Diplomarbeit

Recurrent Nevus vs. Recurrent Melanoma

Differences in Epidemiology, BRAF-Mutation Status and Review of the Literature

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Graz, am 22.11.2015 *Nora Maria Woltsche eh.*

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Zusammenfassung

Einleitung: Rezidiv-Nävi sind viel häufiger als Rezidiv-Melanome, wobei die Unterscheidung der beiden Entitäten oftmals schwierig ist. Ziel dieser Studie ist es, Unterschiede zwischen Rezidiv-Nävi und Rezidiv-Melanomen hinsichtlich Epidemiologie sowie BRAF V600E-Mutationsstatus zu untersuchen.

Material & Methodik: Mittels der Datenbank der Dermatohistopathologie wurden die im Zeitraum 2010 bis 2015 an der Univ.-Klinik für Dermatologie & Venerologie Graz behandelten PatientInnen mit Rezidiv-Nävi und Rezidiv-Melanomen gesucht und hinsichtlich epidemiologischer Faktoren analysiert. Die BRAF V600E Mutationsanalyse wurde bei 50 PatientInnen am vorliegenden Präparat des Primärnävus sowie des Rezidivnävus und bei 13 PatientInnen am vorliegenden Präparat des Primärmelanoms sowie des Rezidivmelanoms durchgeführt.

Ergebnisse: Insgesamt 177 PatientInnen mit Rezidiv-Nävi sowie 28 PatientInnen mit Rezidiv-Melanomen wurden in die epidemiologische Analyse inkludiert. Das mittlere Alter der Nävi-PatientInnen lag bei 31 Jahren, bei Melanom-PatientInnen betrug es 65 Jahre. Rezidiv-Nävi waren gehäuft am Rücken und den Extremitäten lokalisiert, während Rezidiv-Melanome am häufigsten im Kopf/Hals-Bereich auftraten. Die Mehrheit der Nävi rezidivierte in den ersten 12 Monaten, der Gipfel der Rezidive beim malignen Melanom lag bei 13-36 Monaten nach Therapie des primären Tumors. BRAF V600E Mutationsstatus der Primär- sowie der Rezidivläsion konnte bei 36 dieser Nävi-PatientInnen und 12 dieser Melanom-PatientInnen evaluiert werden. 11/36 Nävi-PatientInnen zeigten die Mutation sowohl in der Primär- als auch in der Rezidiv-Läsion, 12/36 Nävi-PatientInnen zeigten die Mutation nur in der Primär-Läsion und 5/36-Nävi-PatientInnen wiesen die Mutation nur in der Rezidiv-Läsion auf. 2/12 Melanom-PatientInnen zeigten die Mutation nur im primären Tumor, wohingegen 3/12 Melanom-PatientInnen die Mutation nur im Rezidiv aufwiesen.

Schlussfolgerung: Rezidiv-Nävi waren assoziiert mit Alter <30 Jahren, Lokalisation am Rücken und Extremitäten und einem kürzeren Zeitraum bis zum Auftreten des Rezidivs, wohingegen Rezidiv-Melanome durch PatientInnen-Alter >65 Jahre,

Lokalisation im Kopf/Hals-Bereich und einen längeren Zeitraum bis zum Auftreten des Rezidivs gekennzeichnet waren. Die BRAF V600E-Mutation war häufiger in Rezidiv-Nävi als in Rezidiv-Melanomen zu beobachten.

Abstract

Introduction: Recurrent nevi are much more common than recurrent melanomas, differentiation often remains challenging. The aim of this study is to evaluate differences between recurrent nevi and recurrent melanoma regarding epidemiologic parameters and BRAF V600E mutation status.

Material & Methods: The data of patients with recurrent nevi and recurrent melanoma treated at the Department of Dermatology & Venereology, Medical University of Graz during 2010 – 2015 was collected and evaluated regarding epidemiologic parameters. BRAF V600E mutation analysis was performed for the primary and the recurrent lesion in 50 patients with recurrent nevi, and for the primary and the recurrent lesion in 13 patients with recurrent melanoma.

Results: A total of 177 patients with recurrent nevi and 28 patients with recurrent melanoma met the inclusion criteria for epidemiologic evaluation. Mean age of the patients with recurrent nevi was 31 years, whereas it was 65 years in patients with recurrent melanoma. The majority of recurrent nevi was located on the upper back and the extremities. Instead, recurrent melanoma was most commonly located on head/neck. The majority of nevi recurred in the first 12 months after initial diagnosis. The peak for recurrence in melanoma was 13-36 months. BRAF V600E mutation status in the primary and the recurrent lesion could be analyzed for 36 nevi patients and 12 melanoma patients. 11/36 nevi patients showed mutation in both, primary and recurrent lesion, 12/36 nevi patients showed mutation only in the primary lesion and 5/36 nevi patients showed mutation only in the recurrent lesion. 2/12 melanoma patients showed mutation in the primary lesion, while 3/12 melanoma patients showed mutation in the recurrent lesion.

Conclusion: In summary, recurrent nevi were associated with age younger than 30 years, location on back and extremities and shorter time to recurrence, whereas recurrent melanomas were associated with age older than 65 years, location head/neck and longer time to recurrence. BRAF V600E mutation was more commonly detected in recurrent nevi than in recurrent melanoma.

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Glossar und Abkürzungen

UV – ultraviolet

SSM – superficial spreading melanoma

NMM – nodular malignant melanoma

LMM – Lentigo-maligna melanoma

ALM – acral lentiginous melanoma

AMM – amelanotic malignant melanoma

AJCC – American Joint Committee on Cancer

SLNB – sentinel lymph node biopsy

DNA – deoxyribonucleic acid

PCR – polymerase chain reaction

OS – overall survival

PFS – progression-free survival

BRAFi – BRAF inhibitor therapy

TE – total/complete excision

EB – epidermolysis bullosa

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1 Introduction

1.1 Melanocytic tumors

1.1.1 Classification of benign melanocytic tumors

The term “melanocytic nevus” comprises a wide range of benign melanocytic proliferations that differ with respect to their epidemiology, morphology, genetics and associated melanoma risk. Basically two main groups of nevi can be differentiated: congenital melanocytic nevi (CMN) and acquired melanocytic nevi (AMN). The former refers to nevi that are present since birth or develop during the first two years of postnatal life, while the latter term comprises basically all nevi that develop later in life [1].

1.1.1.1 Congenital melanocytic nevi

Based on their clinical diameter and associated melanoma risk, CMN are commonly further subdivided into small <1.5cm, intermediate 1.5cm to 19.9cm and large ≥20cm CMN, whereby the risk for melanoma development increases with the clinical size [2]. The prevalence of CMN ranges from 1% to 6% in newborns [3], the incidence of CMN measuring at least 10cm in diameter is about 1:20 000, and 1:500 000 for large CMN [2]. They present as large, homogeneous light- to dark-brown pigmented maculae or plaques with sharp boarders, smooth surface and hypertrichosis [1]. During childhood, they show proportional increase in size according to growth of the skin [4]. CMN are often accompanied by smaller satellite nevi, which may be present on the whole integument. Patients with more than twenty satellite nevi show an increased risk for neurocutaneous melanosis [5]. Neurocutaneous melanosis is defined as leptomeningeal melanocytosis or leptomeningeal melanoma associated with one large CMN ($^2/_3$ of patients) or more than three smaller CMN ($^1/_3$ of patients) [6], [7]. Symptomatic neurocutaneous melanosis is characterized by signs of increased intracerebral pressure, psychomotoric retardation or signs of spinal compression [8], and has a very bad prognosis [9].

1.1.1.2 Acquired melanocytic nevi

In contrast to CMN, which are classified based on their history and clinical diameter, AMN are generally subclassified on histopathological criteria. As such,

AMN can be basically divided into four main subtypes, which are blue nevi, Spitz nevi, intradermal nevi of the Unna and Miescher type and Clark nevi. However, it must be admitted that this classification is not perfect as blue nevi may also be present at birth or as Clark and intradermal nevi may show congenital-like features overlapping with true congenital nevi [1].

Blue nevi are round to oval papules or nodes with blue-grey or blue-black pigmentation, developed throughout the entire life with a certain predilection for childhood and adolescence [1], [3]. The incidence in Caucasians is estimated about 1% [3]. Two subtypes can be distinguished: “common blue nevus”, characteristically <10mm, and “cellular blue nevus”, typically 10-30mm [3]. Malignant transformation is very rarely observed - only large blue nevi on the scalp show an increased risk of malignant transformation [1].

Spitz nevi (formerly “juvenile melanoma”) are benign melanocytic tumors with signs of atypia, differentiation to melanoma often remains challenging [10]. They present as rapid-growing melanocytic lesions, usually solitary, round nodules and nodes with smooth surface and red to brown color, in children and adolescents, commonly located on the face with an incidence of less than 1% [1]. 50% of Spitz nevi are diagnosed in children younger than 10 years [3]. Different subtypes of Spitz nevi can be distinguished: classic/desmoplastic Spitz nevi, pigmented Spitz/Reed nevi and atypical Spitz/Reed nevi [11]. Spitz/Reed nevi can occur de novo or within a pre-existing nevus spilus [12]. Up to 47% of patients with Spitz nevi show positive sentinel node biopsies, however, with a perfect prognosis [13].

Two different entities of intradermal acquired nevi can be divided: Unna’s nevus presenting as adventitial lesion in the papillary and perifollicular dermis and Miescher’s nevus, diffusely infiltrating adventitial and reticular dermis [14]. Unna’s nevi are commonly diagnosed in the 8th decade on the trunk , while Miescher’s nevi are common in the 6th decade in the head/neck region [15]. Their counts were proven to be elevated in melanoma patients [15].

Internationally, there is no consensus on the exact definition of atypical and dysplastic nevi (Clark nevi) [1], which is the most frequently encountered type of nevus in an Caucasian population. Generally, “atypical” nevus is the clinical description, while “dysplastic” nevus represents the histopathologic nomenclature [1]. Clark nevi typically show irregularity in color and shape with a size >5mm, however, this classification is disputed, as clinically common acquired nevi may

show signs of dysplasia histopathologically and also vice versa [3]. Clark nevi typically develop around puberty, reach their peak in number around the 4th decade of life and thereafter decrease in number. The latter phenomenon appears to be linked to the process of apoptosis or involution [1].

Although the great majority of melanocytic nevi will have a benign course throughout all lifetime, some nevi may cause diagnostic difficulties in the differential diagnosis of melanoma. This is in parts due to the fact that some nevi may indeed harbor an increased risk of malignant progression (i.e., true precursors), or identify a given person at increased risk to develop melanoma either de novo or associated with a nevus (i.e. indicators of melanoma) or mimic clinically and/or histopathologically melanoma (i.e., simulators of melanoma). In the following, these subcategories of nevi will be discussed more in detail.

1.1.1.3 Precursors of melanoma – CMN and AMN

Congenital melanocytic nevi:
The risk for malignant transformation is definitely known for large CMN, however, also in small and intermediate CMN, malignant transformation has been reported [3]. According to a systematic review, patients with large CMN show a 465-times higher risk of developing melanoma in childhood and adolescence [16]. The malignant potential of large CMN ranges from 5 – 15% [17] and seems to be directly related to the lesion's size [3]. In large CMN, two thirds of cutaneous melanomas or other malignant tumors do not develop in the epidermis, but in the dermis or subcutis [18], which leads to later diagnosis and worse prognosis. Interestingly, the risk for malignant transformation is 0.7/1 000 000 in children under the age of 10 years, but 13.2/1 000 000 in adolescents with age 15 to 19 years [17].

Acquired melanocytic nevi:
Histopathological studies constantly report that about 1/3 of all melanomas will be associated with a nevus, whereby the most common types of nevi are intradermal nevi, Clark nevi and nevi with congenital-like features [19]. Recently, a prospective study analyzing a high-risk cohort consisting of 832 patients reported 54% of melanomas to be associated with a nevus [20]. In multivariate analysis, the authors found high nevus count, low risk for melanoma, melanoma in situ

developed in the past and female sex to be significant independent factors for the development of melanoma associated with a nevus [20]. Another recent study, evaluated a rate of 32% of nevus-associated melanomas in 1.190 melanoma patients, which were associated with intermittent sun exposure, SSM and a lower Breslow thickness [21]. These data are in line with the report of Purdue et al. describing a rate of 36% for nevus-associated melanomas in 932 patients [22].

1.1.1.4 Indicators for melanoma – Numerous and atypical nevi

The number of common melanocytic nevi is an independent risk factor for development of melanoma [1]. Patients showing more than 50 nevi have a 4 to 5 times higher risk, while patients showing more than 100 nevi have an even 8 to 10 times higher risk for developing melanoma [1]. There is a risk of 1:200 to 1:500 for development of melanoma associated with an atypical/dysplastic/Clark nevus, furthermore, the presence of a higher number of atypical melanocytic nevi is associated with a higher risk for developing melanoma [1].

1.1.1.5 Simulators of melanoma – Spitz nevi and Recurrent nevi

Simulators of melanoma are defined as benign melanocytic lesions mimicking melanoma clinically, dermoscopically and/or histologically [1]. This group consists of different types of nevi and can be summarized as following: (i) Nevi with site related atypia including nevi of the scalp, ears, face, milk line, body folds, palms and soles, nail, genital mucosa and knee; (ii) spitzoid neoplasms including atypical Spitz nevus and atypical Spitz tumor; (iii) exogenously altered nevi and nevi in pregnant women or in patients under immunosuppression and (iv) recurrent/persisting nevi [1].

1.1.1.5.1 Recurrent Nevi

Recurrent nevi, also called persisting nevi, are benign melanocytic nevi that re-grow after partial biopsy or trauma. In the early 50ies it has been hypothesized that partial removal of melanocytic nevi might induce changes towards melanoma. However, this theory has never been proven [23]. In 1957, a study including 75 patients with melanocytic nevi primarily undergoing shave technique followed by electrodissection and afterwards complete excision three to twelve months later, was conducted [24]. No evidence of malignant change neither in the primary nor in the recurrent lesion could be found in the 75 patients [24]. In 1958 a study

including 19 patients with recurrent nevi confirmed this finding [25]. In 1975 the term “pseudomelanoma” was introduced for recurrent nevi because they resembled both clinically and histopathologically, superficial spreading malignant melanoma in some cases [26]. In 1986, a case of “pseudomelanoma” after CO_2 laser ablation [27] was described followed by various reports on melanocytic lesions with “pseudomelanomatous” alterations after Solcoderm treatment, dermabrasion, acid, electrocautery therapy, radiotherapy and chronic non-traumatic irritation [25], [28]–[32].

Recurrence in melanocytic nevi usually occurs rapid, within 6 months after of the primary biopsy [26], [33]. Typically, hyperpigmentation starts within the macular area of the scar [26], [33]. Dermoscopy often reveals hyperpigmentation and hypopigmentation, linear streaks, halos and stippled or diffuse pigmentation patterns [26], [33]. A trizonal histopathologic pattern for recurrent nevi was established: junctional melanocytic component, fibrous scar tissue and deep, residual nevus cells without an increased mitotic index [34]. Problematic is the histopathologic overlap of recurrent nevi with melanoma showing signs of regression [35]. Only in about 20% of cases recurrent nevi originate from dysplastic nevi (Clark) [35], whereas recurrence in Spitz nevi is very rare with a reported overall incidence of 0.9% [36]. The term “sclerosing nevus with pseudomelanotous features” is used equally to “nevus with regression-like fibrosis” and results of unnoticed trauma or friction, possibly representing a chronically recurrent nevus [23].

Many theories regarding mechanisms of recurrence in melanocytic nevi have been proposed. On the one hand, seeding during removal was suggested [37], while on the other hand junctional stimulation after partial removal [25] or growth stimulation through residual nevus cells [38], repopulation from remaining adnexal structures [25], [34] or regrowth from the remaining dermal nevus [39] were hypothesized. Characteristic dermoscopic patterns of recurrent nevi are: radial lines, symmetry and a centrifugal growth pattern [40]. Furthermore, recurrent nevi are associated with age younger than thirty years and a short time to recurrence (less than six months) [40].

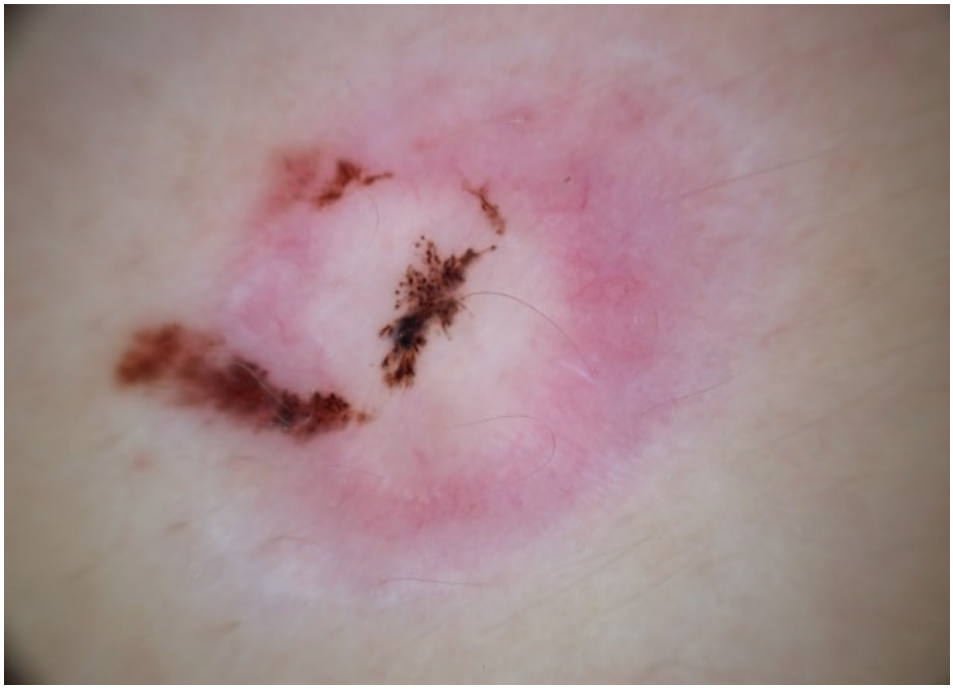

Figure 1: Dermoscopic image of a recurrent nevus, occurring within a few weeks within the traumatized area.

The main problem in daily routine with recurrent nevi is their differential diagnosis to recurrent melanoma. This is particularly the case if a histopathological diagnosis of the primary excised lesion has not been performed. In these cases, excision is mandatory to rule out recurrent melanoma.

1.1.2 Recurrent Melanoma

The classification of two, temporally different melanomas at the same anatomic localization as whether related, recurrent or separate primary lesions, can be difficult [41]. Currently, no diagnostic molecular tool for answering this question exists, however, mutational analysis at various loci in the primary and the recurrent lesion might be helpful [41].

Generally, recurrent malignant melanoma is defined to arise lateral to the scar of primary excision . In contrast to recurrent nevi, recurrent malignant melanoma is associated with age older than thirty years and a longer time to recurrence [40], [42]. Characteristic dermoscopic signs of recurrent melanoma are: circles (especially on the face), eccentric hyperpigmentation at the periphery, chaotic growth pattern, non-continuous growth pattern and pigmentation beyond the scar's edge [40]. Moreover, recurrent melanomas are more often located on the head and neck area than recurrent nevi [40], [43].

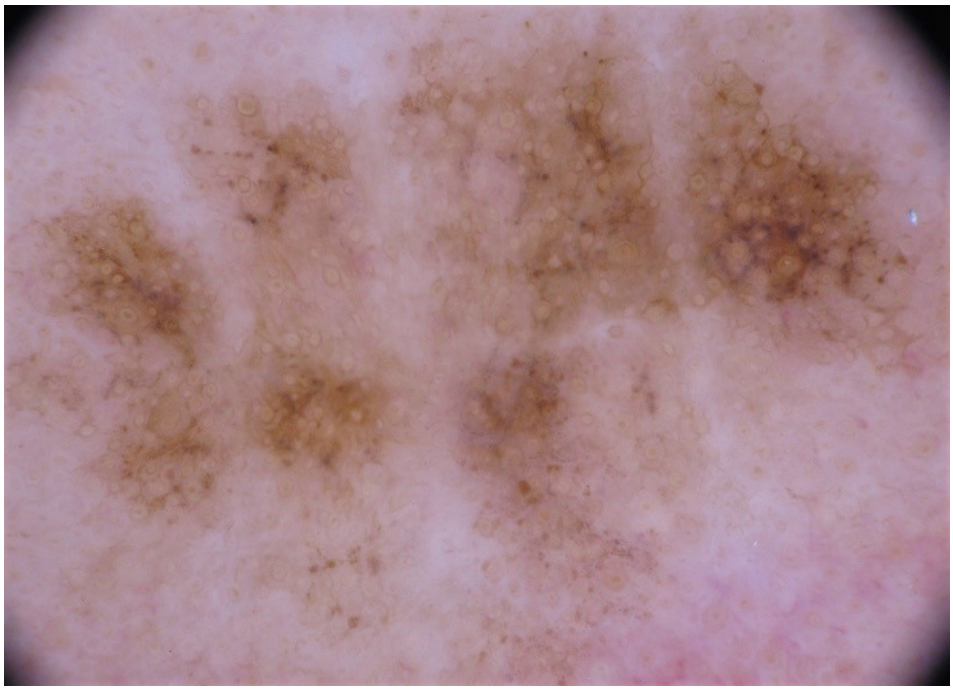

Figure 2: Dermoscopic image of recurrent melanoma, occurring within months to years lateral to the scar.

1.2 Genetical alterations in melanocytic tumors

During the past decades, much of research focused on the identification of molecular pathways involved in the development of melanocytic nevi and melanoma.

Melanomas show about 13 to 17 mutations per Mbp of DNA, which was the highest frequency evaluated among 21 cancer types [44].

Mutations in the mitogen-activated protein kinase (RAS/RAF/MEK/ERK) pathway influencing cell proliferation, differentiation and migration are commonly observed in melanoma and nevi [45]. RAS mutations, most commonly involving N-RAS, were described in about 56% of congenital nevi compared to 33% of primary melanomas and 26% of metastatic melanomas [46]. BRAF is a component of the MAPK pathway downstream of RAS. The highest incidences of mutated BRAF in cancers were found in melanoma (27-70%) [47], [48]. BRAF mutations were also detected in 63% of benign nevi [44]. The BRAFV600E mutation is the most common accounting for 92% of BRAF mutations in sporadic melanoma and about 82% in benign nevi [47], [48]. MEK mutations are rare in human cancers, also in melanoma (3-8%) [49].

Mutations in the PI3K/AKT pathway resulting in PTEN loss (11-60%) and AKT amplification are common events in melanoma [50].
Mutations in the CDKN2A/P16INK4A/ARF pathway are reported in both, familiar and spontaneous melanomas and are frequent mutations in melanoma (88%) [44]. These mutations were also reported in dysplastic nevi [44].
Further common genetic and epigenetic “driver” mutations and amplifications in melanoma affect TERT (71%), TBX2 (44%), APAF1 (37-42%), CDKN2B (36%), MYC (34%), PTPRD (23%), PREX2 (16%), APC (16%), TP53 (12.5%), GRM3 (12.1%), ERBB4 (12.1%), MITF (11.8%), NF1 (11.2%), ARID2 (10%), PPP6C (7.1%) and RAC1 (5.8%) [44].
To sum up, BRAFV600E mutation is the most common mutation in melanoma and nevi.

1.2.1 BRAF and its cellular function

B-RAF belongs to the family of serine-threonine protein kinases, which are important mediators in the MAPK (mitogen-activated protein kinase) signaling cascade through phosphorylation and activation of MEK (mitogen-activated protein kinase kinase) [51]. The V600E mutation, a substitution of glutamic acid for valin, in exon 15 is the most popular BRAF mutation in a variety of tumors resulting in the RAS-independent activation of B-RAF [52]. Consecutively, the pathway is hyperactivated through evasion of the inhibitory feedback loop leading to an increase of ERK, which is responsible for proliferative and anti-apoptotic signaling [53].

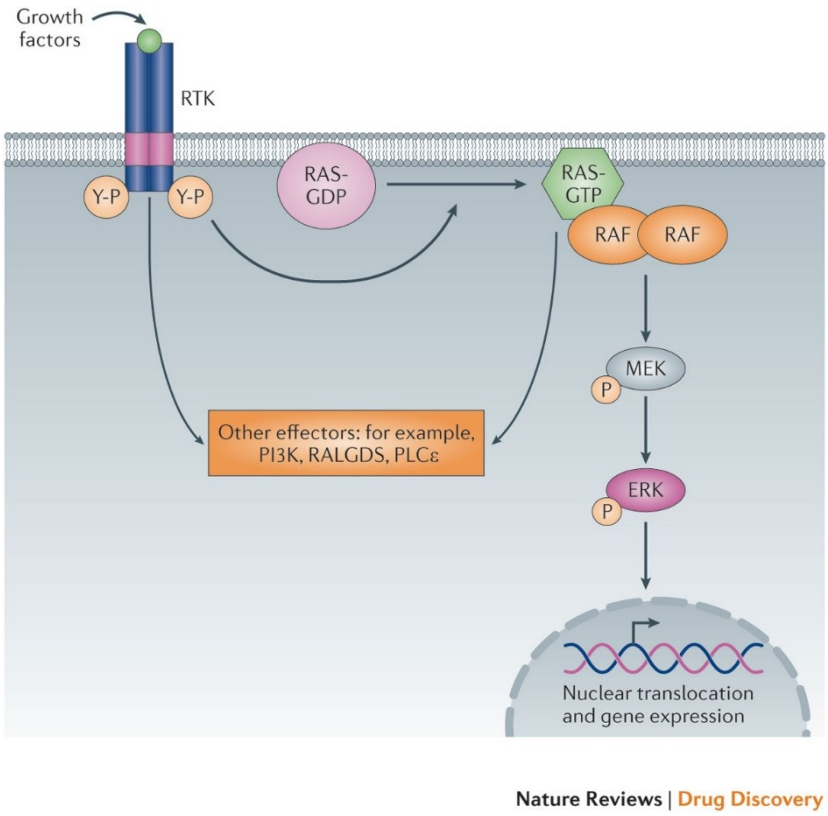

Figure 3:
RAS-RAF-MEK-ERK-pathway [54].

1.2.2 BRAF and nevogenesis

Oncogenic BRAF V600E mutation was shown in about 80% of melanocytic nevi [48]. It is common among acquired nevi such as Clark nevi and small nevi with congenital-like features, however, intermediate to large congenital nevi, Spitz nevi and blue nevi do not show BRAF V600E mutations but mutations in NRAS, HRAS and GNAQ [55]. It was hypothesized that intermittent UV exposure induces BRAF mutations in melanocytes stimulating proliferation and formation of neoplastic clones and that in absence of additional genetic mutations, cell cycle arrest and senescence follow due to induction of p16INK4a and acidic ß-galactosidase [56], [57]. BRAF V600E mutated melanocytic nevi expressed high levels of IGFBP7 inducing senescence in melanocytes, which could not be shown for BRAF V600E mutated melanoma [58]. Interestingly, the frequency of oncogenic BRAF shows an increase from lentiginous/junctional nevi to compound and dermal nevi [59]. High rates of BRAF mutations are reported among nevi located on sun-protected body areas like genitalia or mucosa [60]. Nevi showing BRAF mutation seem to be associated with younger age and growth, what leads to the assumption that BRAF

plays a role in early nevogenesis [61]. Moreover, nevi were shown to be polyclonal regarding BRAF mutations [61]. A model of the role of oncogenic BRAF mutations in the evolution of nevi was proposed [62]. During the period of growth, activating BRAF mutations drive the initial proliferation [62]. In the following period of stabilization, growth arrest via oncogenic senescence is induced, while the lesion shows polyclonality for BRAF mutation [62]. In the final period of involution, the BRAF mutation is lost with most cells having entered senescence or undergone apoptosis [62].

1.2.3 BRAF and melanomagenesis

Constitutively activating mutations in the *BRAF* oncogene have been reported in about 50% of primary melanomas and are associated with specific clinical features such as younger age (≤ 50 years), location on the trunk, location on non-chronically sun exposed skin, high nevus count, superficial spreading melanoma, advanced tumor stage, the presence of mitoses and the presence of a single/occult primary malignant melanoma [63], [64]. In melanoma, the V600E mutation accounts for more than 90% of mutations [65], while other genotypes (V600K, V600_K601E, T599dup, K601E, D594N, V600E and K601E, or L597V) are infrequent. Although the exact role of BRAF mutations in melanomagenesis is not fully elucidated, it must be an early event in melanoma development that acts via evasion of senescence and apoptosis, unchecked replication, growth-factor independent proliferation, transformation of immortalized melanocytes, angiogenesis, tissue invasion/metastasis and the evasion of immune response [66], [67]. In the course of progression, further mutations appear to be necessary to drive invasion, growth and metastatic potential [65], [68].

In 2011, polyclonality in BRAF mutations in melanoma was shown and now the theories on the quantity of BRAF mutations in melanoma have to be questioned as most previous studies used PCR and direct sequencing in mutation detection, which was not able to reliably detect all heterozygous BRAF mutations [65]. According to this study, most of the primary melanoma lesions analyzed consisted of melanoma cells containing wild-type BRAF mixed with melanoma cells containing V600E or other BRAF mutations [65]. Characteristic histopathological signs have been described for BRAF-mutated melanomas as large round tumor

cells with nest formation, extensive pagetoid spreading and prominent pigmentation [69].

Generally, initial “driver” mutations, which may either be activations of oncogenes or losses of tumor suppressor genes, start tumoral growth [70]. After this proliferation period, senescence is executed, which leads to termination of tumor expansion [70]. In 2008, overexpression of BRAF V600E in melanocytes was shown to cause growth arrest in melanocytic nevi through upregulation of the tumor suppressor p16INK4A and induction of acidic ß-galactosidase activity and cell-cycle arrest [71]. In spite of the activated MAPK pathway, benign nevi seem to lose their proliferative activity remaining in growth arrest for decades until finally disappearing [70]. If further genetic alterations are present, they can, however, contribute to development into malignant melanoma [72]. Analysis of malignant melanoma associated with melanocytic nevi was and is conducted to improve the knowledge on melanomagenesis, however, until today, the molecular mechanisms of transformation of a melanocytic nevus into malignant melanoma have not been resolved [70]. In 2013, it was reported that BRAF V600E or NRAS Q61 mutated nevi did not show increased risk of transformation into malignant melanoma [73]. P16INK4A is in consideration to play a central role [71]. Furthermore, PI3K pathway activation is now in discussion [74]. In 2015, rapid melanoma growth in mice was observed through activation of mTORC1 and Akt/mTORC2 through Cdnk2a and Lkb1 inactivation in BRAF V600E mutated melanocytes [75].

1.2.4 Targeted therapy for BRAF (dabrafenib, vemurafenib)

Vemurafenib is a highly specific inhibitor of the BRAF kinase showing V600E mutation [76]. Before the discovery of vemurafenib, which was a breakthrough in targeted therapy of melanoma, many attempts to inhibit mutated BRAF failed [76]. Clinical trials for vemurafenib started in 2008, and in 2011, it was approved by the FDA for treatment of late-stage and unresectable melanoma [77]. The phase III trial showed a median overall survival (OS) of 13.2 months in patients treated with vemurafenib compared to an OS of 9.6 months for dacarbazine, the formerly drug of choice for treatment of metastatic melanoma [78]. The most commonly observed adverse effects of vemurafenib are (i) arthralgia, (ii) rash, (iii) photosensitivity skin reactions, (iv) fatigue, (v) nausea, (vi) alopecia, (vii) pruritus, (viii) hyperkeratosis, (ix) diarrhea, (x) headache and (xi) vomiting [76].

Furthermore, cutaneous squamous cell carcinomas and keratoacanthomas were developed under vemurafenib therapy [78].

Dabrafenib is a BRAF inhibitor approved as single-agent treatment for unresectable or metastatic melanoma in 2013 by the FDA [79]. The phase III trial showed progression-free survival (PFS) of 6.7 months for dabrafenib compared to a PFS of 2.9 months for dacarbazine [78]. Most common adverse effects under treatment with dabrafenib are (i) hyperkeratosis, (ii) papillomas, (iii) palmarplantar erythrodysesthesia, (iv) pyrexia, (v) fatigue, (vi) headache and (vii) arthralgia [80]. Moreover, cutaneous squamous cell carcinoma, keratoacanthoma, primary melanoma and phototoxicity were observed [80].

Most patients develop resistance to BRAF inhibitor therapy (BRAFi) after 6 to 8 months [78]. Intrinsic resistance may be caused by abnormalities in the cell cycle regulation involving cyclin D1, PTEN and the interaction between hepatocyte growth factor (HGF) and its receptor CMET [81]–[83]. Acquired resistance via the non-ERK-dependent pathway is due to the activation of PI3K/AKT/mTOR pathway by PDGFR-β (platelet derived growth factor) and IGF-1R (insulin-like growth factor 1 receptor), whereas the ERK-dependent pathway works via potentiation of signaling through the MAPK pathway, also seen as a result of upregulation of RTK (receptor tyrosine kinase) and RAF dimer formation [78], [84]. Increasing the BRAF inhibitor's dose and administration of combinations with immunotherapeutic agents seem to be ways for overcoming resistance [78].

1.2.5 Alterations in melanocytic lesions under BRAF inhibitor therapy

Among a number of cutaneous side-effects during BRAFi are alterations of the melanocyte system. These include involution, color and size changes and progression towards melanoma in pre-existing nevi, as well as the onset of new nevi and primary melanomas [85]. Remarkably, some of these newly developing or changing lesions reveal marked atypia upon histopathological evaluation, making the differential diagnosis between nevus and melanoma challenging [86]. Moreover, newly evolving nevi under BRAFi therapy were shown to be frequently wildtype with regard to BRAF mutations, while stable or involuting nevi commonly harbor the BRAF V600E mutation [87]. Furthermore, the development of BRAFwt melanomas was shown in patients treated with BRAFi [88]. Six early BRAF wildtype primary melanomas evolving from six atypical melanocytic lesions were detected in four patients with metastatic melanoma with BRAF V600E mutation

four to twelve weeks after initiation of vemurafenib therapy [89]. Five superficial melanomas were reported among over 450 patients in phase 2 and 3 trials [90]. Moreover, twelve primary melanomas in eleven patients with BRAF V600E mutant metastatic melanoma, all of them BRAF wildtype, were observed, at least nine of the new primary melanomas evolving from pre-existing nevi [91]. Ten pigmented lesions from two patients with BRAF V600E mutant metastatic melanoma were observed before and after receiving vemurafenib for three months [92]. Four out of the ten observed lesions were excised due to atypical alterations after the three months of vemurafenib treatment, all of these having evolved from formerly benign-appearing nevi and then diagnosed primary melanomas [92].

1.3 Aims of this study

In this study, we aimed first, to assess the epidemiological and clinical factors associated with recurrent melanocytic proliferations and second, to compare the frequency of BRAF mutations in primary nevi and melanomas with their recurring counterparts after surgery in order to gain more insights into the role of BRAF mutations as driver events in melanocytic neoplasia.

While mutations in the BRAF gene are certainly driver events in the early development of both, nevi and melanoma, their exact role remains to be clarified. Recurrent nevi are often called “pseudo-melanomas” due to their atypical appearance [26]. Differentiation between recurrent nevus and recurrent melanoma often remains challenging [40]. BRAF mutations have been shown in about 80% of nevi and 50% of melanoma [48], [64]. While BRAF mutations are considered driver mutations and early events in melanomagenesis, there is hypothesis that BRAF in nevi induces senescence – although only together with other mutations [75]. Since the implementation of “targeted therapies” including the inhibition of the BRAF mutated pathway in melanoma, there are publications showing increased numbers of eruptive nevi and questionable melanomas under BRAF-inhibition [89]–[92]. In this context the questions arises, if these reported atypical melanocytic proliferations are indeed biologically aggressive melanomas or rather activated nevi showing significant atypia histopathologically alike re-growing recurrent nevi. However, up to date there is very little knowledge about the frequency and potential role of BRAF mutations in recurrent nevi and recurrent melanomas.

2 Material and Methods

2.1 Patients and pathology

Cases of “recurrent nevus”, “persisting nevus” and “recurrent melanoma” diagnosed between 2002 and March 2015 were retrieved at the histopathological database of the Dermatopathology Research Unit of the Department of Dermatology and Venereology at the Medical University of Graz. The correspondent original melanocytic tumors were also collected together with relevant clinical data (age, sex, location, time period and type of surgical procedure, follow-up).

Statistical analysis and graphical depiction was performed with Microsoft Office Excel 2010™.

2.2 Analysis of the frequency of BRAF V600E mutations in primary and recurrent melanocytic tumors

2.2.1 Histopathological revision

All selected cases were reviewed by an experienced board certified dermatopathologist (CM). A recurrent melanocytic nevus was defined by the presence of a sharply circumscribed melanocytic proliferation in the epidermis with a pagetoid spread mimicking a melanoma upon the scar tissue of the previous incomplete surgical procedure at the same location. Nevus cells of the original melanocytic nevus could be observed in the dermis. A recurrent melanoma was defined by the presence of an atypical melanocytic proliferation in the epidermis or in the dermis fulfilling histopathological criteria for melanoma and of a scar in the dermis at the site of a previous melanoma excision [93].
The slides of the primary melanocytic nevus or melanoma were also reviewed. Diagnosis of nevus or melanoma were assessed according to the WHO classification of skin tumors [94]. Only cases with complete clinical data, slides for revision and enough material on the paraffin block for PCR analysis of both original and recurrent melanocytic tumor were selected.

2.2.2 Microtome

Slides of a thickness of 5µm were performed for each paraffin block by a microtome and collected in separate 2ml Eppendorf™-tubes. Depending on the

lesion's size, 5 slides were taken in case of lesions measuring more than 5 millimeters and 10 slides were conducted in case of smaller lesions. Macrodissection was performed in excisions containing a great amount of subcutaneous fat tissue. The Eppendorf™-tubes were stored at room temperature protected from sun-exposure until the DNA isolation was performed.

2.2.3 DNA isolation

DNA isolation was performed with Maxwell™ following the institution's protocol (Promega Maxwell FFPE tissue LEV DNA Purificiation Kit; #AS1130). The paramagnetic Silica particles used in this protocol where removed via a magnet before transferring the isolated DNA probes into the plate for further analysis.

2.2.4 Polymerase chain reaction

The template used for PCR was 5µl. We established a gradient PCR to evaluate best conditions for our probes. The PCR was conducted with 38 cycles and an annealing temperature of 58°C with MyCycler from BioRad™ (Bio-Rad Laboratories Inc., California, USA) . The Master-Mix consisted of a 10 : 1 : 1 : 8 proportion of HotStarTaqPlusMM (Qiagen, Venlo, Netherlands) : Primer forward (10pmol/ml) (Eurofins Scientific, Luxembourg) : Primer reverse (10pmol/ml) (Eurofins Scientific, Luxembourg) : DNA template.
We used the forward and reverse primers BRAFex15_V600E-f-TCATAATGCTTGCTCTGAT
AGGA and BRAFex15_V600E-GGCCAAAAATTTAATCAGTGGA (Eurofins Scientific, Luxembourg) for the templates of nevi patients No. 1 until 15 and the short forward and reverse primers Primer-173 F-TGCTTGCTCTGATAGGAAAATG and R-CCACAAAATGGATCCAGACA (Eurofins Scientific, Luxembourg) for the probes of nevi patients No. 16 until 37 and for all melanoma patients' templates. Initial denaturation was conducted at 95°C for 5 minutes, followed by denaturation at 94°C for 30 seconds and hybridization at 58°C for 30 seconds and elongation at 72°C for 45 seconds in 38 cycles. Afterwards the probes were stored at 72°C for 10 minutes, followed by storage at 10°C until taken out of the cycler.

2.2.5 Electrophoresis

The checkgel was performed with 1.5% Agarose. 5µl of the sample and 1µl 6xMass Ruler loading dye (Thermo Fischer Scientific, Waltham, Massachusetts,

USA) were used. As a ladder, 5µl Fast-Ruler low range r-t-u (Thermo Fischer Scientific, Waltham, Massachusetts, USA) were used. Electrophoresis was conducted with 90V over 30 minutes.

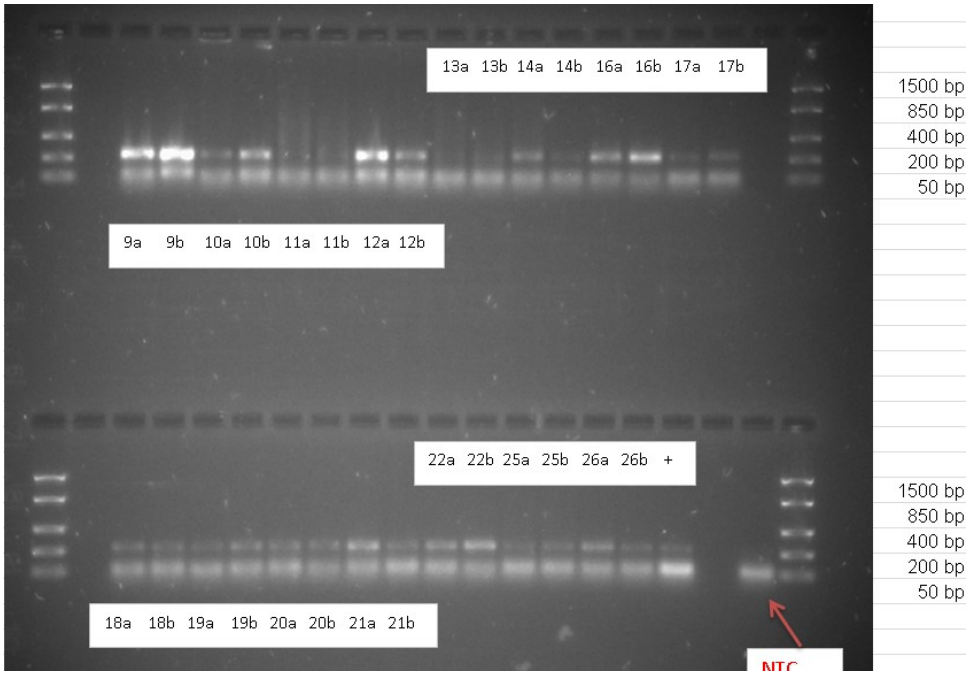

Figure 4: Electrophoresis of the PCR products.

2.2.6 Sequencing

The company Eurofins Scientific Luxembourg conducted purification and sequencing of the samples. The results were sent back to us and analyzed with CodonCode Aligner (CodonCode Cooperation, Massachusetts, USA) by IK.

3 Results

3.1 General data

Overall, 205 cases of recurrent melanocytic lesions were included. These consisted of 177 (86%) recurrent nevi and 28 (14%) recurrent melanomas from 205 patients including 146 women (71%). Mean age of patients with recurrent nevi and melanoma was 31 years (range 10 to 65 years) and 65 (range 33 to 91 years), respectively.

3.2 Assessment of epidemiologic and clinical factors

3.2.1 Nevi

In 75/177 cases (42%) the primary nevi were diagnosed dermal nevi, in 43/177 (24%) atypical/dysplastic nevi, in 4/177 congenital nevi (2%), in 3/177 Spitz nevi (2%) and in 1/177 (1%) compound nevus. In 51 cases (29%) the histopathological diagnosis is unknown.

3.2.1.1 Gender

Among the total of 177 patients with recurrent nevi, there was a remarkable predominance of women (n=133, 75%).

Figure 5: Distribution of sex in the 177 patients with recurrent nevi.

3.2.1.2 Age at the time of recurrence

Mean age of the 177 patients with recurrent nevi was 31 years (range: 10 to 65 years). Thirty one patients were younger than 20 years (18%), 59 patients were aged between 20 and 29 years (33%), 49 patients were between 30 and 39 years old (28%), 27 patients were between 40 and 49 years old (15%), 10 patients were between 50 and 59 years old (6%) while there was only one patient aged 65 years.

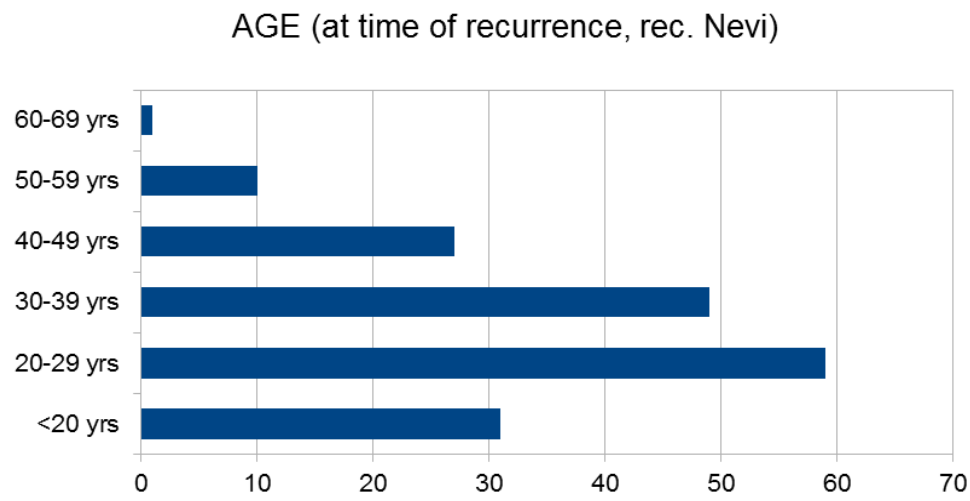

Figure 6: Age at the time of recurrence in the 177 patients with recurrent nevi.

3.2.1.3 Location of lesion

The majority of recurrent nevi were located on the upper back (n=36, 20%) followed by the legs (n=31, 18%), arms/shoulder (n=29, 16%) and chest/axilla (n=28; 16%). Eighteen nevi were located on the lower back (10%) and each 17 nevi were found on the abdomen (10%) or head/neck (10%). In one case, the location of the lesion was not documented.

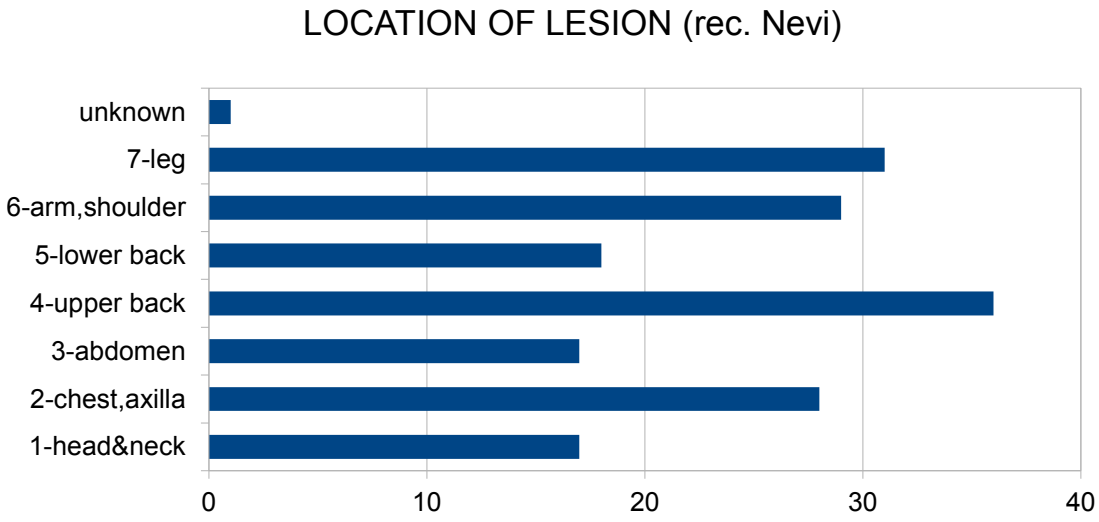

Figure 7: Location of the lesion in the 177 patients with recurrent nevi.

3.2.1.4 Procedure with the primary lesion

In most occasions, the primary nevus was treated by shaving biopsy (n=136 cases, 77%), while total excision was performed only in 25 cases (14%). For 16 cases, the mode of treatment of the primary lesion was not further specified.

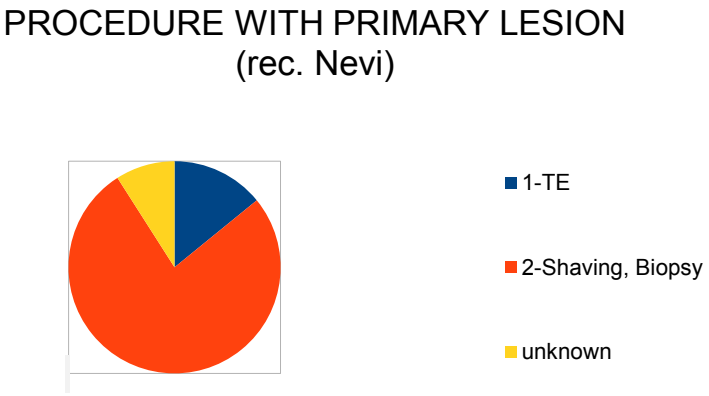

Figure 8: Procedure with the primary lesion in the 177 patients with recurrent nevi.

3.2.1.5 Procedure with the recurrent lesion

In about half of the cases (n=93, 53%) a total excision of the recurrent lesion was performed, while the remaining lesions were re-treated by shaving biopsy (n=54; 31%). For 30 cases, the mode of treatment of the recurrent nevus was missing.

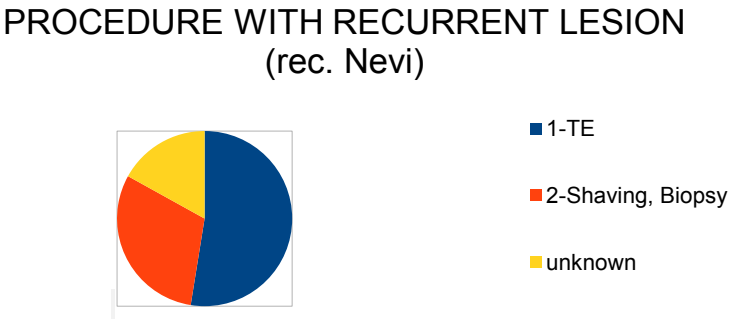

Figure 9: Procedure with the recurrent lesion in the 177 patients with recurrent nevi.

3.2.1.6 Time to recurrence

The majority of nevi (n=98, 55%) recurred within the first 6 months after initial treatment (mean: 11 months, range: 0 to 203 months). In 32 (18%) cases, recurrence was detected 7 to 12 months after initial diagnosis. For 10 patients (6%), recurrence was diagnosed 13 to 18 months after treatment of the primary nevus. Moreover, in 7 patients (4%), nevi recurred between 19 to 24 months, in 6 patients (3%) between 25 and 30 months, in 2 patients (1%) between 31 and 36

months, in one patient (0.6%) between 37 and 48 months, in 5 patients (3%) between 49 and 60 months and in only one patient recurrence was removed more than five years after initial treatment. For 15 patients, the time to recurrence could not be evaluated.

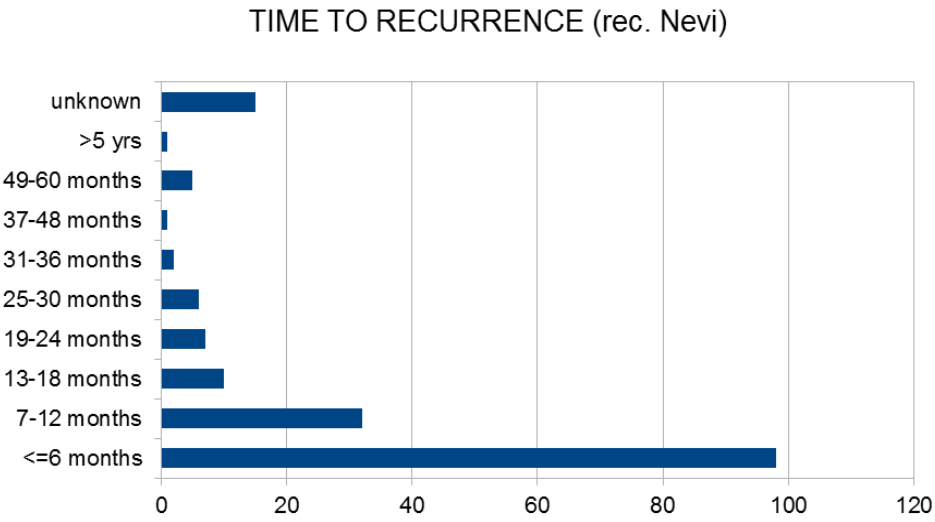

Figure 10: Time to recurrence in the 177 patients with recurrent nevi.

3.2.2 Melanoma

3.2.2.1 Gender

Among a total of 28 patients with a diagnosis of recurrent melanoma were 15 (54%) men and 13 (46%) women.

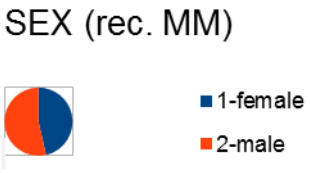

Figure 11: Distribution of sex in the 28 patients with recurrent malignant melanoma.

3.2.2.2 Age at the time of recurrence

The mean age of patients with recurrent melanoma was 65 years (range from 33 to 91 years). The majority of patients (n=10, 36%) were aged 70 and 79 years, eight patients were aged between 60 and 69 years (29%). Only one patient (4%) was 33 years old, two patients were between 40 and 49 years old (7%) and three patients were between 50 and 59 years old (11%). Also three patients were

between 80 and 89 years old (11%), while only one person was aged 91 years (4%).

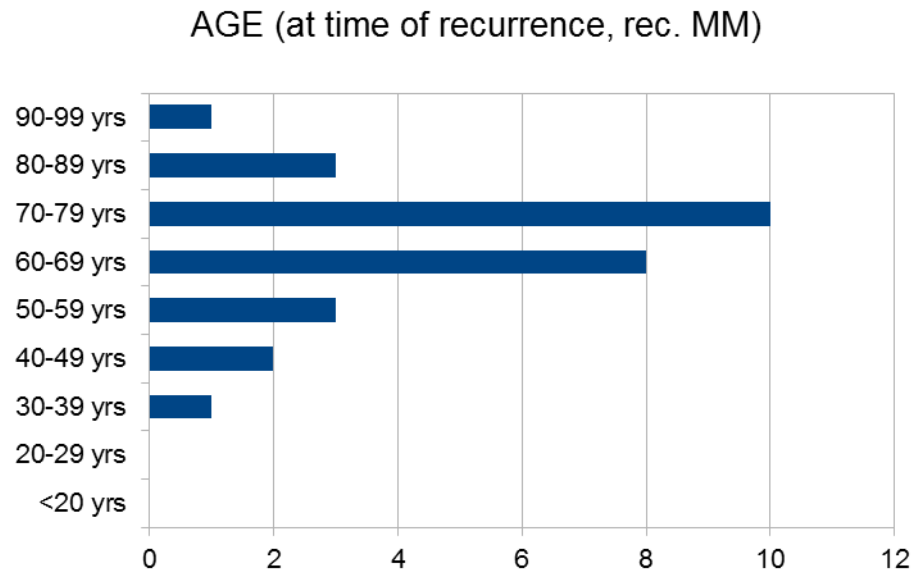

Figure 12: Age at the time of recurrence in the 28 patients with recurrent malignant melanoma.

3.2.2.3 Location of lesion

The majority of recurrent melanomas was located on the head and neck area (n=17, 61%), followed by upper back (n=4, 14%) and legs (n=3, 11%). In two patients each (7% each), the melanoma was located on the chest/axilla and arms/shoulder region.

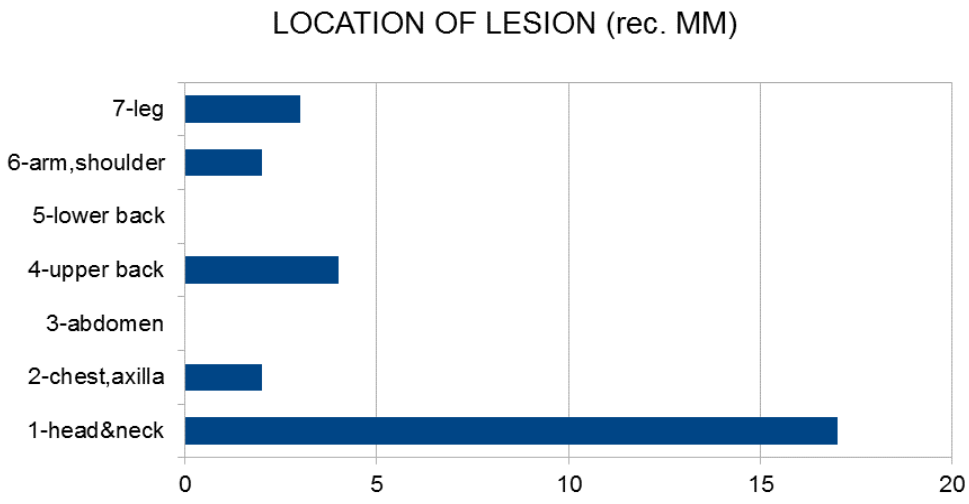

Figure 13: Location of lesion in the 29 patients with recurrent malignant melanoma.

3.2.2.4 Procedure with the primary lesion

In only eleven patients (40%), the primary melanoma was treated by complete excision, whereas in 14 (50%) patients, a shaving biopsy was performed. In 3 patients, the initial treatment procedure was missing.

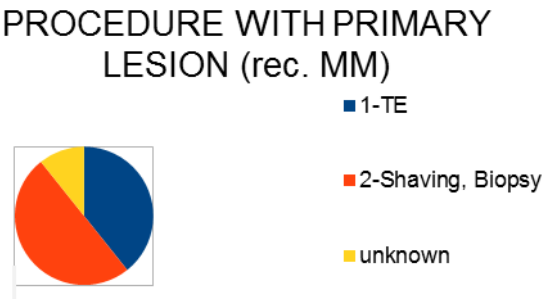

Figure 14: Procedure with the primary lesion in the 28 patients with malignant melanoma.

3.2.2.5 Procedure with the recurrent lesion

In 13 patients (46%) a total excision of the recurrent melanoma was performed, while in 15 (54%) patients a shaving biopsy was done.

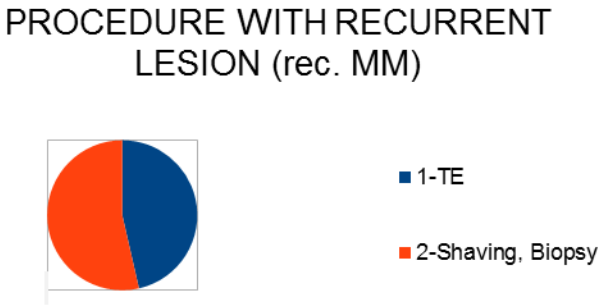

Figure 15: Procedure with the recurrent lesion in the 28 patients with recurrent malignant melanoma.

3.2.2.6 Time to recurrence

The peak for recurrence in melanoma was 13-36 months (mean: 35 months, range: 3 to 136 months). Most patients developed recurrence between 13 to18 months after treatment of the primary lesion (6 patients, 21%), followed by four patients each (14% each) developing recurrence between 25 to 30 months or 7 to 12 months. Three patients were diagnosed recurrence between 31 to 36 months after primary excision (11%). Two patients each (7% each) showed recurrence

between 19 until 24 months, 37 until 48 months or 85 until 96 months, while only one patient each (4%) recurred in a period of less than 6 months, between 49 until 60 months, between 61 until 72 months, between 97 until 108 months or between 133 and 144 months.

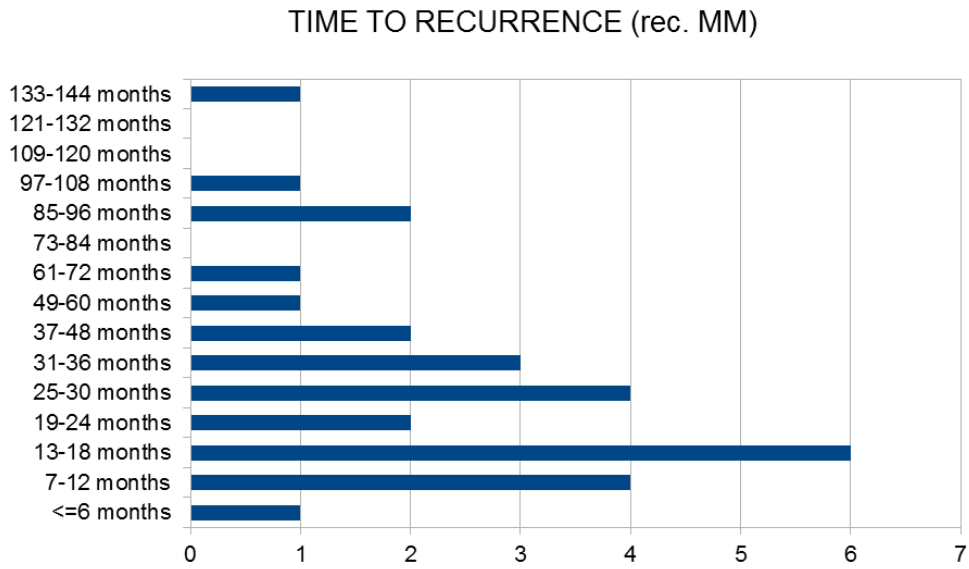

Figure 16: Time to recurrence in the 28 patients with recurrent malignant melanoma.

Summary of data:

Recurrent nevi were associated with age younger than 30 years, location other than head/neck and shorter time to recurrence (mean 11 months), whereas recurrent melanomas were associated with age older than 65 years, location head/neck and longer time to recurrence (mean 35 months).

3.3 Mutational Analysis of BRAF V600E

For a total of 63 cases, we were able to perform a molecular testing for BRAF mutation on the primary and recurrent lesion. In detail, we analyzed a total of 50 primary and recurrent nevi and 13 primary and recurrent melanomas.

3.3.1 Nevi

We gained BRAF V600E mutation status in the samples of primary and recurrent lesion in 36 patients with recurrent nevi.

Eleven patients (31%) showed BRAF V600E mutation in both, primary and recurrent lesion. Twelve patients (33%) had BRAF V600E mutation only in the primary lesion, but not in the recurrent lesion. Five patients (14%) showed BRAF V600E mutation only in the recurrent lesion. Eight patients (22%) did not show BRAF V600E mutation in any of their lesions. The patients' and lesions characteristics and their mutational status are depicted in Table 2.

The 11 cases (31%) showing BRAFV600E mutation in the primary and the recurrent lesion were characterized by mean age of 29 years (range 13 to 45 years), mean time to recurrence of 6 months (range 2 to 12 months) and by locations of the lesions on head/neck, chest, abdomen and back. None of these lesions was located on the extremities. 8/11 cases (73%) showed a congenital pattern in histology. 9/11 (82%) were dermal nevi and 2/11 (18%) were compound nevi.

In 12 cases (33%), BRAFV600E mutation was shown in the primary, but not in the recurrent lesion. Mean age was 29 years (range 13 to 45 years), mean time to recurrence was 9 months (range 1 to 32 months). Of those 5 lesions (42%) were located on the extremities, 2 lesions (17%) showed a congenital pattern in histology. 2 nevi (17%) were atypical/dysplastic nevi, 8 (66%) were dermal nevi and 2 (17%) were compound nevi.

In 5 cases (14%), BRAFV600E mutation was evaluated in the recurrent, but not in the primary lesion. Mean age was 29 years (range 10 to 55 years), mean time to recurrence was 13 months (range 4 to 29 months). 3/5 lesions (60%) were located on the extremities. 2 lesions (40%) showed a congenital pattern in histology. 1 Spitz nevus (20%) and 1 atypical/dysplastic nevus (20%) were part of this group.

8 cases (22%) were wildtype in both, primary and recurrent, lesions. Mean age was 35 years (range 25 to 41 years), mean time to recurrence was 13 months

(range 0 to 59 months). 5 lesions (63%) were located on the upper back or chest. 2 lesions (25%) showed a congenital pattern in histology. 4 nevi (50%) were atypical/dysplastic nevi.

3.3.2 Melanoma

In 12 patients with recurrent melanoma, BRAF V600E mutation status could be obtained in the samples of primary and recurrent lesion and eventual additional excisions following non-in-sano resections. Remarkably, none of the patients showed BRAF V600E mutation in both, primary and recurrent lesion. Two patients (17%) had BRAF V600E mutation in the primary lesion, but not in the recurrent lesion. Three patients (25%) showed BRAF V600E mutation only in the recurrent lesion. The remaining patients (n=7, 58%) were wt regarding BRAF. The patients' and lesions characteristics are listed in Table 3.

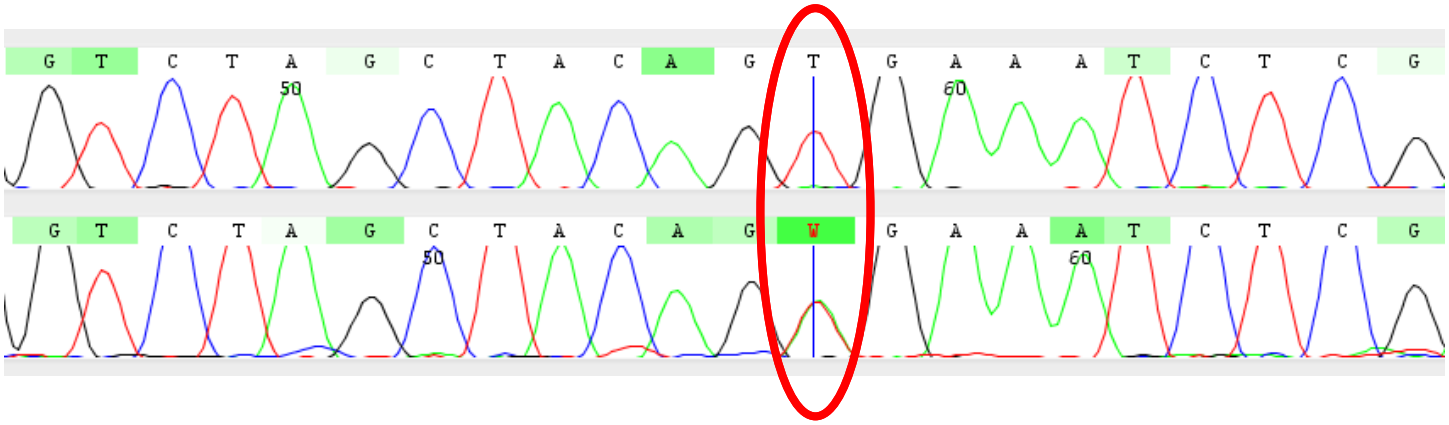

Figure 17: BRAF V600E mutation status analyzed with CodonCode Aligner. Codon 600 is marked with a red ellipse. In the first row, there is a wiltype constitution (T/T) – while in the second row there is a T/A transition.

No.	Sex	Age at recurrence (years)	Location	Histopathological diagnosis	Congenital pattern	BRAF status – primary lesion	BRAF status - recurrence	Time to recurrence (months)
1	male	38	lower back	dermal nevus	yes	mutation	mutation	11
2	female	22	chest/axilla	dermal nevus	no	mutation	wildtype	23
3	female	42	chest/axilla	dermal nevus	no	mutation	wildtype	7
4	female	33	upper back	compound nevus	yes	mutation	wildtype	5
5	female	32	head/neck	dermal nevus	yes	mutation	mutation	2
6	female	16	legs	dermal nevus	yes	mutation	wildtype	6
7	female	34	arms/shoulders	junctional nevus	no	wildtype	mutation	10
8	female	16	abdomen	dermal nevus	no	mutation	mutation	12
9	male	17	upper back	compound nevus	yes	mutation	mutation	7
10	female	55	upper back	compound nevus	yes	wildtype	mutation	17
11	female	45	chest/axilla	dermal nevus	yes	mutation	mutation	4
12	female	21	upper back	dermal nevus	no	mutation	mutation	2
13	male	16	upper back	dermal nevus	no	mutation	wildtype	5
14	male	30	arms/shoulders	dysplastic nevus (Clark)	no	wildtype	mutation	5
15	female	38	legs	atypical nevus	no	mutation	wildtype	6
16	male	13	lower back	dermal nevus	yes	mutation	mutation	2
17	male	42	arms/shoulders	dermal nevus	no	mutation	wildtype	5
18	female	38	upper back	nevus sebaceus	no	wildtype	wildtype	19
19	female	38	upper back	dermal nevus	yes	mutation	mutation	4
20	female	18	upper back	compound nevus	no	mutation	wildtype	3
21	male	41	upper back	dysplastic nevus (Clark)	no	wildtype	wildtype	2
22	female	35	arms/shoulders	dermal nevus	no	mutation	wildtype	5
23	female	27	arms/shoulders	atypical nevus	yes	wildtype	wildtype	13
24	female	18	abdomen	dermal nevus	yes	wildtype	mutation	29
25	male	26	abdomen	dermal nevus	no	mutation	wildtype	32
26	female	43	abdomen	dermal nevus	yes	mutation	mutation	4
27	female	40	abdomen	dysplastic nevus (Clark)	yes	wildtype	wildtype	59
28	female	19	upper back	compound nevus	no	mutation	mutation	8
29	female	41	upper back	compound nevus	no	wildtype	wildtype	5
30	male	29	chest/axilla	dermal nevus	no	mutation	wildtype	6
31	female	28	arms/shoulders	dermal nevus	no	wildtype	wildtype	5
32	female	36	arms/shoulders	dysplastic nevus (Clark)	no	mutation	wildtype	1
33	female	10	legs	Spitz nevus	no	wildtype	mutation	4
34	female	32	head/neck	dermal nevus	yes	mutation	mutation	7
35	female	25	upper back	dermal nevus	no	wildtype	wildtype	2
36	female	37	chest/axilla	atypical nevus	no	wildtype	wildtype	0

Table 1: Characteristics of the 36 patients with recurrent nevi analyzed regarding BRAF V600E mutation.

No.	Sex	Age at recurrence (years)	Location	Histopathological diagnosis	BRAF status – primary lesion	BRAF status - recurrence	Time to recurrence (months)
I	female	60	head/neck	Lentigo maligna (AJCC 2009: T1a)	mutation	wildtype	105
II	male	76	head/neck	Lentigo maligna with nodular component (AJCC 2009: T2a)	wildtype	wildtype	20
III	female	70	legs	M.i.s. (AJCC 2009: T1a)	wildtype	wildtype	87
IV	male	74	arms/shoulders	M.i.s. (AJCC 2009: T1a)	wildtype	mutation	86
V	male	70	chest/axilla	malignant melanoma	wildtype	wildtype	35
VI	male	61	head/neck	spindle cell melanoma with desmoplastic component (AJCC 2002: T1a)	wildtype	wildtype	17
VII	female	69	leg	M.i.s. (AJCC 2009: T1a)	mutation	wildtype	18
VIII	female	78	head/neck	M.i.s. (AJCC 2009: T1a)	wildtype	wildtype	72
IX	female	62	upper back	malignant melanoma	wildtype	mutation	51
X	female	73	head/neck	M.i.s. (AJCC 2009: T1a)	wildtype	wildtype	80
XI	male	45	head/neck	M.i.s. (AJCC 2009: T1a)	wildtype	mutation	44
XII	female	85	head/neck	Lentigo maligna	wildtype	wildtype	16 (1^{st}) / 40 (2^{nd})

Table 2: Characteristics of the 12 patients with recurrent malignant melanoma analyzed regarding BRAF V600E mutation.

4 Discussion

Our study provides novel insights regarding the clinical characteristics and molecular pathways of recurrent melanocytic nevi and melanomas.

Recurrent nevi in our study were associated with age younger than 30 years, location other than the head/neck area and a shorter time to recurrence compared with recurrent melanomas, which mainly affected the head/neck region of patients older than 65 years and revealed a longer time to recurrence. Our findings are in line with a previous study identifying age older than 30 years and location on head/neck as independent factors associated with recurrent melanoma [40]. Mean age of the patients with recurrent nevi analyzed herein was 31 years compared to 30.2 years in the study by Blum et al., in patients with recurrent melanoma, mean age was 65 years in our series and 63.1 years in the cohort reported by Blum et al. [40].
Moreover, also similar to our findings, they reported on a shorter time interval of recurrence for nevi compared to melanomas in their sample [40]. The mean time interval between the first and the second treatment was 11 months and 8 months for patients with recurrent nevi in our and their cohort, respectively, while mean time to recurrence in melanoma patients was 35 months in our series and 25 months in their study [40].

Of particular interest is the fact that albeit BRAF V600E mutations are currently considered early driver events in the development of both, nevi and melanomas, up to date data are lacking regarding the frequency of BRAF mutations among re-growing nevi and melanomas.

The lack of data is even more surprising in the light of evidence suggesting the onset of new melanocytic lesions, either nevi or primary melanomas, under BRAFi therapy [85]. It has been shown that some of these lesions reveal marked clinical and histopathological atypia, making a confident differential diagnosis between nevus and melanoma difficult or even impossible [85]. The marked atypia of BRAFi induced melanocytic neoplasia is particularly interesting as also recurrent nevi are well documented melanoma simulators from a morphological perspective

[26]. In other words, the question arises whether BRAFi induced melanocytic lesions can be regarded equivalent to recurrent/activated nevi.

In order to gain more insights into the pathways driving re-growth in melanocytic neoplasia, we tested the BRAF V600E mutational status in a subset of recurrent nevi and recurrent melanomas and compared these findings with those of the primary tumors of each category. The rational to test for BRAF is based on several lines of evidence suggesting first, that this mutation is the most frequent in both, nevi and melanomas [44], [47], [48] and second, that newly developing melanocytic neoplasia and involuting nevi under BRAFi are usually wildtype and mutated, respectively [85]–[92].

Our genetical analysis confirms previous data regarding the frequency of BRAF mutations in relation to age and location. In line with previous reports, BRAF mutations in our study were detected at much higher frequency in primary nevi (78%) compared to primary melanomas (42%). Moreover, our study confirms current knowledge that melanomas arising on the head/neck area and/or in elderly persons show a low frequency with regard to BRAF mutations [63], [64] compared to other body sites and/or younger age.

The finding that BRAF mutations are particularly frequent in melanocytic nevi of young individuals supports the notion that BRAF plays a role in melanocytic growth. However, recent studies demonstrated that the frequency of BRAF mutations is also dependent from the actual growth phase of the nevus itself. In detail, actively growing nevi harbor BRAF mutations at much higher frequency (>90%) compared to stable nevi (around 60%) [59]–[62]. These data along with the findings of a clonal heterogeneity with respect to the mutation are currently explained by BRAF-induced oncogenic senescence. According to this model, BRAF-mutant cells differentially contribute to the life of a nevus, driving initially growth and later, inducing growth arrest via p16INK4a [56], [57].

Assuming that mutant BRAF plays a significant role in the growth of nevi, one could expect a high frequency of BRAF mutations also among recurrent nevi and

melanomas, which represent re-growing melanocytic proliferations after incomplete removal.

With regard to this assumption, our genetical analysis revealed some unexpected and novel findings that require further attention. Of a total of 36 analyzed nevi, 23 (64%) primary but only 16 (45%) recurrent nevi revealed mutations in the BRAF gene. Thereby we detected BRAF mutations in both, the primary and recurrent nevus, in only 1/3 of cases, whereas in another 1/3 BRAF V600E was present only in the primary nevus but not in the recurrent nevus. Vice versa, BRAF V600E mutation was detected in only 14% of recurrent nevi, which were initially wildtype for the mutation. In contrast, mutational BRAF was detectable in only 2 (17%) primary and 3 (25%) recurrent out of the 12 analyzed melanomas. Notably, none of the patients showed BRAF V600E mutation in both, primary and recurrent lesion.

Due to the lack of comparable data, we are unable to explain our findings, but there are at least some aspects that should be further discussed:

First, the fact that only a minority of nevi (31%) and none of the melanomas revealed the mutations in both, the primary and recurrent lesion, points towards mutually exclusive pathways resulting in growth/maintenance and re-growth between the primary and recurrent tumor. In fact, progression of BRAF mutant melanoma under BRAFi therapy has lead to the discovery of resistance via activation of alternative pathways [78], [81]–[84]. However, we did not further test for the possible activation of other present mutations or pathways.
On the other hand, the mechanical injury of the epidermis and/or basal layer by incomplete surgical removal itself may also result in an altered keratinocyte/melanocyte interaction or may cause upstream of cell cycle activating proteins. In this context it appears interesting that also blistering diseases such as epidermolysis bullosa (EB) may induce the rapid growth and development of large and bizzare appearing nevi. The exact mechanism of alteration in EB nevi is not known. Speculations exist on (i) the induction by the Koebner phenomenon initiating proliferation during reepitheliazation, (ii) and seeding of a blister cavity by preexisting nevi [95], [96].

Second, the fact that only a few recurrent nevi and melanomas revealed mutations in BRAF argues against the hypothesis that this mutation plays solely a function in driving melanocytic growth. In fact, most eruptive nevi or melanomas under BRAFi therapy are wildtype for the mutation [85]–[92].
Third, it has been shown that the frequency to detect the mutation strongly depends on the method employed to test for the mutation. We used Sanger method, which detects mutations at a cut off level of 20% [65]. Given that recurrent nevi usually contain fewer cells than primary tumors, also the method might have been to weak to detect the mutation equally in the primary and recurrent tumors.

Furthermore, we only evaluated a small group of patients, however, the number of patients developing recurrent malignant melanoma is low.

In conclusion, we demonstrate that the frequency of BRAF mutations differ between primary and recurrent melanocytic neoplasia, which might be explained by the activation of alternative pathways. Further studies are needed to gain more insights into the molecular pathways of recurrent nevi and melanomas in order to understand whether these tumors might function of model to test for nevogenesis and melanomagenesis.

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