelong yearly follow-up (more often when e.g., immunosuppressed, genetic predisposition, history of multiple BCC)	http://www.awmf.org/uploads/tx_szleitlinien/032-021l_S2k_Basalzellkarzinom_2013-verlaengert.pdf
Great Britain (BAD)	2008	Primary BCC: advice on self-monitoring or follow-up in primary care. Patients with history of multiple BCCs: follow-up ≥ 3 years	http://www.bad.org.uk/shared/get-file.ashx?id=45&itemtype=document
France (FSD)	2004	Primary BCC: at least yearly follow-up for 5 years, preferably lifelong	http://www.sfdermato.org/media/pdf/recommandation/cbc-2004-recommandations-96f90a29d135dac081b7053db84cdb57.pdf
European (EDF)	2012	Primary BCC: advice on self-monitoring and ideally a lifelong yearly follow-up. However if unfeasible then follow-up of high-risk patients (i.e., high risk of recurrence, already treated for a recurrence, history of multiple BCCs) every 6-12 months for 3-5 years	http://www.euroderm.org/edf/index.php/edf-guidelines/category/5-guidelines-miscellaneous?download=24:guideline-basal-cell-carcinoma-update-2012
Australia (ACD)	2008	Primary BCC: advice on self-monitoring and lifelong follow-up every 6-12 months	https://www.dermcoll.edu.au/atoz/basal-cell-carcinoma-bcc/
USA (AAD)	2017 (draft version)	Primary BCC: advice on self-monitoring and at least yearly follow-up	https://www.aad.org/File%20Library/Main%20navigation/Practice%20Center/Quality/Quality%20measures/AAD-BCC-Guidelines-FOR-MEMBER-COMMENT.pdf

[1] Conclusion is a summarized version of the guideline's follow-up conclusions/recommendations, focusing on multiple BCCs

Abbreviations: NVDV, Dutch Society of Dermatology and Venereology; ADO, Arbeitsgemeinschaft Dermatologische Onkologie; DK, Deutschen Krebsgesellschaft; DDG, Deutschen Dermatologischen Gesellschaft; BAD, British Association of Dermatologists; FSD, French Society of Dermatology; EDF, European Dermatology Forum; ACD, Australasian College of Dermatologists; AAD, American Academy of Dermatologists.

future, as BCC incidence is still increasing. Identifying and focusing on BCC patients at high risk of developing mBCC will optimize BCC care and reduce the strain on dermatological care. This thesis can assist dermatologists and general practitioners to accomplish this as it presents research on non-genetic and genetic epidemiology of mBCC patients, including prediction models to identify these patients. However, replication and external validation of these models and larger genetic studies on mBCC are needed.

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Chapter 11

Summary / Samenvatting

In **hoofdstuk 1** geef ik een algemene inleiding op dit proefschrift waarin ik uiteenzet waarom ik onderzoek heb gedaan naar multipele/metachrone basaalcelcarcinomen (mBCCs) en welke onderzoeksvragen er beantwoord gaan worden. Daarnaast geef ik kort aan uit welke hoofdstukken mijn thesis bestaat, wat hierin besproken wordt en wat voor data ik gebruikt heb.

In **hoofdstuk 2** worden alle facetten van de epidemiologie van het BCC besproken in een uitgebreid non-systematisch review. Hieruit komt naar voren dat het BCC de meest voorkomende kanker is bij de blanke mens (>1:2 Australiërs ontwikkelen minimaal 1 BCC) en de incidentiecijfers wereldwijd nog steeds toenemen (jaarlijks >5% toename in Europa). Daarbij zijn de ziektelast en zorgkosten significant toegenomen. Vanwege deze problemen en het verhoogde risico (>30%) op een volgende ultraviolette straling (UV)-gerelateerde huidkanker zorgen BCC patiënten voor een toenemende druk op het zorgsysteem. Ook wordt duidelijk dat het BCC een complexe ziekte is waarbij het samenspel tussen omgevingsfactoren (o.a. UV), fenotype (o.a. UV-gevoeligheid) en genotype (o.a. mutaties en polymorfismen) een sleutelrol speelt in de ontstaanswijze. Preventieprogramma's die continu worden verbeterd, kunnen van grote waarde zijn in het aanpakken van het groeiende huidkankerprobleem. Om de meest adequate BCC-zorg te leveren dienen artsen 'shared decision-making' toe te passen en hun beleid zorgvuldig te kiezen.

In **hoofdstuk 3** wordt middels een commentaar op een ander wetenschappelijk artikel over multipele keratinocyten huidkankers (mKCs, BCCs en plaveiselcarcinomen) uit Australië uiteen gezet waarom patiënten met een eerste KC een interessante doelgroep voor preventieprogramma's zijn. Daarnaast wordt duidelijk gemaakt dat investeren in grote en goed opgezette cohort studies/consortia noodzakelijk is om de betrouwbaarheid van de resultaten te verhogen en de basale wetenschap hopelijk stimuleert om meer onderzoek te doen naar onderliggende mechanismen.

In **hoofdstuk 4** worden de niet-genetische en genetische predictoren van patiënten met een superficieel eerste BCC onderzocht in een prospectieve cohort studie, omdat er mogelijk verschillen zouden bestaan in het risicofactor profiel tussen patiënten met een superficieel eerste en niet-superficieel eerste BCC en hun risico op een volgende BCC. De data werden verzameld door gebruik te maken van de deelnemers van het Erasmus Rotterdam Gezondheid Onderzoek (ERGO / 'Rotterdam Study') en de koppeling tussen ERGO en het Pathologisch Anatomisch Landelijk Geautomatiseerd Archief (PALGA). ERGO is een langlopend (1989-heden) prospectief bevolkingsonderzoek onder mensen van veelal noordwesters Europese afkomst van 45 jaar en ouder, woonachtig in de wijk Ommoord in Rotterdam. PALGA is de nationale histopathologie databank

en bestaat sinds 1971 en heeft sinds 1991 volledige landelijke dekking. De follow-up/koppelingsperiode liep van 01-07-1989 t/m 31-12-2013. Er werden 14.628 deelnemers gekoppeld waarvan uiteindelijk 948 BCC patiënten geschikt waren voor niet-genetische - en 1.014 voor genetische analysen. We includeerden 11 fenotypische -, omgevings- en tumor-specifieke karakteristieken als variabelen voor de niet-genetische analysen en 20 enkel nucleotide polymorfismen (SNPs, 'single nucleotide polymorphisms') als variabelen voor de genetische analysen, waarbij we binaire logistische multivariabele regressie-analysen verrichtten met als uitkomstmaat het wel/niet hebben van een superficieel eerste BCC. We ontdekten dat patiënten met een superficieel eerste BCC significant jonger zijn, ongeveer tweemaal vaker vrouw en 12-18 keer vaker een BCC op de romp of extremiteiten ontwikkelen dan patiënten met een niet-superficieel eerste BCC. Daarnaast vonden we geen significante genetische verschillen tussen beide BCC groepen. Patiënten met een superficieel eerste BCC verschillen dus van patiënten met een niet-superficieel eerste BCC met betrekking tot fenotypische (leeftijd en geslacht) en tumor-specifieke kenmerken (locatie). Ook zou er nog steeds een niet ontdekt genotypisch verschil kunnen bestaan. Aangezien patiënten met een superficieel eerste BCC hun eerste BCC op jongere leeftijd ontwikkelen, zouden ze een hoger levenslang risico op een volgende huidkanker (e.g., BCC) kunnen hebben en daardoor een interessante groep zijn voor secundaire preventie.

In **hoofdstuk 5** wordt middels een commentaar op een ander wetenschappelijk artikel over de absolute risico's op het ontstaan van nieuwe KCs in een Amerikaans cohort uiteen gezet waarom rekening houden met mortaliteit in overlevingsstatistiek belangrijk is. We laten zien door gebruik te maken van ERGO-data, dat een overschatting van het absolute risico op een volgende KC kan ontstaan als men geen rekening houdt met het concurrerende risico op dood binnen een cohort. We vergelijken de 'foutieve' Kaplan-Meier methode met de cumulatieve incidentie curve waarbij duidelijk wordt dat de verschillen in kansen op een volgende KC uiteen kunnen lopen van 74% (Kaplan-Meier methode) tot 52% (cumulatieve incidentie curve) na 20 jaar follow-up, omdat het probleem van het concurrerende risico op dood groter wordt. Dit probleem kan voor alle uitkomstmaten (behoudens algemene mortaliteit) voorkomen in overlevingsstatistiek en wordt vooral gezien in studies met een oudere populatie en een lange follow-up.

In **hoofdstuk 6** wordt een model ontwikkeld voor het voorspellen van het absolute risico op een tweede BCC, omdat ongeveer 30% van alle patiënten met een BCC minimaal één volgende BCC in de tijd ontwikkelen en we niet precies weten wie dit zijn. De data werden verzameld door gebruik te maken van de deelnemers van de prospectieve ERGO-studie en de koppeling met PALGA. Er werden 13 omgevings-, fenotypische

- en tumor karakteristieken als predictoren geselecteerd. Het predictiemodel werd gebaseerd op het Fine en Gray regressie model waarbij rekening werd gehouden met het concurrerend risico op dood. Onder de 1.077 geïncludeerde ERGO-deelnemers met minimaal 1 BCC, ontwikkelden er 293 minimaal een tweede BCC na gemiddeld 3 jaar. Enkele welbekende risico factoren voor een eerste BCC waren niet prognostisch voor een tweede BCC, terwijl het hebben van meer dan 1 BCC bij initiële presentatie de sterkste predictor was. Het discriminerend vermogen van het model was redelijk (bootstrap gevalideerde c-index= 0.65 op 3 jaar). Er werden 3 risico groepen gemaakt, waarbij de risico's op een tweede BCC na 3 jaar uiteen liepen van 7-28%. Een combinatie van gemakkelijk verkrijgbare klinische karakteristieken kan met redelijke betrouwbaarheid patiënten identificeren die een hoog risico hebben op een tweede BCC. Externe validatie en uitbreiding met sterkere predictoren is wenselijk om het predictiemodel te verbeteren.

In **hoofdstuk 7** wordt voortgeborduurd op het predictiemodel uit hoofdstuk 6, omdat het onduidelijk is wie er meer dan 2 BCCs ontwikkelen, hoe hoog dit risico is en hoe snel dit gebeurt. De data werden wederom verzameld door gebruik te maken van de deelnemers van de prospectieve ERGO-studie en de koppeling met PALGA. De follow-up werd uitgebreid tot en met het 5^{e} nieuwe BCC. Er werden 14 omgevings-, fenotypische - en tumor karakteristieken als predictoren geselecteerd, waarbij het aantal voorgaande BCC diagnosen als nieuwe predictor werd toegevoegd. Het predictiemodel werd gebaseerd op het Fine en Gray regressie model waarbij rekening werd gehouden met het concurrerend risico op dood. Bootstrapping werd gebruikt om het model verder te verbeteren en valideren. Onder de 1.077 geïncludeerde ERGO-deelnemers ontstonden er tweede tot en met vijfde BCCs in respectievelijk 293, 122, 58 en 36 patiënten, na gemiddeld 3,0 , 2,1 , 1,7 en 1,8 jaar follow-up na de vorige BCC. Het risico op een volgende mBCC was hoger voor patiënten met meer voorgaande BCCs. Het hebben van meer dan 1 BCC tijdens een diagnose moment was een andere sterke predictor van mBCC. Het discriminerende vermogen van het model was redelijk met een voor optimisme gecorrigeerde c-index van 0,70 na 3 jaar. Wanneer het aantal voorgaande BCC diagnosen wordt gecombineerd met andere gemakkelijk verkrijgbare klinische kenmerken in een predictiemodel, kunnen patiënten met een hoog risico op een volgende BCC geïdentificeerd worden.

In **hoofdstuk 8** wordt onderzocht of er genetische aanleg bestaat voor het ontwikkelen van mBCC, omdat er nog nauwelijks genetische onderzoeken zijn verricht naar patiënten met meerdere BCCs en het niet bekend is of de reeds ontdekte BCC loci ook geassocieerd zijn met mBCC. De data werden verzameld door gebruik te maken van de deelnemers van de prospectieve ERGO-studie en de koppeling met PALGA.

Er werd zowel een kandidaatgen analyse (CGA, 'candidate gene approach') met 19 kandidaat SNPs verspreid over 17 loci, als een genoomwijde associatie studie (GWAS, 'genome-wide association study') verricht. Na kwaliteitschecks bleven er 7.260.691 markers over voor genetische analysen. Er werden 1.219 deelnemers met ten minste 1 BCC geïncludeerd, waarvan er 472 (38,7%) mBCC hadden. De CGA werd verricht middels een SNP- en gen-gebaseerde logistische regressie analyse waarbij patiënten met 1 BCC vergeleken werden met patiënten met mBCC, gecorrigeerd voor leeftijd, geslacht en 4 principale componenten (PCs). De Bonferroni methode werd gebruikt om aan te passen voor 'multiple testing'. Er werden geen significante genetische verschillen gevonden tussen beide groepen m.b.t. de geïncludeerde kandidaat SNPs/genen. De pilot GWAS, d.m.v. logistische regressie aangepast voor leeftijd, geslacht en 4 PCs, leverde een aantal opvallende niet-significante associaties op, waarvan de meest significante SNP rs78857623 (P-waarde $1,2\times10^{-7}$) was, gelegen in een intron op het tumorsuppressor gen *FHIT*. Het lijkt er dus op dat genetische loci die geassocieerd zijn met BCC niet geassocieerd zijn met mBCC. Daarnaast leverde de GWAS enkele interessante associaties op, maar deze bevindingen moet worden gerepliceerd in andere cohorten gezien de beperkte power.

In **hoofdstuk 9** wordt voortgeborduurd op de genetische associatie analysen uit hoofdstuk 8 waarbij ditmaal mKC als uitkomstmaat wordt genomen (twee typen huidkanker die veel overeenkomsten vertonen) ten einde de power te vergroten. De data werden verzameld door niet alleen gebruik te maken van de deelnemers van de prospectieve ERGO-studie en de koppeling met PALGA, maar ook een samenwerking aan te gaan met enkele Amerikaanse prospectieve cohortonderzoeken, te weten de Nurses' Health Study (NHS), NHS II, Health Professionals Follow-Up Study en de Framingham Heart Study. Er werd een GWAS verricht met hierin >8 miljoen SNPs bij 1.666 patiënten met mKC en 1.950 controles met een enkele KC. De meest significante (P-waarde $5,5x10^{-6}$) SNPs (40) uit de ontdekkingsfase werden geïncludeerd in de replicatiefase in een onafhankelijk cohort van de ERGO-studie van 574 patiënten met mKC en 872 met een enkele KC. Er werden geen genoomwijde significante SNPs gevonden. Ook de reeds 19 bekende BCC loci werden getest voor eventuele associatie met mKC in een CGA en het bleek dat rs1805007 (*MC1R* locus) significant (P-waarde $2,80x10^{-4}$) geassocieerd was met mKC. Om de nieuwe genetische markers te vinden voor mKC zijn er grotere consortia nodig.

In **hoofdstuk 10** geef ik antwoord op mijn eerder (hoofdstuk 1) gestelde onderzoeksvragen door gebruik te maken van mijn onderzoeksresultaten en bespreek ik de beperkingen van de door ons verrichtte studies. Tot slot bespreek ik de mogelijke implicaties van mijn bevindingen met het oog op de toekomst.

Chapter 12

List of abbreviations

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LIST OF ABBREVIATIONS

BCC	basal cell carcinoma
mBCC	metachronous / multiple basal cell carcinomas
GWAS	genome-wide association study
PALGA	Dutch nationwide network and registry of histopathology and cytopathology
UVR	ultraviolet radiation
SCC	squamous cell carcinoma
KC	keratinocyte carcinoma
CI	confidence interval
OR	odds ratio
ERGO / RS	Rotterdam Study
SNP	single nucleotide polymorphism
c-index	concordance index
HR	hazard ratio
CGA	candidate gene approach
MAF	minor allele frequency

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LIST OF PUBLICATIONS

in chronological order, including publications not included in this thesis

Verkouteren JA, Nijsten T. Smoking, a Dangerous Habit for the Skin: comment on "Smoking and the Risk of Nonmelanoma Skin Cancer". *Arch Dermatol. 2012 Aug;148(8):946.*

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Verkouteren JAC, L.M. Pardo, A.G. Uitterlinden, T. Nijsten. Predictors of a superficial first basal cell carcinoma. *submitted to J Eur Acad Dermatol Venereol.*

CURRICULUM VITAE

Joris (Arnoldus Cornelis) Verkouteren is op 30 maart 1987 geboren te Tholen. In 2005 behaalde hij zijn gymnasium diploma aan de Regionale Scholengemeenschap 't Rijks te Bergen op Zoom. Ditzelfde jaar werd hij middels decentrale selectie toegelaten tot de studie geneeskunde aan de Erasmus Universiteit Rotterdam en verhuisde hij naar deze stad. Tijdens zijn studie was hij vanaf 2007 tot en met 2010 werkzaam in het studententeam van de afdeling Urologie en Vrouwenziekten van het Erasmus MC te Rotterdam. Na zijn reguliere co-schappen begon hij in 2011 aan zijn afstudeeronderzoek op de afdeling dermatologie van het Erasmus MC en later dat jaar voltooide hij ook zijn oudste co-schap aldaar. Op 9 december 2011 behaalde hij zijn arts-examen en op 1 januari 2012 begon hij aan zijn promotietraject op de afdeling dermatologie van het Erasmus MC. Vanaf 2012 tot en met 2016 hield hij zich hoofdzakelijk bezig met zijn proefschrift en het klinische werk voor het Erasmus Rotterdam Gezondheid Onderzoek onder begeleiding van prof. dr. Tamar Nijsten en dr. Luba Pardo. In 2015 werd hij aangenomen tot de opleiding tot dermatoloog en per 1 januari 2016 is hij gestart met zijn klinische stages. Tijdens zijn studententijd leerde hij zijn huidige partner Marlot van 't Hof kennen en inmiddels wonen zijn 6 jaar samen in Rotterdam.

PhD PORTFOLIO

Name PhD student: J.A.C. Verkouteren
Erasmus MC Department: Dermatology
Research School: NIHES/MolMed

PhD period: 2012-2018
Promotor: Prof. dr. T.E.C. Nijsten
Supervisor: Dr. L.M. Pardo

PhD training	Year	Workload Hours	ECTS
General courses			
Basiscursus Regelgeving en Organisatie voor Klinisch onderzoekers (BROK)	2012		1,0
Integrity in research	2014		0,3
BROK re-certification	2015	3	
DOO-course hospital management	2017		1,0
Specific courses / workshops / seminars			
Principles of Research in Medicine and Epidemiology (NIHES)	2012		0,7
Principles of Genetic Epidemiology (NIHES)	2012		0,7
Advanced Genetic Association Analysis	2012		1,0
Classical Methods for Data-analysis (NIHES)	2012		5,7
Dermoscopy training	2012	5	
Genome Wide Association Analysis (NIHES)	2013		0,7
Pathway and Network Analysis	2013	7	
Microsoft Excel 2010: Basic (MolMed)	2013		0,3
Presentation techniques	2014	3	
Genome Browsing with Ensembl and Viewing Variation (MolMed)	2015	1	
Oral presentations			
Basal cell carcinoma: who are truly at risk. 2020 Meeting Erasmus MC, Netherlands	2013		1,0
Genome-wide association study identifies a novel candidate locus associated with risk of multiple basal cell carcinomas. 14th Annual Scientific Meeting NVED, Lunteren, Netherlands	2014		1,0
Predicting the risk of a second primary basal cell carcinoma. 23rd EADV Congress, Amsterdam, Netherlands	2014		1,0
Biostatistics. PhD-weekend, Dermatology department Erasmus MC, Netherlands	2014		1,0
Extremophiles. PhD-weekend, Dermatology department Erasmus MC, Netherlands	2015		1,0
Predictiemodel voor een tweede basaalcelcarcinoom. NVDV Scientific Meeting, Rotterdam, Netherlands	2015		1,0
Predicting the risk of a second basal cell carcinoma. ISDS 37th Annual Meeting, Amsterdam, Netherlands	2016		1,0
Predicting the risk of a subsequent BCC. 1st Cells 2 Surgery, Rotterdam, Netherlands	2017		1,0
Poster presentations			
Common genetic variations of multiple basal cell carcinomas. 44th Annual ESDR Meeting, Copenhagen, Denmark	2014		1,0
Predicting the risk of metachronous basal cell carcinomas. IDEA-KeraCon Conference, Aurora, USA	2016		1,0

Attendance of (inter)national conferences / symposia etc.			
PhD Day. Erasmus MC, Netherlands	2012	5	
14th Annual Scientific Meeting NVED. Lunteren, Netherlands	2013		1,0
IID. Edinburgh, Scotland	2013		1,0
AAV Wetenschapsmiddag. Erasmus MC, Netherlands	2013	5	
23rd EADV Congress. Amsterdam, Netherlands	2014		1,0
PhD-weekend. Dermatology department Erasmus MC, Netherlands	2014		1,0
15th Annual Scientific Meeting NVED. Lunteren, Netherlands	2014		1,0
NVDV Scientific Meeting. Rotterdam, Netherlands	2015	8	
AAV Wetenschapsmiddag. Erasmus MC, Netherlands	2015	5	
Quantitative methods in medical research. Erasmus MC, Netherlands	2015	3	
PhD-weekend. Dermatology department Erasmus MC, Netherlands	2015		1,0
45th Annual ESDR Meeting. Rotterdam, Netherlands	2015		1,0
KNAW Genetic Screening: Who, Why and When? Amsterdam, Netherlands	2015	6	
PhD-weekend. Dermatology department Erasmus MC, Netherlands	2016		1,0
IDEA-KeraCon Conference. Aurora, USA	2016		1,0
ISDS 37th Annual Meeting. Amsterdam, Netherlands	2016		1,0
1st EDEN Forum. Madrid, Spain	2017		1,0

Teaching	**Year**	**Workload**	
		Hours	**ECTS**
EADV fostering course on Clinical Research and Epidemiology. Erasmus MC, Rotterdam, Netherlands	2014	4	
EADV fostering course on Clinical Research and Epidemiology. Erasmus MC, Rotterdam, Netherlands	2016	8	
EDEN preconference course on Skin Cancer Epidemiology (survival analysis). Madrid, Spain	2017		1,0

Other	**Year**	**Workload**	
		Hours	**ECTS**
PhD-weekend committee. Dermatology department Erasmus MC, Netherlands	2014		1,0
Occasional reviewer for - Acta Dermato-Venereologica - British Journal of Dermatology - Journal of the European Academy of Dermatology and Venereology - Cancer Epidemiology	2012-2016		1,0
TOTAL		**63**	**35,4**

WORD OF THANKS / DANKWOORD

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