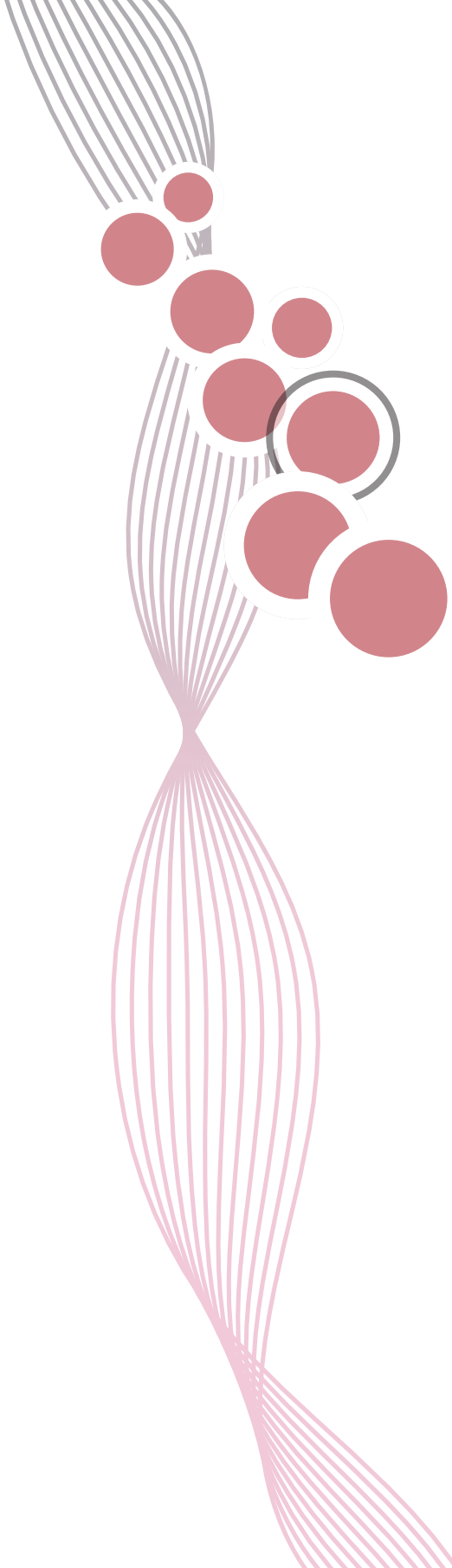
Basal cell carcinoma



Chapter 6

Discussion & valorization

BCC is the most common cancer worldwide and its incidence is still rising, by 3-10% annually. However, as there are few adequate skin cancer registries, the actual increase might be even higher. Although BCCs rarely metastasize and mortality is extremely low, they can cause significant morbidity due to locally aggressive behavior. Half of all BCC arise primarily on the face and (bald) scalp. Surgical excision, which is the current gold standard of treatment, has disadvantages, such as the risk of bleeding, infection, impaired healing and scarring, which can be disfiguring and cause patients to experience a significantly decreased quality of life.

The high incidence of BCC combined with the associated high costs of treatment and the attendant workload is causing a significant and mounting impact on the health-care system. Consequently, BCC is an important public health problem. For that reason, there is an urgent medical and societal need for a patient friendly and cheap (targeted) non-invasive treatment, and preferably one that can be performed by patients themselves. To develop such a treatment, a profound understanding of BCC pathogenesis is essential. The research described in this thesis aims to profile genetic and epigenetic alterations in both non-aggressive and locally advanced BCC in order to enhance our understanding of BCC pathogenesis. This will be described in **part one**. **Part two** discusses new topical and systemic drugs in the treatment of BCCs.

Part one: pathogenesis of BCC

Epigenetics and more in BCC

Epigenetics encompasses heritable changes in gene expression without changes in the DNA sequence. These changes can be present at the DNA (DNA methylation), RNA (RNA interference by non-coding RNAs), and protein (histone modifications, polycomb group proteins, and chromatin remodeling) level. The continuous interplay of these epigenetic regulations determines the organism's phenotype, which is referred to as the epigenome.[1,2] The studies presented in this thesis focus on DNA-methylation, one of the most important and well-studied epigenetic alterations. DNA methylation consists of adding a methyl group to cytosine nucleotides, primarily when they are followed by a guanine, the so-called CpG dinucleotide. Aberrant DNA methylation patterns are seen in many cancers (o.a. colon, breast, kidney and prostate cancer)[1] and other human diseases, such as autoimmune and neurological disorders.[3] In BCCs, however, only a few studies have so far looked at DNA methylation. Also, these studies focused on predefined tumor suppressor genes and did not provide evidence that any observed changes in methylation patterns were relevant for BCC carcinogenesis.[4-9] Additional evidence shows that ultraviolet (UV) radiation A and UVB may alter epigenetic modifications[10] and UV-signature mutations seem to preferentially occur at methylated C-phosphate-G (^{m}CpG) dinucleotides.[11] As BCCs mainly occur at sun-exposed areas of the skin[12], they might be expected to show extensive methylation changes compared to non-diseased skin.

In a first exploration of promoter hypermethylation in BCCs, we assessed the promoter CpG island methylation status of nine candidate tumor suppressor genes (TSG). CpG islands

are regions with a higher frequency of CpG dinucleotides that are mostly found around the promoter region of the gene.[13] As described in **chapter 2.1**, nested methylation specific polymerase chain reaction (MSP) was performed on bisulfite-modified genomic DNA of BCCs and healthy skin with primers specific for methylated DNA, respectively unmethylated DNA. Significantly more DNA methylation in BCCs was found in the gene promoters of *SHH, APC* and *SFRP5*, all key regulators of the SHH and WNT signaling pathways, and of *RASSF1A. RASSF1A* is a well-known tumor suppressor of which epigenetic inactivation is previously described in BCCs[9], but also in malignant melanoma[14] and Merkel cell carcinoma.[15] Interestingly, healthy sun-exposed (SE) skin samples showed methylation patterns similar to sun-protected (SP) skin, suggesting that the methylation we observed is more tumor-specific than locus or patient specific, a finding that is in line with literature to date.[16] It is of interest that methylation patterns in sun-exposed skin did not resemble those in BCC. Our findings therefore do not support a contribution of UVB exposure to the observed promoter hypermethylation of *SHH, APC, SFRP5* and *RASSF1A*. However, since the epigenetic changes that we found parallel genetic changes driving BCC growth, it seems likely that gene silencing by promoter hypermethylation is relevant to BCCs.

In this study we used nested MSP because it is highly sensitive and can detect 1 methylated allele in >50,000 unmethylated alleles. This makes it exquisitely suitable for methylation analysis of archived formalin-fixed paraffin-embedded (FFPE) DNA, as this generally yields small amounts of poor quality DNA.[17] Since biopsy or excision tissue samples taken during regular care are commonly FFPE and stored in the archives of the pathology department, there is wealth of tissue available for research. On the down side, (nested) MSP is a 'yes or no' approach that does not provide quantitative results. Also, it can only be used to screen pre-defined candidate genes. Fortunately, novel techniques such as comprehensive probe-based, bisulfite whole- genome sequencing, and enrichment-based technologies[18-20] allow unbiased genomewide analysis of DNA methylation (the "methylome"). This is a major advantage compared to locus-specific, low-throughput approaches that are typically PCR based. However, the latter are still used for validation purposes.[19]

Chapter 2.2 describes the first analysis of the BCC methylome by genome-wide ^{m}CpG DNA enrichment sequencing (MethylCap-seq).[21] MethylCap-seq is based on capture of methylated DNA on a genome-wide scale with the methyl-DNA binding domain (MBD) domain of the protein MeCP2. As only methylated DNA is subsequently sequenced, the complexity of the analysis is reduced and high sequence coverage can be obtained.[22] MethylCap-seq is a robust, highly reproducible approach, especially for larger sample cohorts.[22]

We identified 32979 methylated regions in 22121 genes, 13 of which were differentially methylated in sporadic BCCs compared to healthy skin, with a False Discovery Rate (FDR) of <0.1 (**chapter 2.2**). As methylation can regulate gene expression levels[23], we performed rRNA depleted, strand specific, total RNA sequencing (RNA-seq henceforth) and correlated these data to the methylome. This correlation provides information on the functional methylome: i.e. those methylation events that affect gene expression. Of note, there was little overlap

between the top-ranked differentially methylated loci and differentially expressed genes (FDR<0.1), even when preselecting for genes that might be differentially expressed in BCC. At present, we cannot explain this finding. The full spectrum of functions that DNA-methylation has remains to be delineated, as is its full impact on RNA expression levels. IFI30 was one of the hits that was both differentially methylated and differentially expressed, a finding that points to the importance of immune signaling in BCC. IFI30 is known to play a critical immunoregulatory role in melanoma by mediating antitumor immunity via $CD4^+$ T-cells.[24]

We compared the results of our first analysis in BCC with those obtained from methyl-Cap-seq and RNA analysis of a facial laBCC (**chapter 2.3**). We identified considerable agreements and differences between the non-aggressive and aggressive BCCs. The most striking difference was a dramatic increase in expression of matrix metalloproteinase 13 (MMP13) in the laBCC, which could explain the more aggressive behavior of this tumor.[25]

Additional pathway analysis of the RNA-seq data was performed with SPIA software[26], showing that pathways activated in BCC are all involved in immune signaling: cytokine-cytokine receptor interaction signaling (KEGG hsa04060), chemokine signaling (Chemokine) (KEGG hsa04062) and Toll-like receptor signaling (KEGG hsa04620). Pathway enrichment analysis of the differentially methylated genes also revealed cytokine-cytokine receptor interaction as the most significant pathway. The importance of immune signaling in BCCs is further supported by results from our independent clinical trial, described in **chapter 4.1**. Previous work has also found increased expression of innate immune genes in squamous cell carcinomas (SCCs) and in BCCs.[27]

Apparently, SHH signaling might not be the sole driver in sporadic BCCs. In fact, BCC pathogenesis seems to be rather complex, and this is further confirmed by the whole-exome sequencing of BCCs described by Jayaraman *et.al.*[28] Surprisingly, although BCC can be considered as the least malignant human cancer, they harbor the largest mutational burden compared to any other known human malignancy. The vast majority of mutations (75.7%) showed a clear UV signature. However, Jayaraman *et.al.* could not identify any novel genes as drivers in BCC. The authors hypothesized that the resulting high level of mutated proteins might increase the hosts' immune response, resulting in a less aggressive phenotype.[28] It is of interest that the incidence of nonmelanoma skin cancer (NMSC) in immunosuppressed patients, such as organ transplant recipients (OTR), is up to 250-fold higher than in the general population. For common cancers like gastrointestinal, lung, breast, bladder, prostate, and gynecological, this risk is only two to five-fold higher.[29,30] In OTR, SCCs occur 4-10 times more often than BCCs and tend to behave in a more aggressive manner with increased metastatic potential, and higher recurrence rates compared to SCC in non-immunocompromised people.[30,31] This increased risk of SCCs and also BCCs may be due to the higher rates of human papillomavirus (HPV) infection in immunosuppressed populations, however the most convincing evidence is available for the link between HPV and SCC. An etiologic association between HPV and BCC remains to be elucidated.[32] In addition to viral oncogenic infections, decreased immune-surveillance and the direct effect of the immunosuppressive medications

used by OTR causes them to be more vulnerable to NMSC development.[32] The influence of an immune response in BCC development was previously reported and shows that transplantation of human BCC biopsies is only successful in mice deficient of T, B and natural killer (NK) cells.[33] However, BCC nest are frequently, but not always, surrounded by an infiltrate consisting of T cells and only very few B lymphocytes and NK cells that do generally not invade the tumor nests.[34] Although complete tumor eradication is rare[30], higher number of T cells is seen in actively regressing BCCs.[34] Generally, BCCs express no or low levels of MHC class I (MHC-I) molecules and as a consequence there is an absence of infiltrating $CD8^+$ effector T cells. Following treatment with topical imiquimod, a Toll-like receptor-7 antagonist, significantly higher expression levels of MHC-I accompanied by an increase of peritumoral $CD8^+$ T cells were seen.[30] This pro-inflammatory Th1 initiated anti-tumor immune response has also been implicated in the well-established spontaneous regression of BCC after a biopsy.[35] Also, A Th2 anti-inflammatory response has been described in BCCs, which suggests a dynamic state in where pro- and anti-inflammatory immune response coexist and compete.[36] The exact mechanisms underlying an effective tumor specific immune response is unclear as there are still many missing links. In our study described in **chapter 4.1**, an extensive infiltrate was seen in the BCCs treated with topical diclofenac, in the majority of the cases even resulting in complete histological tumor regression. The nature of this immuneresponse will be analyzed in a future study in an attempt to unravel the role of the BCC immuneresponse.

DNA methylation in BCC and the future

As epigenetic alterations are potentially reversible, drugs have been developed to inhibit enzymes that catalyze epigenetic modifications. In the case of DNA methylation, these are the DNA methyltransferases (DNMTs), consisting of 3 main subtypes. DNMT1 maintains existing methylation during DNA replication, while DNMT3A and DNMT3B are required for ***de novo*** methylation. To date, the FDA has approved two demethylating drugs for the treatment of higher-risk myelodysplastic syndrome: 5-azacytidine (5-aza-CR; manufactured by Celgene as Vidaza) and 5-aza-2′-deoxycytidine (5-aza-CdR; manufactured by Eisai as Dacogen). However, these drugs have substantial side effects, which makes them less useful for BCC treatment, as the advantage of being a non-invasive treatment will not outweigh these side effects.[37] The systemic side effects could be avoided by using the 5-aza's topically. Unfortunately, they are both pharmalogically unstable agents, which impedes their topical formulation.

Whereas DNA methylation changes in cancer constitute a potential target for treatment, they could also be used to develop biomarkers for more accurate detection of cancer, a better insight in tumor prognosis, and even to predict treatment response.[2] In contrast to genetic biomarkers that detect the numerous different point mutations occurring throughout the length of the gene, DNA hypermethylation consistently affects a small promoter region, facilitating analysis. Also, DNA methylation alterations occur in higher percentages of tumors than genetic variations, resulting in a higher sensitivity.[37] The utility of biomarkers for BCC treatment can be debated, as these tumors are generally characterized by indolent growth, an excellent prognosis and easy accessibility

for assessment and treatment. However, given the large number of patients that will develop at least one or more subsequent BCC after the first BCC, a prognostic biomarker that could predict the individual risk of (recurrent) BCC could be extremely useful. It would enable classification of patients as having a low, an intermediate or a high risk of developing BCC. Using this information, follow-up schemes could be tailored to the patient's individual risk, thereby making care for BCC more efficient and cost-effective.

Beyond DNA methylation

In our work, we focused on DNA (cytosine) methylation, because it is the best-defined epigenetic change in malignancy. Hydroxymethylation of cytosine (5-hmC) is a more recently described modification, that affects the flexibility and stability of DNA differently from methylation. The 5-hmC level is strongly reduced in tumors of the brain, kidney, lungs, skin and liver, from which it was suggested that 5-hmC loss could serve as biomarker for the detection of cancers. However, the exact role of 5-hmC in the development of cancer remains unknown.[38,39]

Additional epigenetic modifications that have been found to be involved in human cancers include histone modifications, chromatin remodeling and the activity of non-coding RNAs (ncRNAs).[3,40] These different processes work together to establish and maintain the global and local condensed or decondensed chromatin states that eventually determine gene expression.[2] DNA is tightly compacted around histones to the nucleosome which is the basic unit of the chromatin.[40] These histones can be modified post-transcriptionally by (hyper) acetylation, methylation, phosphorylation and ubiquination. Those different processes affect, among others, transcriptional regulation, DNA repair, DNA replication, alternative splicing, and chromosome condensation.[3] Hyperacetylation of the lysines in histone tails is the best described modification and is facilitated by Histone Acetyltransferases (HATs). This results in a decondensed chromatin confirmation that can be actively transcribed. On the other hand, histone deacetylases (HDACs) restore the condensed state, resulting in gene silencing by preventing binding of transcription factors and RNA polymerases.[40] In the case of tumor suppressor genes, this means that tumor development is facilitated. Thus, HDAC inhibition could be used to treat cancer. Indeed, Vorinostat (SAHA). a synthetic HDAC inhibitor (HDACi) was approved by the FDA in 2006 for the treatment of cutaneous T-cell lymphoma.[41] Two more (romidepsin (Istodax) and belinostat (Beleodaq)) have followed in the past few years and many HDACis are in different stages of clinical trials for various hematological and solid tumors.[42] Apart from the direct effect of HDCAis on acetylation resulting in re-expression of the gene, HDCAis can also target processes involved in tumor progression, cell cycle control, apoptosis, angiogenesis and cell invasion.[42] Interestingly, HDACis have been shown to suppress SHH activity through Gli1, Gli2 and Gli3 in vitro.[43] A newly designed chimeric compound, NL-103, which integrates the functional inhibitory group of the HDACi vorinostat and SHH inhibitor vismodegib, downregulates the expression of Gli2 in a NIH3T3-12Gli mouse embryo fibroblast cell line. It was concluded that NL-103 can effectively inhibit SHH and that dual inhibition of HDACs and SHH signaling could help to overcome vismodegib[44], a phenomenon that is described in more detail in part two.

The dynamic process of chromatin remodeling is not only regulated by histone acetylation/deacetylation, but also through ATP-dependent protein complex formation, histone modification by polycomb proteins, and by interaction with ncRNAs.[40] ncRNAs are RNA fragments that are not translated into proteins of which several subtypes can be identified such as miRNA, siRNA, piRNA, XiRNA and long ncRNAs.[40] RNA interference (RNAi) is a part of a small regulatory RNA, including siRNA and miRNA, that regulates gene silencing. RNAi based therapies are already being tested in phase 1 studies as treatment in several solid tumors (brain, breast) and considered to be safe in use. These drugs have the benefit of being highly specific and they have only little side effects.[45] A comprehensive description of all possible epigenetic modification processes is beyond the scope of this thesis. But it is evident that the rise in knowledge on epigenetic modifications in human cancers like BCC can yield considerable diagnostic, therapeutic and even predictive opportunities in the (near) future.

mTOR signaling in BCC

Whereas most research on BCC has been focused on SHH and WNT signaling, it is important to think ***out of the box*** in order to unravel the pathogenesis of this tumor completely. As an example of such thinking, when looking for therapeutic targets we considered that BCCs are thought to be hair follicle tumors.[46] The hair follicle is considered as a moderately to severely hypoxic microenvironment, while the dermis is well oxygenated and the epidermis is only modestly hypoxic.[47] Therefore we hypothesized that hypoxia response pathways could be involved in the pathogenesis of hair follicle tumors such as BCCs. Hypoxia-inducible transcription factor 1 (HIF1) is a primary mediator of hypoxia-induced gene expression in human hair follicles.[48] HIF1 transcription can be regulated by mechanistic/mammalian target of rapamycin 1 (mTORC1) via 4E-binding protein 1 (4E-BP1), ribosomal protein S6 kinase-1 (S6K1) and signal transducer and activation of transcription 3 (STAT3).[49] mTORC1 in turn is stimulated by PI3K-Akt signaling pathways and once activated, it promotes several processes like angiogenesis, cell growth and tumorigenesis.[50] The importance of mTORC1 in the pathogenesis of different cancer types, including renal carcinoma and colon carcinoma is well recognized and has already resulted in the development of treatments that target mTORC-dependent signaling events.[51] Interestingly, activated mTORC signaling is also associated with the development of benign skin tumors like angiofibromas in patients with tuberous sclerosis.[48]

If HIF and mTORC1 pathways do contribute to the growth of hair follicle tumors, new opportunities for targeted therapy and diagnostics could emerge. In **chapter 3.1** we provide a systematic analysis of HIF and mTORC1 signaling in BCC and its benign counterpart, trichoepithelioma (TE). TEs strongly resemble BCC both on the macroscopic and microscopic level, but do not exhibit invasive growth. Differentiation between these two hair follicle-derived tumors is important because of their distinct biologic behavior and therapeutic approach. Immunohistochemical analysis of HIF1, mTORC1 and their most important target genes showed that both HIF and mTORC1 signaling seem to be active in BCC as well as in TE. However, there were no appreciable differences between these two tumors with respect to pathway activity. To date, immunohistochemical analyses of HIF, mTORC1 and their tar-

get genes does not provide a reliable diagnostic tool for the discrimination of BCC and TE.

In conclusion, we and others have shown that sporadic BCCs are tumors driven by a variety of derailed signaling systems, beyond SHH signaling. This observation is in line with the fact that sporadic BCC exhibit a mostly moderate response to targeted SHH inhibitors. There are thus several potential drug targets already, and doubtlessly many more remain to be discovered.

Part two: new topical and systemic treatment modalities for BCC

Vitamin D and NSAIDs as anticancer agents

Targeted non-invasive treatments: vitamin D

The primary aim of BCC treatment is complete tumor eradication. However, maximal preservation of function and cosmesis at the treatment site are very important aspects that must be considered when choosing an appropriate therapy. The treatment of choice largely depends on clinical and histopathological characteristics. The risk of recurrence should be carefully evaluated in order to avoid overtreatment of low-risk BCCs or under-treatment of high-risk BCCs.[52] Conventional surgical excision is the gold standard in treatment of all BCCs, with success rates varying from 90-98% for primary (untreated) BCCs.[53,54] For sBCCs, non-invasive treatment modalities such as photodynamic therapy (PDT) (photochemical), imiquimod (immune-modulating) and 5-fluorouracil (5-FU) cream (chemotherapeutic) are well studied and frequently prescribed.[55] Long term tumor-free survival rates in sBCCs are high, varying from 72.8-84.0% in PDT, 83.4-87.3% for Imiquimod and tumor-free survival rates of 80.1% were found after treatment with 5-flourouracil.[55,56] This illustrates that cure rates following surgical excision are still much higher. At the time of the clinical trial described in **chapter 4.1**, there were no pertinent data to support non-invasive treatments for nBCCs. Likewise, according to the Dutch 'evidenced-based guideline treatment of the basal cell carcinoma', non-invasive treatment modalities were reserved for sBCCs only. We concluded that there was still an opportunity for (the development of) alternative or additional non-invasive treatment modalities for sBCCs as well as nBCCs.

Current research in oncology is focusing on the development of targeted cancer treatments that are specifically aimed at key signaling pathways instead of the more generally used, non-specific cytotoxic agents. Interestingly, sometimes drugs already available for the treatment of one disease turn out to be effective against a different disease (including human cancers), which is known as drug repurposing. The active metabolite of vitamin D, 1α,25(OH)2D3 (calcitriol) is of particular interest in this regard. Preclinical research has indicated that this metabolite or other vitamin D analogues might have potential as anticancer agents because of their antiproliferative effects, activation of apoptotic pathways and inhibition of angiogenesis.[57] Vitamin D is known to be a steroid hormone crucial in calcium homeostasis and regulation of bone metabolism. It also has many other biological actions that are still not fully characterized.[58] In the skin, calcitriol is mainly synthesized under influence of UVB exposure.

For that reason, sun protection advice given by dermatologists in order to prevent skin aging and skin cancer is often disobeyed because patients fear becoming vitamin D deficient. Indeed, sunscreens do block the vitamin D production, however in daily practice, typically only little amounts of sunscreen are applied to the skin and vitamin D deficiency therefore is not normally an issue. Mean daily exposure during 15-30 minutes (depending on latitude and season) of the face, hands and underarms without any sun protection would generate the required amount of vitamin D in people with Fitzpatrick skin type I-III. However, dermatologists and cancer groups, including the Skin Cancer Foundation of the United States discourage such unprotected UV exposure, as all unprotected UV exposure contributes to cumulative DNA damage and thus to a further increased risk of skin cancer development. Their advice is to substitute vitamin D orally. In a time where vitamin D (deficiency) seems to be a real hype, it is important to realize that neither the influence of vitamin D insufficiency nor the health benefit of maintaining high serum vitamin D levels has been established for the general population.[59] Animal and cellular studies strongly suggest a role for vitamin D in the prevention and treatment of various human cancers. So far, however, clinical studies in most cancers have not yet delivered compelling evidence to support the use of vitamin D supplements in daily practice.[60]

In keratinocytes, the vitamin D receptor (VDR) interferes in both SHH- and WNT signaling and that way seems to be crucial in the tumorigenic response.[61] Also, normal keratinocytes and melanocytes respond to calcitriol with a reduction in proliferation and an increase in differentiation.[62] There is some evidence to suggest that high vitamin D serum levels might protect against BCC development.[63] Conversely, an association with higher serum vitamin D levels and a higher risk of BCC has also been shown in two studies.[64,65] This higher risk may well be explained by the fact that whereas UVB exposure does lead to higher vitamin D serum levels, it will also cause the DNA damage that results in skin cancer.[61] Additionally, previous *in vitro* studies found that high doses of calcitriol can inhibit keratinocyte proliferation, while lower doses may stimulate proliferation.[62,66] Apparently, the balance between the positive and negative effects of vitamin D is a very delicate one. In our study, described in **chapter 4.1,** topical calcitriol applied twice daily to sBCCs and nBCCs during 8 weeks did not have any effect. No histopathological tumor clearance was found, nor was there any effect on the proliferation marker Ki-67 and anti-apoptosis marker Bcl-2. However, based on our results we cannot completely rule out that vitamin D analogs could treat BCC. The lack of response we observed might have been due to calcitriol being unable to reach sufficiently high concentrations in the tumors.

Targeted non-invasive treatments: diclofenac

Nonsteroidal anti-inflammatory drugs (NSAIDs) are well-known and frequently used drugs with analgesic, antipyretic and anti-inflammatory effects. There are many different types of NSAIDs. The main class effect is the inhibition of cyclooxygenases 1 (COX-1) and 2 (COX-2), with a consequent decrease in prostaglandin synthesis. COX-1 is a housekeeping gene that is constitutively expressed and controls normal physiological functions such as maintenance of the gastric mucosa and platelet function, vascular homeostasis, as well as renal blood flow. In contrast, COX-2 is an immediate-early gene, and expression is generally be-

low detection levels.[67] COX-2 modulates cell proliferation, angiogenesis and suppression of apoptosis.[68] In BCCs, COX-2 is commonly highly expressed under influence of UV radiation and multiple other inflammatory stimuli, whereas in normal skin COX-2 is usually undetectable.[67,68] Diclofenac is the most widely prescribed non-selective COX inhibitor worldwide.[69] Over the past three decades, epidemiologic, clinical, and experimental studies have established a chemopreventive effect of NSAIDs in various tissues. However NSAIDs are not used as chemopreventive agent because of incomplete efficacy and toxicities.[70] The effect of the oral selective COX-2 inhibitor Celecoxib on the development of BCCs was studied in a double-blinded, randomized controlled clinical trial in 60 patients with BCNS. After Celecoxib (200 mg, twice daily, 24 months) a trend towards reduction in BCC burden in the treatment group in all subjects was sugested.[71] A recent meta-analysis did not find a statistically significant chemopreventive effect of NSAIDs on NMSC, not even after stratification for the different NSAIDs and for BCCs and SCCs.[72] Thus, there is no convincing evidence to date that regular use of systemic NSAIDs could serve as chemopreventive agent in the development of BCCs. However, as the local bioavailability is expected to be higher after local application, and considering the fact that NSAIDs can target the WNT signaling pathway that is activated in BCCs[73], we assumed that topically applied diclofenac could be effective in the treatment of (low-risk) BCCs. Moreover, diclofenac both directly and indirectly induces apoptosis.[74,75] In **chapter 4.1** we showed that application of diclofenac 3% gel with 2.5% hyaluronic acid for 8 weeks twice daily (under occlusion) to sBCC resulted in a complete histologic regression in 64.3% of all tumors. These results further support inflammation as a possible driver of BCC growth (see also **part one**). The histological clearance found was not as high as can be obtained with currently available non-invasive treatment modalities, however it should be noted that we made use of a diclofenac compound (Solaraze®) that is already FDA approved for the treatment of actinic keratosis (AK).[76] Previous dose-response studies for its specific use in BCC were not performed. Optimized dosage regimens might further improve efficacy. Apart from more targeted mode of action, further advantages of topical diclofenac are that it is cost-efficient, easy in use and that it has limited side effects. This makes topical diclofenac a promising drug for the treatment of (low-risk) BCCs.

Topical diclofenac as chemopreventative agent

Because of the promising results of topical diclofenac as treatment of sBCC and the ever larger number of patients affected, it is of interest to determine whether topical diclofenac could serve as a chemopreventative agent. About one third of the patients diagnosed with their first primary BCC will develop at least one subsequent BCC. This risk is highest within the first 6 months after diagnosis, but remains substantially high even after a follow-up period of 5 years. In particular patients with red hair, a higher socioeconomic status, and/or those with a BCC on their upper extremities have a higher risk of developing multiple BCCs.[77]

Topically applied 3% diclofenac gel with 2.5% hyaluronic acid (the same formula used in the clinical trial in **chapter 4.1**) has already been used in the treatment of AK and resulted in complete clinical clearance of 41-60% of AKs after 3 months of treatment.[76] Topical diclofenac in

twice-daily doses is approved by the FDA for the treatment of AK over a period of 60-90 days and it is prescribed for this purpose in many European countries, although its use is not recommended by the 'Dutch guideline actinic keratosis'. Given the inhibitory effect of diclofenac on cell proliferation and the promising response rates both in AK and BCC, topical diclofenac could presumably help to reduce the risk of new or subsequent BCC development. The side effects are usually mild to moderate (e.g. erythema, pruritus and erosion) and resolve without sequelae (**chapter 4.1**). Therefore, it might be of interest to explore whether diclofenac could be used as a preventive additive to daily skin care creams. With a lower concentration or a reduced frequency of application, known side effects could be reduced whilst retaining efficacy.

Targeted non-invasive treatments and future perspectives of BCC management

In contrast to most cancers, skin tumors are readily accessible for clinical examination and tissue samples for (immuno)histochemical (IHC) evaluation can easily be obtained. It might be argued that accurate clinical follow-up instead histological examination could be considered when assessing new topical treatments in clinical trials. However, immunohistochemical stains on pre- and post-treatment tissue samples can provide insight in the mode of action or efficacy of new treatment modalities. In quantifying the expression levels of specific immunohistochemical markers, percentages of positive tumor cells need to be determined. Manual pathologist-based quantification (MQ) is still the most frequently used technique for IHC evaluation[78] and is considered as the gold standard. However, MQ is time-consuming and labor-intensive.[79] It is also a subjective approach that may have high intra- and inter-observer variability.[80-82] Larger studies with more immunohistochemical stains to be investigated inevitably generate a large number of slides to be assessed, rendering MQ impractical.

Automated, digital image analysis might offer a solution and has been used since the 1980s. Several different methods are available.[83] We used a so-called operator-dependent semi-automated quantification (SAQ) computerized thresholding technique (Leica Qwin version 3.5.1, Leica Microsystems, Cambridge, UK), which is an extensively evaluated method, especially for mammographic density.[84,85] SAQ is time saving, provides easily accessible data and generates good reproducible results, even by generally inexperienced young researchers (**chapter 4.2**). However, we found large discrepancies between MQ and SAQ, that point to the need for studies in which consensus evaluation by more than one pathologist is compared with SAQ measurements. Evidence from a well-designed study that SAQ is a reproducible and accurate method for assessing immunohistochemical stains would be very relevant for research. Studies assessing changes in expression values in pre- versus post-treatment tissue samples are especially suitable for SAQ, as the change in expression is less susceptible for random errors.

In **chapter 4.1**, two pathologists manually assessed all pre- and post-treatment tissue samples. In contrast to the more promising results in sBCCs, in nBCCs we did not detect significant changes in proliferation, apoptosis or total tumor clearance after treatment with diclofenac, calcitriol or a combination of both. The difference in response between nBCCs and

sBCCs is in agreement with reports from other studies investigating non-invasive therapies. sBCCs spread from the epidermis into the papillary dermis, which makes the tumors more accessible to topical treatment. In contrast, nBCCs show deep, compact, and nodular growth[86] presumably leading to insufficient penetration of topically applied drugs. Only recently, high 3-year success rates (81.8%) were found for nBCCs treated with imiquimod for 12 weeks, 7 days a week.[54] This is an intensified treatment schedule compared to the presently licensed schedule of 5 days a week for 6 weeks. Imiquimod has several antitumor activities. The major biological effect of imiquimod is the activation of nuclear factor-kappa B (NF-κB) through toll-like receptors 7 (TLR7) and 8 (TLR8). Induction of NF-κB results in the production and secretion of pro-inflammatory cytokines and chemokines, stimulating a strong Th1-mediated antitumoral cellular immune response.[87] Additionally, imiquimod directly stimulates antitumoral activation of dendritic cells (DCs). DCs are important (tumor) antigen presenting cells in the skin and they are thought to be crucial in generating an aggressive immune response against cutaneous cancers.[51] Interestingly, a recent study in a *Patched1* deficient murine BCC cell line suggests that imiquimod may actually serve as a targeted therapy by directly inhibiting SHH signaling downstream of SMO. Imiquimod can cause GLI phosphorylation and subsequent reduction in GLI target genes.[88] The fact that imiquimod, apart from its immunomodulating effect, also seems to directly inhibit SHH signaling, could explain why it is the most effective topical treatment currently available.[55] Indeed (pre-) clinical evidence has suggested that simultaneously targeting SHH and other signaling pathways may have a synergistic effect.[89] In **chapter 4.1** we hypothesized that combining diclofenac gel and calcitriol ointment would enhance their individual efficacy. In our clinical trial, the combination did not show the expected synergistic effect. The observed clinical effect of the "combination therapy" arm was probably attributable to diclofenac, as there was no effect at all in the calcitriol arm. Since calcitriol ointment was applied five minutes after application of diclofenac gel, dilution or suboptimal absorption could explain the observed reduced efficacy compared to diclofenac monotherapy in sBCCs. Of note, in the combination therapy group, there were four non-responding tumors that appeared to have a nodular BCC component although they were initially diagnosed as sBCC on the biopsy. Since we also found that nodular BCCs did not respond to topical therapy, treatment failure in these patients was probably related to the histological subtype. Due to the sample size of 16 tumors per arm, those 4 non-responders with a different histological subtype had a large impact on the overall treatment outcome. Nevertheless, the promising results of topical diclofenac in sBCCs suggest new possibilities for the management of BCCs. Further optimization with dose-response trials and studies combining topical diclofenac with available (targeted) non-invasive therapies are needed to test whether topicals can obtain cure rates comparable to those of the gold standard, surgery. Based on our present understanding, treatment with diclofenac gel and imiquimod cream is a promising combination that we are currently investigating.

Non-invasive treatments in general can be expected to have several advantages, making them worth pursuing. They will generally give a better cosmetic outcome, with an increased quality of life for patients as a result.[54] Patients will be able to treat themselves at home, which makes them less dependent on hospital care. A second advantage of this home-

based treatment is that the workload for dermatologists will decrease, resulting in lower overall healthcare costs. Since the skin cancer epidemic is yet to peak, the workload for dermatologists is expected to increase. General practitioners might be able to help in this increase in demand. However, according to a self-administered questionnaire filled in by 268 general practitioners, over 50% of the general practitioners felt the need to know more about skin cancer care.[90] Intensified training programs on recognition and management of skin cancer are therefore essential to facilitate treatment by non-specialists. General practitioners are already used to take biopsies of suspicious lesions, but they generally do not perform therapeutic surgical excisions of BCCs (yet). Also, in general, they are not familiar with prescribing non-invasive treatments. Good collaborations between the general practitioners and dermatologists are necessary to improve care of the patient with (pre-) skin cancer, and is essential in order to reduce health care costs. The department of dermatology (MUMC) already collaborates with general practitioners in the so called '1.5th line of care' or 'stadspoliklinieken', where dermatologists visit the general practitioner one day a week in order to facilitate dermato(onco-)logical care by reducing the number of unnecessary referrals to the hospital. Another way to make current dermatological practice more efficient is the employment of specialized nurse practitioners and physician assistants, who could participate in the management of skin cancer. Of course, oncological care of the skin should then be thoroughly implemented in their educational training. With the appropriate knowledge, these paramedics could not only educate patients about skin cancer prevention, but they would also be able to recognize (pre-) skin malignancies and potentially even treat AK and low-risk BCCs.[91] Of note, high risk BCC, SCC and malignant melanoma should always be treated by a dermatologist, as they are associated with higher morbidity and even mortality. However, paramedics could serve as a first point of call for BCC patients and help manage further care. It should be stressed that paramedics should always collaborate with a dermatologist.

Targeted SMO-inhibitors in BCC

Vismodegib and (acquired) resistance

BCC is characterized by abberant activation of the SHH pathway, mostly due to inactivating mutations in the tumor suppressor gene *PTCH1* or activating mutations in the *SMO* oncogene.[92] SHH-inhibitors specifically target SMO, but GLI or other downstream pathway components may be targeted in the near future.[93] Vismodegib (Erivedge™, also known as GDC-0449) is an orally active synthetic SMO inhibitor and was approved by the FDA in 2012 for the treatment of laBCC and mBCC.[94] In July 2015, a second SMO inhibitor, sonidegib (Odomzo™, also known as LDE255), was approved by the FDA for laBCC not amenable to curative surgery or radiation therapy.[95] As the mode of action of these Hedgehog Pathway Inhibitors (HPIs) is identical, similar efficacy rates and adverse events are seen (class effect).[95,96] Recently, preplanned interim results of the largest international, open label trial to the safety and efficacy of vismodegib in laBCC and mBCC (STEVIE study) were published.[97] After at least 12 months of treatment, complete and partial response was seen in 32% of laBCC and 33% of mBCC patients. Twenty-seven percent of all patients treated had stable disease and progressive

disease was seen in 3% of the patients. The mean duration of treatment was 36.3 weeks for laBCCs and 52.0 weeks for mBCCs. Treatment was discontinued in no less than 80% of all patients treated in this study. Almost all patients reported one or more adverse events (99%), of which the most important events where muscle spasms, alopecia and dysguesia (loss of taste). These adverse events were the most important reason for treatment discontinuation. The authors concluded that the safety profile described in the STEVIE study was consistent with previous analyses of vismodegib in patients with laBCC and mBCC.[97] The department of dermatology of the MUMC participated in this STEVIE trial and included 6 patients.

Initial response to vismodegib followed by secondary tumor progression has been observed and is suggested to occur in 20% of the patients within the first year of treatment.[98-102] The mechanisms underlying resistance to vismodegib in BCCs may clinically be divided into primary resistance (no response to treatment) or secondary (acquired) resistance (progression after an initial response).[103] We purposely speak of a clinical classification, because it is still unclear whether the non-responding tumor cells are already present in the primary tumor and grow out to visible tumor due to selection, or whether HPIs may even induce mutations that lead to resistance. Acquired resistance to vismodegib has also been described in medulloblastoma, caused by a heterozygous *SMO* mutation that appeared in the metastatic medulloblastoma during treatment.[104] In this thesis, two patients with laBCCs who had developed respectively primary and secondary resistance to vismodegib are outlined. **Chapter 5.1** describes two heterozygous mutations ((p.Trp281Leu and p.Val321Met) in newly developed tumor tissue after 20 weeks of treatment in a laBCC patient who initially had a good clinical response to vismodegib. Neither *SMO* mutation was found in the laBCC biopsy specimen obtained before initiation of vismodegib therapy, or in the biopsy specimens of responding sclerotic skin during therapy. Analysis of a buccal swab revealed no mutations at all. It is of particular interest that the same novel somatic *PTCH1* mutation was detected in both the primary and the resistant BCC, and was absent in clinically and histologically responding tissue. This observation implies that cells from the original tumor had survived. Thus, even though the resistant tumors arose from the same, clonal primary tumor, a clonal origin of the resistant BCC nests is precluded, a finding that supports tumor heterogeneity. Interestingly, the *SMO* codon 321 that we found was previously described as a critical binding site for vismodegib binding[102], but codon 281 has never been related to vismodegib resistance. It appears that resistance to vismodegib in BCC can be caused by several different acquired *SMO* mutations that interfere with drug binding, a finding that is also described by Sharpe ***et.al.***[92] Also, in a few resistant cases, recurrent copy number variants in the downstream effectors *SUFU* and Gli2 were detected. There is a complex interplay between mutual and heterogeneous resistance mechanisms to SMO inhibition in BCCs, even within the same tumor.[92] The fact that sporadic BCC is a very heterogeneous tumor is confirmed by whole-exome sequencing of the genetic landscape of BCC, showing that BCCs harbor the most mutations of all known human cancers.[28] From this point of view, it could be suggested that the use of oral SMO inhibitors like vismodegib should be reserved for BCNS patients only, given the fact that these BCC are presumed to be obligatory and solely SHH driven, in contrast with sporadic BCC that appears to be more multifactorial (see **part one** of this discussion).

A better understanding of the tumorigenesis of sporadic BCC and the mechanisms underlying vismodegib resistance is important for the development of alternative treatment strategies. Sequential mutation analysis on pre-treatment and recurrent tumor tissue may help to anticipate on the type of resistance (either primary or secondary) in order to actively alter therapy. Then, treatment schedules and combination therapies can be customized to both patient and tumor characteristics, in an example of so-called personalized medicine. Overall, possible therapeutic approaches are sequential or rotational therapy with non-treatment periods ('drug holidays') in order to obtain a better tolerability for side effects, combination therapies, or alternating different therapies. Combining different systemic therapies in laBCC or mBCC may have the advantage of synergistic or additional therapeutic effects, lower dosage application and, possibly, the prevention of (acquired) resistance.[105] In addition, systemic itraconazole and nicotinamide might be promising agents in BCC treatment.[106,107]

Vismodegib, SCC and future analyses

Chapter 5.2 discusses a patient with a 15-year history of a recurrent infiltrative BCC of the nose and right cheek, who was included in the STEVIE study and treated with vismodegib. No clinical improvement was observed and we even feared for progression into SCC as impressive hyperkeratosis developed after 7 weeks of treatment. Formation of SCC within the initial BCC has been reported in several case studies describing patients treated with vismodegib, as has development of SCC and keratoacanthoma on other body sites.[108-113] In our patient, histologic examination of tissue specimens acquired during surgery performed 5 months after initiation of vismodegib treatment, revealed residual BCC cells with positive staining for staining Ber-Ep4 (BCC marker). No signs of basosquamous carcinoma or SCC were found. Also, there were epidermal cysts that stained negatively for Ber-Ep4, excluding the possibility that these were BCC remnants that had survived of the original tumor. A possible explanation for the emergence of the benign squamous neoplasms could be the effect of vismodegib on keratinocyte differentiation. Vismodegib can mimic the loss of Indian hedgehog (IHH), a member of the HH ligand family the loss of which was shown to promote the progression of benign papillomas to SCC.[114] In the case of SCCs emerging upon SMO inhibition, it has been hypothesized that a phenotypic switch from BCC to SCC, caused by new mutations (*NOTCH1/2* and *KMTC2*), could occur as a mechanism of tumor escape.[115] Secondly, SMO inhibition might promote carcinogenesis by selecting tumor cells driven by other molecular pathways, like RAS/MAPK signaling. Activation of RAS/MAPK signaling can override the oncogenic addiction of the tumor to SHH signaling and thereby enabling proliferation of resistant tumors with enhanced metastatic behavior.[116] However, further functional analysis is needed if the complex effects of vismodegib on keratinocyte differentiation and proliferation. Unfortunately, we were not able to perform any mutational analyses in our patient, mostly because no pre-treatment fresh frozen tissue samples had been obtained.

In daily dermatological and pathological practice, tissue samples acquired for diagnostic considerations are generally formalin-fixed paraffin-embedded (FFPE). DNA extracted from FFPE is fragmented and can also contain sequence artifacts arising from DNA dam-

age that can be difficult to distinguish from true mutations, especially in highly heterogeneous tumors such as BCC. These sequence variants are more often seen in FFPE than in fresh frozen tissue.[117] One of the potential techniques to overcome this problem is the use of molecular inversion probes (MIPs). MIPs provide a practical, highly sensitive method for the detection of low-frequency mutations and subclonal variations in FFPE, requiring low amount of sample input, while per-sample costs are very low.[118] This technique makes it possible to retrospectively perform pre- and post-treatment analysis. Such approaches will help to further unravel mechanisms of resistance, not only to vismodegib in BCC, but also to other small molecule inhibitors used to treat human malignancies such as melanoma.

Vismodegib and its place in daily dermatological practice

Systemic targeted treatments such as vismodegib are associated with potential toxicities and the number of patients reporting adverse events is considerably high, making its use limited to laBCCs and mBCCs. However an international multicenter clinical trial to the efficacy and safety of two different vismodegib regimens in patients with multiple BCC (MIKIE) (NCT01815840, clinicaltrials.gov) is currently ongoing. This study already anticipates on the fact that this group of patients will require a long-term treatment by introducing rotational therapy in which 'drug-holidays' of 8 weeks are implemented in an attempt to minimize adverse events. It can be speculated that also the occurrence of resistance may be diminished following rotational therapy. The interim results of the STEVIE study showed a median time to first onset of the most common adverse events of 2.83 months for muscle spasms, 5.55 months for alopecia, and 6.51 months for dysgeusia.[96] The side effects are cumulative and tend to get worse with increasing duration of the treatment. Temporary treatment stops e.g. every 3 months will lower or even prevent the burden of adverse events and is specially desirable in patients with multiple BCCs and BCNS. The possibility to use vismodegib in alternating treatment schedules in order to support a longer maintenance on treatment would provide an extra indication for FDA approval for this indication. However, experts in the field already implement drug-holidays, especially in BCNS patients, in which the treatment regimen is personalized to the patient and mainly patient driven rather than doctor driven. Interesting is a phase II, single-armed, multicenter trial (the NICCI trial), assessing the utility of vismodegib as neo-adjuvant treatment prior to surgery in laBCC and rBCC.[119] Although currently still off-label, limited case reports already describe results of neo-adjuvant treatment of laBCCs with vismodegib.[98,120,121] Neo-adjuvant therapy with vismodegib was effective in reducing the surgical defect, leading to less scarring and morbidity. However, long term follow-up is lacking and BCC remnants were found during Mohs surgery in the majority of the cases after three to six months of treatment with vismodegib.[98,120,121] One should be aware of the fact that reduction of BCC nests following vismodegib not necessarily occurs from the periphery to the center of the tumor. Thus when performing Mohs surgery of only the center of the clinically remaining tumor, (subclinical) scattered tumor nests in het former tumor area are probably missed. This inevitably results in inadequate treatment and a high risk of recurrence. If therapy is optimized possibly by combination therapy, and the percentage of resistance for therapy is decreased, we do agree that planning patients for surgical excision

may reduce the chance of recurrence and could offer interesting further treatment options. Currently, the use of vismodegib as systemic treatment is limited to laBCCs and mBCCs because of potential toxicities which do not outweigh the disadvantages of available treatments of regular low-risk BCCs. In these low risk tumors, topical SMO inhibitors would have a broader applicability, however the results of their effectiveness in humans are rather disappointing. Topical CUR61414 had little or no effect on proliferation and apoptosis of BCCs, assessed by Ki-67 and cleaved caspase-3 immunohistochemical stains respectively, probably due to low penetration or rapid clearance of the active component.[122] LDE225 0.75% cream is a different topical SMO inhibitor and although clinical regression is seen in almost all BCCs of eight BCNS patients after 4 weeks of treatment, histological examination of post-treatment biopsy samples of all tumors revealed that tumor nests were still present. A major advantage of topical application is the excellent safety profile with no local or systemic side effects.[123] Topical SMO inhibitors could emerge as an attractive non-invasive alternative for low-risk BCCs. However, future studies are needed to improve their efficacy and provide confident evidence for their use in daily practice.

The development and availability of small-molecule (targeted) inhibitors as vismodegib offer a new area in the management of BCC. As the dermatologist is the specialists with the most experience in treatment of different forms of skin cancer and the only specialist that has accessibility to all available treatment options, the dermatologist should always be involved in setting the indication for such treatments. In any case a multidisciplinary team in which the dermatologist, medical oncologist, plastic surgeon, radiotherapist and ENT-specialist closely cooperate should be involved. As laBCC/mBCC is generally a disease of the elderly, comorbidities and polypharmacy are not uncommon. The management of these patients requires a more holistic approach. Some have argued that the prescription and treatment with vismodegib should be restricted to medical oncologists. However, most dermatologists are experienced in prescribing systemic drugs and even cytostatic drugs and they are capable of dealing with possible side effects and adverse events. Treatment should be supervised by the physician who is experienced in the patient's ill-health and it is likely that the physician who decides to start vismodegib is the main therapist. In mBCCs, this could be the medical oncologist, but in laBCCs treatment by the dermatologists seems more logical. A good collaboration between all members of the multidisciplinary team is a key point. As the number of patients is limited, it is important to gain and share experiences with all team members and discuss cases regularly.

References

1. Esteller M. Epigenetics in cancer. The New England journal of medicine 2008;358:1148-59.
2. Rodriguez-Paredes M, Esteller M. Cancer epigenetics reaches mainstream oncology. Nature medicine 2011;17:330-9.
3. Portela A, Esteller M. Epigenetic modifications and human disease. Nature Biotechnology 2010;28:1057-68.
4. Goldberg M, Rummelt C, Laerm A, Helmbold P, Holbach LM, Ballhausen WG. Epigenetic silencing contributes to frequent loss of the fragile histidine triad tumour suppressor in basal cell carcinomas. The British journal of dermatology 2006;155:1154-8.
5. Heitzer E, Bambach I, Dandachi N, Horn M, Wolf P. PTCH promoter methylation at low level in sporadic basal cell carcinoma analysed by three different approaches. Experimental dermatology 2010;19:926-8.
6. Sathyanarayana UG, Moore AY, Li L, et al. Sun exposure related methylation in malignant and non-malignant skin lesions. Cancer letters 2007;245:112-20.
7. van Doorn R, Gruis NA, Willemze R, van der Velden PA, Tensen CP. Aberrant DNA methylation in cutaneous malignancies. Seminars in oncology 2005;32:479-87.
8. Wu W, Zhang J, Yang H, Shao Y, Yu B. Examination of AKAP12 promoter methylation in skin cancer using methylation-sensitive high-resolution melting analysis. Clinical and experimental dermatology 2011;36:381-5.
9. Stamatelli A, Vlachou C, Aroni K, Papassideri I, Patsouris E, Saetta AA. Epigenetic alterations in sporadic basal cell carcinomas. Archives of dermatological research 2014;306:561-9.
10. Katiyar SK, Singh T, Prasad R, Sun Q, Vaid M. Epigenetic alterations in ultraviolet radiation-induced skin carcinogenesis: interaction of bioactive dietary components on epigenetic targets. Photochemistry and photobiology 2012;88:1066-74.
11. Ikehata H, Ono T. The mechanisms of UV mutagenesis. Journal of Radiation Research 2011;52:115-25.
12. Flohil SC, Seubring I, van Rossum MM, Coebergh JW, de Vries E, Nijsten T. Trends in Basal Cell Carcinoma Incidence Rates: A 37-Year Dutch Observational Study. The Journal of investigative dermatology 2012.
13. Schuebel KE, Chen W, Cope L, et al. Comparing the DNA hypermethylome with gene mutations in human colorectal cancer. PLoS genetics 2007;3:1709-23.
14. Helmbold P, Richter AM, Walesch S, et al. RASSF10 promoter hypermethylation is frequent in malignant melanoma of the skin but uncommon in nevus cell nevi. The Journal of investigative dermatology 2012;132:687-94.
15. Helmbold P, Lahtz C, Enk A, et al. Frequent occurrence of RASSF1A promoter hypermethylation and Merkel cell polyomavirus in Merkel cell carcinoma. Molecular carcinogenesis 2009;48:903-9.
16. Esteller M. Aberrant DNA methylation as a cancer-inducing mechanism. Annu Rev Pharmacol Toxicol 2005;45:629-56.
17. Derks S, Lentjes MH, Hellebrekers DM, de Bruine AP, Herman JG, van Engeland M. Methylation-specific PCR unraveled. Cell Oncol 2004;26:291-9.
18. Harris RA, Wang T, Coarfa C, et al. Comparison of sequencing-based methods to profile DNA methylation and identification of monoallelic epigenetic modifications. Nature Biotechnology 2010;28:1097-105.
19. Mensaert K, Denil S, Trooskens G, Van Criekinge W, Thas O, De Meyer T. Next-generation technologies and data analytical approaches for epigenomics. Environ Mol Mutagen 2014;55:155-70.
20. Sandoval J, Heyn H, Moran S, et al. Validation of a DNA methylation microarray for 450,000 CpG sites in the human genome. Epigenetics 2011;6:692-702.
21. De Meyer T, Mampaey E, Vlemmix M, et al. Quality evaluation of methyl binding domain based kits for enrichment DNA-methylation sequencing. PloS one 2013;8:e59068.
22. Brinkman AB, Simmer F, Ma K, Kaan A, Zhu J, Stunnenberg HG. Whole-genome DNA methylation profiling using MethylCap-seq. Methods 2010;52:232-6.

23. van Vlodrop IJ, Niessen HE, Derks S, et al. Analysis of promoter CpG island hypermethylation in cancer: location, location, location! Clinical cancer research : an official journal of the American Association for Cancer Research 2011;17:4225-31.
24. Rausch MP, Hastings KT. GILT modulates CD4+ T-cell tolerance to the melanocyte differentiation antigen tyrosinase-related protein 1. The Journal of investigative dermatology 2012;132:154-62.
25. Ciurea ME, Cernea D, Georgescu CC, et al. Expression of CXCR4, MMP-13 and beta-catenin in different histological subtypes of facial basal cell carcinoma. Romanian journal of morphology and embryology = Revue roumaine de morphologie et embryologie 2013;54:939-51.
26. Tarca AL, Draghici S, Khatri P, et al. A novel signaling pathway impact analysis. Bioinformatics 2009;25:75-82.
27. Muehleisen B, Jiang SB, Gladsjo JA, Gerber M, Hata T, Gallo RL. Distinct innate immune gene expression profiles in non-melanoma skin cancer of immunocompetent and immunosuppressed patients. PloS one 2012;7:e40754.
28. Jayaraman SS, Rayhan DJ, Hazany S, Kolodney MS. Mutational landscape of basal cell carcinomas by whole-exome sequencing. The Journal of investigative dermatology 2014;134:213-20.
29. Kasiske BL, Snyder JJ, Gilbertson DT, Wang C. Cancer after kidney transplantation in the United States. American journal of transplantation : official journal of the American Society of Transplantation and the American Society of Transplant Surgeons 2004;4:905-13.
30. Walter A, Barysch MJ, Behnke S, et al. Cancer-testis antigens and immunosurveillance in human cutaneous squamous cell and basal cell carcinomas. Clinical cancer research : an official journal of the American Association for Cancer Research 2010;16:3562-70.
31. Zavos G, Karidis NP, Tsourouflis G, et al. Nonmelanoma skin cancer after renal transplantation: a single-center experience in 1736 transplantations. International journal of dermatology 2011;50:1496-500.
32. Wheless L, Jacks S, Mooneyham Potter KA, Leach BC, Cook J. Skin cancer in organ transplant recipients: more than the immune system. Journal of the American Academy of Dermatology 2014;71:359-65.
33. Carlson JA, Combates NJ, Stenn KS, Prouty SM. Anaplastic neoplasms arising from basal cellcarcinoma xenotransplants into SCID-beige mice. Journal of cutaneous pathology 2002;29:268-78.
34. Urosevic M, Dummer R. Immunotherapy for nonmelanoma skin cancer: Does it have a future? Cancer 2002;94:477-85.
35. Fujimura T, Kakizaki A, Kambayashi Y, Aiba S. Basal cell carcinoma with spontaneous regression: a case report and immunohistochemical study. Case Reports in Dermatology 2012;4:125-32.
36. Kaporis HG, Guttman-Yassky E, Lowes MA, et al. Human basal cell carcinoma is associated with Foxp3+ T cells in a Th2 dominant microenvironment. The Journal of investigative dermatology 2007;127:2391-8.
37. Heyn H, Esteller M. DNA methylation profiling in the clinic: applications and challenges. Nature reviews Genetics 2012;13:679-92.
38. Orr BA, Haffner MC, Nelson WG, Yegnasubramanian S, Eberhart CG. Decreased 5-hydroxymethylcytosine is associated with neural progenitor phenotype in normal brain and shorter survival in malignant glioma. PloS one 2012;7:e41036.
39. Shukla A, Sehgal M, Singh TR. Hydroxymethylation and its potential implication in DNA repair system: A review and future perspectives. Gene 2015;564:109-18.
40. Romani M, Pistillo MP, Banelli B. Environmental Epigenetics: Crossroad between Public Health, Lifestyle, and Cancer Prevention. BioMed research international 2015;2015:587983.
41. Marks PA, Breslow R. Dimethyl sulfoxide to vorinostat: development of this histone deacetylase inhibitor as an anticancer drug. Nat Biotechnol 2007;25:84-90.
42. Mottamal M, Zheng S, Huang TL, Wang G. Histone deacetylase inhibitors in clinical studies as templates for new anticancer agents. Molecules 2015;20:3898-941.
43. Canettieri G, Di Marcotullio L, Greco A, et al. Histone deacetylase and Cullin3-REN(KCTD11) ubiquitin ligase interplay regulates Hedgehog signalling through Gli acetylation. Nature cell biology 2010;12:132-42.

44. Zhao J, Quan H, Xie C, Lou L. NL-103, a novel dual-targeted inhibitor of histone deacetylases and hedgehog pathway, effectively overcomes vismodegib resistance conferred by Smo mutations. Pharmacology Researcg and Perspectives 2014;2:e00043.
45. Mansoori B, Sandoghchian Shotorbani S, Baradaran B. RNA interference and its role in cancer therapy. Advanced Pharmaceutical Bulletin 2014;4:313-21.
46. Peterson SC, Eberl M, Vagnozzi AN, et al. Basal cell carcinoma preferentially arises from stem cells within hair follicle and mechanosensory niches. Cell Stem Cell 2015;16:400-12.
47. Evans SM, Schrlau AE, Chalian AA, Zhang P, Koch CJ. Oxygen levels in normal and previously irradiated human skin as assessed by EF5 binding. The Journal of investigative dermatology 2006;126:2596-606.
48. van Steensel MA, van Geel M, Badeloe S, Poblete-Gutierrez P, Frank J. Molecular pathways involved in hair follicle tumor formation: all about mammalian target of rapamycin? Experimental dermatology 2009;18:185-91.
49. Dodd KM, Yang J, Shen MH, Sampson JR, Tee AR. mTORC1 drives HIF-1alpha and VEGF-A signalling via multiple mechanisms involving 4E-BP1, S6K1 and STAT3. Oncogene 2015;23;34(17):2239-50.
50. Wataya-Kaneda M. Mammalian target of rapamycin and tuberous sclerosis complex. Journal of dermatological science 2015;79:93-100.
51. Cargnello M, Tcherkezian J, Roux PP. The expanding role of mTOR in cancer cell growth and proliferation. Mutagenesis 2015;30:169-76.
52. Marzuka AG, Book SE. Basal Cell Carcinoma: Pathogenesis, Epidemiology, Clinical Features, Diagnosis, Histopathology, and Management. Yale Journal of Biology and Medicine 2015;88:167-79.
53. Evidenced-based guideline Treatment of the basal cell carcinoma. at http://www.nvpc.nl/uploads/stand/150414DOC-MB-Definitieve_richtlijn_Basaalcelcarcinoom_2014_goedgekeurd_ALV_14_april_2015154.pdf.)
54. Bath-Hextall F, Ozolins M, Armstrong SJ, et al. Surgical excision versus imiquimod 5% cream for nodular and superficial basal-cell carcinoma (SINS): a multicentre, non-inferiority, randomised controlled trial. The lancet oncology 2014;15:96-105.
55. Arits AH, Mosterd K, Essers BA, et al. Photodynamic therapy versus topical imiquimod versus topical fluorouracil for treatment of superficial basal-cell carcinoma: a single blind, non-inferiority, randomised controlled trial. The lancet oncology 2013;14:647-54.
56. Roozeboom MH, Arits AH, Nelemans PJ, Kelleners-Smeets NW. Overall treatment success after treatment of primary superficial basal cell carcinoma: a systematic review and meta-analysis of randomized and nonrandomized trials. The British journal of dermatology 2012;167:733-56.
57. Deeb KK, Trump DL, Johnson CS. Vitamin D signalling pathways in cancer: potential for anticancer therapeutics. Nature reviews Cancer 2007;7:684-700.
58. Mabey T, Honsawek S. Role of Vitamin D in Osteoarthritis: Molecular, Cellular, and Clinical Perspectives. International Journal of Endocrinol 2015;2015:383918.
59. Reddy KK, Gilchrest BA. What is all this commotion about vitamin D? The Journal of investigative dermatology 2010;130:321-6.
60. Bikle DD. Vitamin D and cancer: the promise not yet fulfilled. Endocrine 2014;46:29-38.
61. Bikle DD. The vitamin D receptor: a tumor suppressor in skin. Discovery Medicine 2011;11:7-17.
62. Bikle DD, Oda Y, Xie Z. Vitamin D and skin cancer: a problem in gene regulation. The Journal of steroid biochemistry and molecular biology 2005;97:83-91.
63. Tang JY, Parimi N, Wu A, et al. Inverse association between serum 25(OH) vitamin D levels and non-melanoma skin cancer in elderly men. Cancer Causes Control 2010;21:387-91.
64. Asgari MM, Tang J, Warton ME, et al. Association of prediagnostic serum vitamin D levels with the development of basal cell carcinoma. The Journal of investigative dermatology 2010;130:1438-43.
65. Eide MJ, Johnson DA, Jacobsen GR, et al. Vitamin D and nonmelanoma skin cancer in a health maintenance organization cohort. Archives of dermatology 2011;147:1379-84.

66. Bollag WB, Ducote J, Harmon CS. Biphasic effect of 1,25-dihydroxyvitamin D3 on primary mouse epidermal keratinocyte proliferation. Journal of cellular physiology 1995;163:248-56.

67. Muller-Decker K. Cyclooxygenase-dependent signaling is causally linked to non-melanoma skin carcinogenesis: pharmacological, genetic, and clinical evidence. Cancer metastasis reviews 2011;30:343-61.

68. Reinau D, Surber C, Jick SS, Meier CR. Nonsteroidal anti-inflammatory drugs and the risk of nonmelanoma skin cancer. International journal of cancer 2015;137:144-53.

69. McGettigan P, Henry D. Use of non-steroidal anti-inflammatory drugs that elevate cardiovascular risk: an examination of sales and essential medicines lists in low-, middle-, and high-income countries. PLoS Medicine 2013;10:e1001388.

70. Gurpinar E, Grizzle WE, Piazza GA. NSAIDs inhibit tumorigenesis, but how? Clinical cancer research : an official journal of the American Association for Cancer Research 2014;20:1104-13.

71. Tang JY, Aszterbaum M, Athar M, et al. Basal cell carcinoma chemoprevention with nonsteroidal anti-inflammatory drugs in genetically predisposed PTCH1+/- humans and mice. Cancer prevention research (Philadelphia), Pa;3:25-34.

72. Zhang B, Liang X, Ye L, Wang Y. No chemopreventive effect of nonsteroidal anti-inflammatory drugs on nonmelanoma skin cancer: evidence from meta-analysis. PloS one 2014;9:e96887.

73. Roos J, Grosch S, Werz O, et al. Regulation of tumorigenic Wnt signaling by cyclooxygenase-2, 5-lipoxygenase and their pharmacological inhibitors: A basis for novel drugs targeting cancer cells? Pharmacology and Therapeutics 2015;november.

74. Fecker LF, Stockfleth E, Nindl I, Ulrich C, Forschner T, Eberle J. The role of apoptosis in therapy and prophylaxis of epithelial tumours by nonsteroidal anti-inflammatory drugs (NSAIDs). The British journal of dermatology 2007;156 Suppl 3:25-33.

75. Barker N, Clevers H. Mining the Wnt pathway for cancer therapeutics. Nature reviews 2006;5:997-1014.

76. Cayirli M, Kose O, Demiriz M. Clinical, dermoscopic and immunohistochemical assessment of actinic keratoses and evaluation of the effectiveness of diclofenac therapy with immunohistochemical analysis. Archives of dermatological research 2013;305:389-95.

77. Kiiski V, de Vries E, Flohil SC, et al. Risk factors for single and multiple basal cell carcinomas. Archives of dermatology 2010;146:848-55.

78. Camp RL, Chung GG, Rimm DL. Automated subcellular localization and quantification of protein expression in tissue microarrays. Nature medicine 2002;8:1323-7.

79. Daniel K, Maria A, Amelie L, et al. Somatostatin receptor immunohistochemistry in neuroendocrine tumors: comparison between manual and automated evaluation. International journal of clinical and experimental pathology 2014;7:4971-80.

80. Taylor CR, Levenson RM. Quantification of immunohistochemistry--issues concerning methods, utility and semiquantitative assessment II. Histopathology 2006;49:411-24.

81. Rubin MA, Zerkowski MP, Camp RL, et al. Quantitative determination of expression of the prostate cancer protein alpha-methylacyl-CoA racemase using automated quantitative analysis (AQUA): a novel paradigm for automated and continuous biomarker measurements. The American journal of pathology 2004;164:831-40.

82. Polley MY, Leung SC, McShane LM, et al. An international Ki-67 reproducibility study. Journal of the National Cancer Institute 2013;105:1897-906.

83. Matos LL, Stabenow E, Tavares MR, Ferraz AR, Capelozzi VL, Pinhal MA. Immunohistochemistry quantification by a digital computer-assisted method compared to semiquantitative analysis. Clinics (Sao Paulo, Brazil) 2006;61:417-24.

84. Yaffe MJ. Mammographic density. Measurement of mammographic density. Breast cancer research : BCR 2008;10:209.

85. Lobbes MB, Cleutjens JP, Lima Passos V, et al. Density is in the eye of the beholder: visual versus semi-automated assessment of breast density on standard mammograms. Insights into imaging 2012;3:91-9.

86. Rippey JJ. Why classify basal cell carcinomas? Histopathology 1998;32:393-8.
87. Schon MP, Schon M. Imiquimod: mode of action. The British journal of dermatology 2007;157 Suppl 2:8-13.
88. Wolff F, Loipetzberger A, Gruber W, Esterbauer H, Aberger F, Frischauf AM. Imiquimod directly inhibits Hedgehog signalling by stimulating adenosine receptor/protein kinase A-mediated GLI phosphorylation. Oncogene 2013;32:5574-81.
89. Brechbiel J, Miller-Moslin K, Adjei AA. Crosstalk between hedgehog and other signaling pathways as a basis for combination therapies in cancer. Cancer treatment reviews 2014.
90. van Rijsingen MC, van Bon B, van der Wilt GJ, Lagro-Janssen AL, Gerritsen MJ. The current and future role of general practitioners in skin cancer care: an assessment of 268 general practitioners. The British journal of dermatology 2014;170:1366-8.
91. Reefman E BA, Kukutsch N, Bergman W. Huidige en toekomstige rol huidtherapeuten in huidoncologische zorg. Nederlands Tijdschrift voor Dermatologie en Venereologie 2015;25:341-2.
92. Sharpe HJ, Pau G, Dijkgraaf GJ, et al. Genomic analysis of smoothened inhibitor resistance in basal cell carcinoma. Cancer cell 2015;27:327-41.
93. Liu LS, Colegio OR. Molecularly targeted therapies for nonmelanoma skin cancers. International journal of dermatology 2013;52:654-65.
94. Axelson M, Liu K, Jiang X, et al. U.S. Food and Drug Administration approval: vismodegib for recurrent, locally advanced, or metastatic basal cell carcinoma. Clinical cancer research : an official journal of the American Association for Cancer Research 2013;19:2289-93.
95. Burness CB. Sonidegib: First Global Approval. Drugs 2015;75:1559-66.
96. Basset-Seguin N, Hauschild A, Grob JJ, et al. Vismodegib in patients with advanced basal cell carcinoma (STEVIE): a pre-planned interim analysis of an international, open-label trial. The lancet oncology 2015;16:729-36.
97. Basset-Seguin N, Sharpe HJ, de Sauvage FJ. Efficacy of Hedgehog Pathway Inhibitors in Basal Cell Carcinoma. Molecular cancer therapeutics 2015;14:633-41.
98. Chang AL, Atwood SX, Tartar DM, Oro AE. Surgical excision after neoadjuvant therapy with vismodegib for a locally advanced basal cell carcinoma and resistant basal carcinomas in Gorlin syndrome. JAMA dermatology 2013;149:639-41.
99. Chang AL, Oro AE. Initial assessment of tumor regrowth after vismodegib in advanced Basal cell carcinoma. Archives of dermatology 2012;148:1324-5.
100. Fecher LA. Systemic therapy for inoperable and metastatic basal cell cancer. Current treatment options in oncology 2013;14:237-48.
101. Madan V, Lear JT, Szeimies RM. Non-melanoma skin cancer. Lancet 2010;375:673-85.
102. Dijkgraaf GJ, Alicke B, Weinmann L, et al. Small molecule inhibition of GDC-0449 refractory smoothened mutants and downstream mechanisms of drug resistance. Cancer research 2011;71:435-44.
103. Pricl S, Cortelazzi B, Dal Col V, et al. Smoothened (SMO) receptor mutations dictate resistance to vismodegib in basal cell carcinoma. Molecular Oncology 2015;9:389-97.
104. Yauch RL, Dijkgraaf GJ, Alicke B, et al. Smoothened mutation confers resistance to a Hedgehog pathway inhibitor in medulloblastoma. Science 2009;326:572-4.
105. Flaherty KT, Infante JR, Daud A, et al. Combined BRAF and MEK inhibition in melanoma with BRAF V600 mutations. The New England journal of medicine 2012;367:1694-703.
106. Kim DJ, Kim J, Spaunhurst K, et al. Open-label, exploratory phase II trial of oral itraconazole for the treatment of basal cell carcinoma. Journal of clinical oncology : official journal of the American Society of Clinical Oncology 2014;32:745-51.
107. Chen AC, Martin AJ, Choy B, et al. A Phase 3 Randomized Trial of Nicotinamide for Skin-Cancer Chemoprevention. The New England journal of medicine 2015;373:1618-26.

108. Iarrobino A, Messina JL, Kudchadkar R, Sondak VK. Emergence of a squamous cell carcinoma phenotype following treatment of metastatic basal cell carcinoma with vismodegib. Journal of the American Academy of Dermatology 2013;69:e33-4.

109. Saintes C, Saint-Jean M, Brocard A, et al. Development of squamous cell carcinoma into basal cell carcinoma under treatment with Vismodegib. Journal of the European Academy of Dermatology and Venereology : JEADV 2015;29:1006-9.

110. Orouji A, Goerdt S, Utikal J, Leverkus M. Multiple highly and moderately differentiated squamous cell carcinomas of the skin during vismodegib treatment of inoperable basal cell carcinoma. The British journal of dermatology 2014;171:431-3.

111. Aasi S, Silkiss R, Tang JY, et al. New onset of keratoacanthomas after vismodegib treatment for locally advanced basal cell carcinomas: a report of 2 cases. JAMA dermatology 2013;149:242-3.

112. Poulalhon N, Dalle S, Balme B, Thomas L. Fast-growing cutaneous squamous cell carcinoma in a patient treated with vismodegib. Dermatology 2015;230:101-4.

113. Zhu GA, Sundram U, Chang AL. Two different scenarios of squamous cell carcinoma within advanced Basal cell carcinomas: cases illustrating the importance of serial biopsy during vismodegib usage. JAMA dermatology 2014;150:970-3.

114. Kakanj P, Reuter K, Sequaris G, et al. Indian hedgehog controls proliferation and differentiation in skin tumorigenesis and protects against malignant progression. Cell Reports 2013;4:340-51.

115. Ransohoff KJ, Tang JY, Sarin KY. Squamous Change in Basal-Cell Carcinoma with Drug Resistance. The New England journal of medicine 2015;373:1079-82.

116. Zhao X, Ponomaryov T, Ornell KJ, et al. RAS/MAPK Activation Drives Resistance to Smo Inhibition, Metastasis, and Tumor Evolution in Shh Pathway-Dependent Tumors. Cancer research 2015;75:3623-35.

117. Do H, Dobrovic A. Sequence artifacts in DNA from formalin-fixed tissues: causes and strategies for minimization. Clinical Chemistry 2015;61:64-71.

118. Hiatt JB, Pritchard CC, Salipante SJ, O'Roak BJ, Shendure J. Single molecule molecular inversion probes for targeted, high-accuracy detection of low-frequency variation. Genome Res 2013;23:843-54.

119. Leiter U HU, Gutzmer R, Haenssle H, Haefner HM, Mohr P, Garbe C. A phase II, single-armed, multicenter trial of neoadjuvant vismodegib in patients with large and/or recurrent basal cell carcinoma: NICCI. Journal of Clinical Oncology 2014;2014:suppl; abstr TPS9116.

120. Ally MS, Aasi S, Wysong A, et al. An investigator-initiated open-label clinical trial of vismodegib as a neoadjuvant to surgery for high-risk basal cell carcinoma. Journal of the American Academy of Dermatology 2014;71:904-11 e1.

121. Alcalay J, Tauber G, Fenig E, Hodak E. Vismodegib as a neoadjuvant treatment to Mohs surgery for aggressive basal cell carcinoma. Journal of drugs in dermatology : JDD 2015;14:219-23.

122. Tang T, Tang JY, Li D, et al. Targeting superficial or nodular Basal cell carcinoma with topically formulated small molecule inhibitor of smoothened. Clinical cancer research : an official journal of the American Association for Cancer Research 2011;17:3378-87.

123. Skvara H, Kalthoff F, Meingassner JG, et al. Topical treatment of Basal cell carcinomas in nevoid Basal cell carcinoma syndrome with a smoothened inhibitor. The Journal of investigative dermatology 2011;131:1735-44.