

# Basal cell carcinoma develops in contact with the epidermal basal cell layer – a three-dimensional morphological study

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## Abstract

Basal cell carcinoma is the most common malignant tumor of the skin, and it develops most frequently on the areas of the body that make its treatment and care extremely difficult, especially in cases of neglecting or aggressive growth and invasion. Both typical mild cases as well as locally aggressive tumor types do not tend to metastasize, and it has been postulated that they should share some common biological and morphological features that might explain this behavior. In this study, we have utilized a high-resolution three-dimensional reconstruction technique on pathological samples from 15 cases of common aggressive (fibrosing and adenoid types) and mild (superficial type) basal cell carcinomas, and showed that all these types shared contact points and bridges with the underlying basal cell layer of the epidermis or with the outmost layer of the hair follicle. The connections found had in fact the highest number for fibrosing type (100%), compared to the superficial (85.71%) and adenoid (55%) types. The morphology of the connection bridges was also different, adjacent moderate to abundant inflammatory infiltrate seeming to lead to a loss of basaloid features in these areas. For the adenoid type, tumor islands seemed to be connected also to each other more strongly, forming a common “tumor lace”, and while it has been showed that superficial and fibrosing types have higher recurrence risks, all together these data might iterate a connection between the number of bridging points and the biological and clinical manifestation of this skin tumor.

**Keywords:** basal cell carcinoma, tumor continuity, epidermal continuity, three-dimensional reconstruction.

## Introduction

Basal cell carcinoma (BCC) is the most common skin cancer [1]. Most clinical and pathological types of BCC consist of slow growing tumors with a local evolution and only exceptionally with the occurrence of metastases [2]. However, there have also been described histopathological aggressive variants that can invade and destroy the skin towards deeper structures and tissues [2, 3]. Some variants, as the morphea-like or sclerosing type for example, can increase in size, invade and destroy the skin, skin appendages and the underlying adjacent tissues. Most BCC occur on the head and neck regions, aggressive ones leading to both dramatic esthetic and functional disabilities and also to important surgical treatment limitations. Examples of intracranial invasion towards the brain or the eyes have been reported in the literature, in some of them the tumor spread even leading to the death of the patients [4–11].

It has been postulated that BCC might derive from the basal cell layer of the epidermis or from the skin appendages, a plethora of cell biology-based studies supporting the derivation of tumor cells from this niches [12–15]. Cytokeratin expression and the expression of different transcription factors is also suggesting a common pattern between the basal cell layer of the epidermis or the outer cell layer of the hair shaft [16]. Morphological

studies have also been performed with the aid of different microscopy techniques, but they either lacked the processing power to render high-resolution reconstructions, or utilized too little number of cases.

If proven, this purely morphological description would not only shed more light onto the link between morphology and variate aggressive clinical behaviors of different BCC types, but resolving the technical challenges faced by such large volumetric analyses might make this applicable in other related areas such as pre-surgery superficial tumor growth evaluation and patient follow-up after treatment.

In this view, we have examined here the dermal–epidermal junction in 15 cases of superficial, adenoid and fibrosing BCC by utilizing a high-resolution three-dimensional (3D) reconstruction approach starting from continuous series of seriate sections, a technique that allowed us to investigate morphological changes in depths as much as 200 µm.

## Materials and Methods

### Tissue processing

Formalin-fixed, paraffin-embedded archived tissue blocks were selected from head and neck confirmed basal cell carcinoma cases from the Archive of Department of Pathology from Râmnicu Vâlcea County Hospital, Vâlcea

County, Romania, during 1999–2007 [17]. From this casuistry, we have randomly selected for this study five cases from superficial, adenoid and fibrosing BCC with head and neck localization.

After diagnostic reconfirmation, from each block were cut series of 50 seriate sections (5  $\mu\text{m}$ -thick) on a HM350 rotary microtome coupled with a waterfall-based section transfer system (STS) (Thermo Fisher Scientific, Walldorf, Germany), that allowed continuous cutting without losing any intermediate sections. All sections were first flattened on a water bath with added alcohol at 40°C for three seconds and then loaded on poly-lysine charged glass slides and dried overnight at 45°C. The slides were deparaffinized and re-hydrated through a series of graded alcohol baths of decreasing concentration, followed by a routine Hematoxylin and Eosin (HE) staining. After dehydration and overnight clearance in xylene, the sections were coverslipped in DPX mountant (Sigma-Aldrich Chemie GmbH, Munich, Germany). All protocols were kept constant, and all of the seriate sections in each batch were processed in one session.

### Image analysis

All slides were next photographed on a Nikon 55i microscope (Nikon GmbH, Wien, Austria) equipped with a 5 Mp cooled color CCD camera and the Image ProPlus AMS image analysis package (Media Cybernetics, Bethesda, MA, USA), under constant contrast and illumination conditions.

Only regions of interest (ROI) including tumor areas as well as adjacent epidermis were next targeted and followed up during the whole series of seriate sections, based on identifiable common reference points (*i.e.*, blood vessels, skin appendages, particular shapes of the tumor epithelia, etc.). From each considered case, four to five ROIs were selected for analysis. Consecutive images have been stacked into multi-layered *.tiff* files and individual planes were rotated and translated relatively to each other in order to produce a continuous follow-up of each ROI in Adobe Photoshop software (Adobe Systems Incorporated, San Jose, CA, USA). Images have been next segmented for their nuclear RGB profile, and after filtering out individual cells outside palisading tumor areas, based on an adjustable size-based morphological filter, the stromal components have been removed from the data sets Image ProPlus AMS (Figure 1). After rotation, stacks were imported again in Image ProPlus AMS, binarized and inverted. In order to increase the compactness and reduce the edges' ruggedness, stacks were subjected to a blind deconvolution algorithm with five iterations (Auto Deblur, Image ProPlus AMS). Finally, the stacks were converted to three-dimensional renderings using the 3D Constructor module (Image Pro AMS package), and volumes have been visually inspected. Contact points between tumor nests and basal layer of epidermis or hair follicle have been identified, counted and averaged for each tumor type, both on seriate sections and 3D renderings.

### Results

For fibrosing (morphea-like) BCC, serial sections follow-up revealed rare epithelial bridges between the superficial tumor nests and the basal cell layer of the

adjacent epidermis in all studied areas (100% of the 23 ROIs) from the five studied cases (Figure 2). These continuity areas spanned over no more than two sections (8  $\mu\text{m}$ ), and they tended to appear once at each 21 consecutive sections ( $\pm 15.62$  sections). In most of the instances, the tumor areas were connected to the basal epidermal layer (82.36%), but we also identified connection points with the basement layer of initial segments of the hair follicle appendage (the remaining 17.64%) (Figure 2). Deeper intradermal hair follicle appendages did not seem to be in direct continuity with the tumor islands, although this form of BCC typically extends into deeper dermis and beyond. Moreover, these epithelial junction bridges did not seem to retain the basaloid palisading pattern of the tumor areas themselves, and consisted mostly of flattened eosinophilic narrow strands of epithelia. Their thickness was around  $8.81 \pm 5.32 \mu\text{m}$  and contained not more than 2–3 rows of cells. Due to abrupt changes even at 4  $\mu\text{m}$  intervals and complicated tumor masses, no clear-cut connections could be observed on each individual slides between all the tumor islands. However, after 3D renderings, it became clear that for all studied areas, 95.74% of the individual tumor islands were connected to each other and all this complex meshwork had at least a communication bridge with the basal layer of the epidermis or of the hair follicle (Figure 2). Thus, peripheral buds or apparently isolated adjacent nests of tumor cells, which exhibit or not a typical palisading arrangement, even associated with different degrees of stromal densities, correspond to tumor extension and connection bridges either between the tumor masses, or between the tumor and the epidermis or skin appendages.

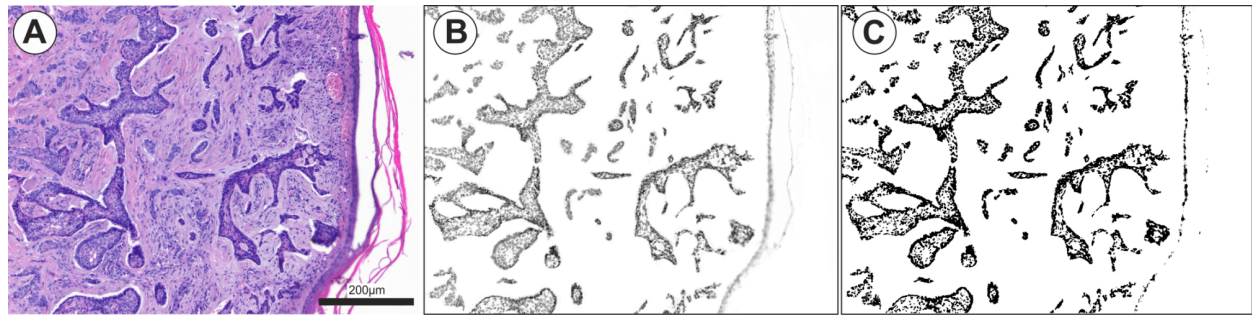
For the five superficial BCCs, again only the most superficial tumor foci showed clear-cut connection bridges with the underlying epithelium or the layers of the hair shaft, and this was encountered in 18 of the 21 studied ROIs (85.71%), for the 200  $\mu\text{m}$  depth analyzed for each stack (Figure 3). The continuity bridges tended to appear at each 23 consecutive sections ( $\pm 17.15$  sections). In these cases, we identified even less contact points with the basement layer of initial segments of the hair follicle (4.75%), as most of the contacts appeared with the basal epidermal layer (95.25%). None of the tumor foci exhibited clear-cut continuity with the deeper portions of the hair follicles, although now the tumors did not reach deeper reticular dermis. As opposite to the morphea-like BCC, these connection bridges retained the palisading basaloid architecture, and had an average thickness of  $14.41 \pm 8.24 \mu\text{m}$ , being thicker and shorter than those that characterized fibrosing BCC. On individual serial sections, more tumor islands seemed to be connected to each other as a “tumor lace” compared to fibrosing BCC, and 3D renderings confirmed here too that more than 95% of the tumor islands were connected to each other, and all these complexes had at least one bridge towards the basal layer of the epidermis or to the superficial portion of the hair follicle (Figure 3).

Although adenoid BCC with its typical lace-like morphology resembled the tumor nests density of the morphea-like pattern, it had a very few connections with the underlying epithelium or layers of the hair shaft (11 out of 20 studied ROIs, or 55%) (Figure 4). The continuity

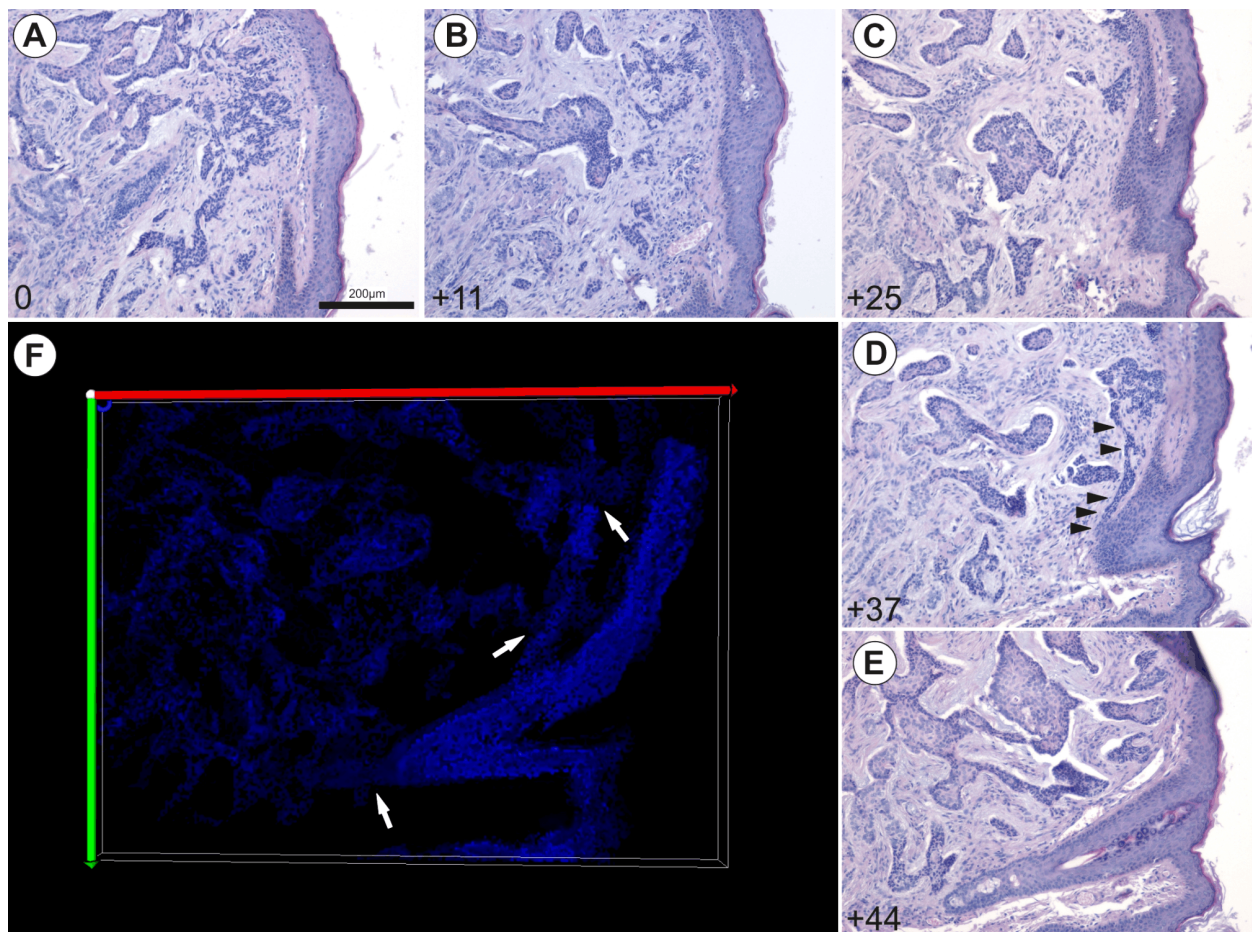
bridges tended to appear at each 29 consecutive sections ( $\pm 15.3$  sections). In fact, this tumor pattern had the smallest number of connections with the basal epidermal layer among all three studies instances. Although, like for fibrosing BCC, the tumor network invaded the deep dermis, there was even lower contact with the superficial or deeper segments of the hair follicle (3.24% of the counted contact points). Again, like for the fibrosing pattern, these epithelial junction bridges did not retain the basaloid palisading pattern of the tumor, and consisted of flattened eosinophilic narrow strands of epithelial cells. Their thickness was around  $5.25 \pm 3.57 \mu\text{m}$  and contained not more than 2–3 rows of cells, these being the thinnest bridging points amongst the

three studied entities. On both seriate sections and 3D renderings, this decreased continuity with the basal layers was evident (Figure 4).

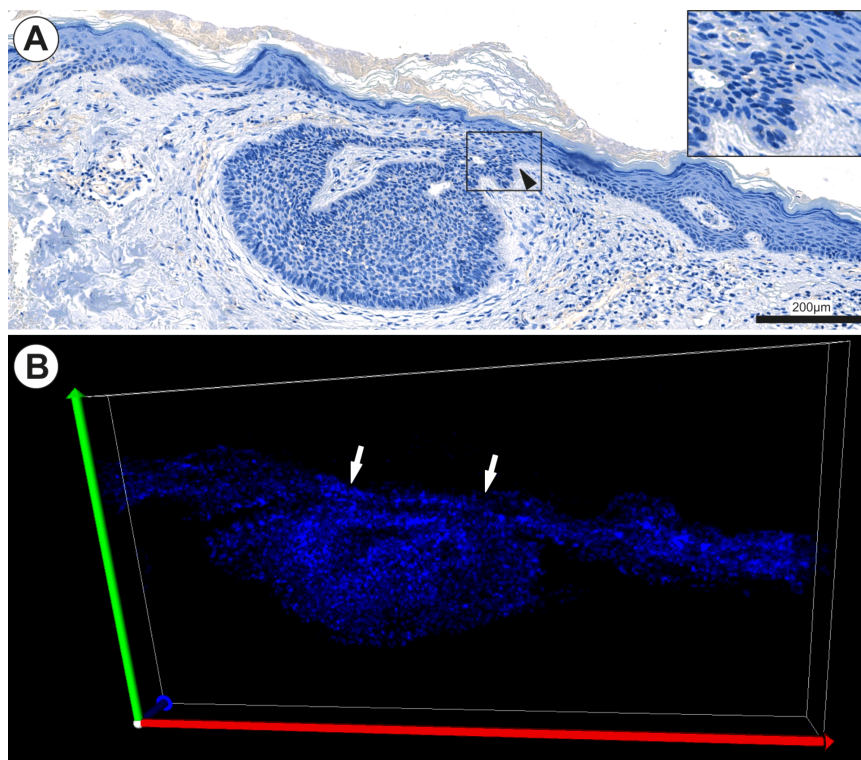
A mixed inflammatory infiltrate has been seen in conjunction mostly with the adenoid [four of five (80%) of cases] (Figure 4) and morphea-like [three of five (60%) of cases] types, in our randomly chosen casuistry none being present in the superficial group of cases. Moreover, the non-palisading pattern of the epithelial bridges joining the tumor islands with the basal cell layer of the epidermis was mostly seen in these cases with adjacent inflammatory infiltrate [in 25 of the 34 (73.5%) studied ROIs in these two types.



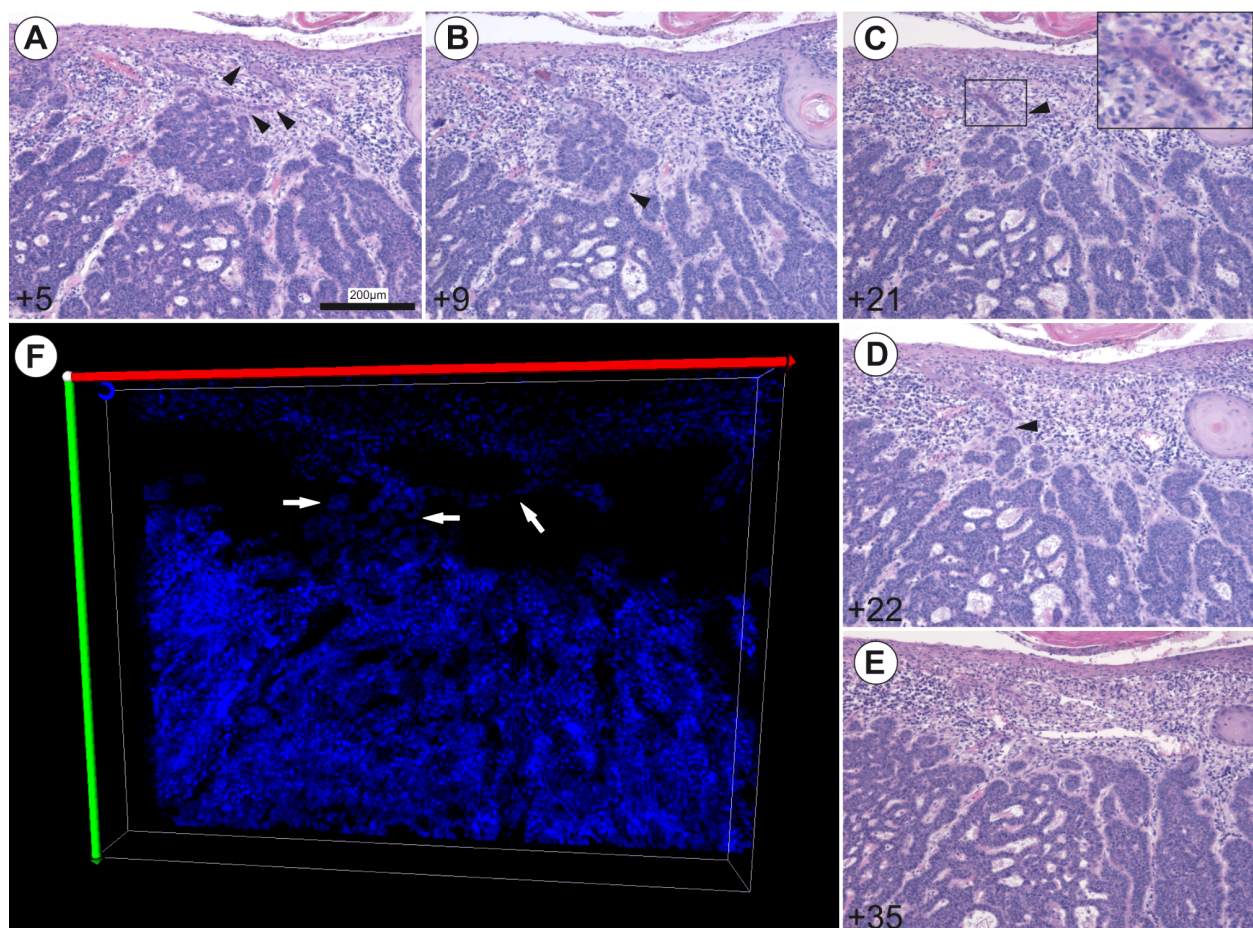
**Figure 1** – Example of the image segmentation algorithm utilized to extract the morphological features of BCC. The original captured image (A) (HE staining) is showed in conjuncture with the result of morphological filtering algorithm that removed the eosin and individual non-connected nuclei (B), and the final binarized image ready for stacking and three-dimensional rendering.



**Figure 2** – Exemplary rendering for a morphea-like BCC. Numbers in the lower left corner in A–E images (HE staining) represent distance in sections from the initial section (0). Arrowheads indicate tumor–epidermal bridges (E), and the arrows in the three-dimensional rendering (F) show connection points, not all apparent in the individual sections due to their oblique sectioning planes. A moderate inflammatory infiltrate is present in the surrounding dermis.



**Figure 3 – Exemplary rendering for a superficial BCC.** Arrowhead indicate one connection point of the tumor nest with the epidermis (A) (HE staining), while the arrows in the three-dimensional rendering (B) show all connection points evident in this volume. The inset in image A shows that bridging area exhibits a typical basaloid pattern.



**Figure 4 – Exemplary rendering for an adenoid BCC.** Numbers in the lower left corner in A–E images (HE staining) represent distance in sections from the initial section (0). Arrowheads indicate tumor–epidermal bridges (A–D), and the arrows in the three-dimensional rendering (F) show connection points, not all apparent in the individual sections due to their oblique sectioning planes. A more abundant inflammatory infiltrate is present in the surrounding dermis and the epidermis is partially eroded. Although the main tumor mass continuity cannot be assessed on individual sections due to its complexity, the rendering shows a clear-cut connection between all the strands of tumor cells (F). The inset in image (C) shows that bridging area exhibits a non-basaloid pattern with eosinophilic appearance.

## Discussion

Basal cell carcinomas (BCC) are slow growing cancers that have only on rare occasions an aggressive evolution, especially if ignored over time [4, 18]. There have been many histopathological classifications of BCC based on the tumor growth patterns and histological differentiation [18–23]. Despite this, due to intra-group variations, there is no common current agreement on the risk of local tumor recurrence for different tumor types after surgical excision, mainly because of their clinical invisible irregular extensions and growth patterns, which cannot be evaluated on individual randomly chosen sections from within the histopathological specimens.

In order to evaluate the growth patterns of BCC beyond the classical single-slide histopathological assessment, a more complex morphological evaluation is needed to draw more conclusions on their patterns of growth and the relationship with the clinical behavior. Imayama *et al.* (1987) published a first study addressing to this conundrum, and utilized scanning electron microscopy (SEM) on two superficial BCCs in order to assess their three-dimensional structure and to identify their growth characteristics [24]. This study showed that superficial BCCs grow beneath and along the epidermal basal cell layer and along the external root sheath of the hair follicle, and transformed tumor cells seemed like proliferating on these structures. The epidermal basal cell layer, under which the tumor had been growing, and with which the tumor nests came in contact, lost its original architecture [24]. The free basal cell layer of the surrounding epidermis presented scattered dendritic cells, with projections toward the dermis, cells that were also presented in another study as melanocytes [25]. For the hair follicles, their external root sheath was covered by the tumor cells, but only for the initial portion of the shaft. In the same lines, the tumor cell islands surrounded also the base of the sweat glands' ducts, while the deeper dermal portion of the hair follicle and the sweat glands' ducts had no contact with the tumor cells despite passing through a tumor volume [24]. In another study on 400 surgically excised BCCs, the connection between the tumor areas and the basal cell layer of the epidermis or of the hair follicles was observed in more than 90% of the cases, without any essential difference between superficial and nodular BCCs [26].

Despite the fact that these studies were rather old and did not benefit from the use of powerful computer-aided morphological analysis, a new growing body of technical possibilities and new imaging approaches would greatly benefit from a continuation of this study. A more recent study showed the potential of the current imaging techniques to resolve a large stack of histological serial sections into a volumetric rendering, and revealed that what might look in single histological sections as isolated tumor islands can be in fact finger-like connected structures growing as the volume of BCC [27, 28]. Although these studies were limited to solid BCC, and did not approach the connection between the tumor and the epidermis, they were a proof of principle for these new methods [27, 28]. In the same lines, optical coherence tomography (OCT) and *in vivo* fluorescent confocal microscopy have been shown to be able to provide contact-free imaging into the

skin and have proved good correspondence with routine histology in BCC [29–31], indicating that a strong connection of the BCC volume with the epidermis might act as a pre-requisite for further growth, and would clearly increase the clinical applicability of these imaging techniques.

We have showed here, first of all, that for morphea-like, superficial and adenoid BCC, high resolution histomorphological three-dimensional reconstruction reveals tumor continuity with the basal cell layer of the epidermis and the initial portion of the external root sheath of the hair follicle, for most of the studied cases (100% for morphea-like, 85.71% for superficial and 55% for adenoid types). This observation broadens the previous perspective that not only superficial and nodular BCC need to maintain their contact with an intact basal epidermal layer/hair follicles [24, 26, 27, 30]. It is not clear whether this contact is needed for further growth and differentiation, or for making metastases exceptional events. It is known that abnormal Hedgehog signaling in BCC leads to induction of Wnt ligands, like  $\beta$ -catenin, and these expression patterns are also present during the development of the embryonic human hair germs [32, 33]. It is still difficult to draw a conclusion here, as most of the published clinical data agree that superficial and morpheaform types have higher recurrence risks, while our study showed that these two forms have had the highest number of contacts with the epidermal basal cell layer. A quick diagnosis of locally extensive BCC may also refer these patients to new emerging therapeutic options based on these data, like the recently Food and Drug Administration (FDA)-approved Erivedge (Vismodegib), which acts by interfering with the Hedgehog signaling [34]. This novel molecule binds to smoothened (SMO) and leads to downstream inhibition of the Hedgehog pathway, with reports in the literature proving that the treatment is able to reduce the tumor sizes in more than 40% of the extensive BCC cases considered for study [34].

BCCs seem to be extending based on the growth of the cells from their most external cellular layers [35], and after the diameter of the tumor areas becomes more than 100  $\mu\text{m}$ , the tumor starts to divide and extend finger-like projections into the surrounding stroma [24, 27, 28]. The source is thought to be pluripotent progenitor cells in adult or epithelial germ cells for those neoplasms arising in childhood. BCCs have been showed to exhibit a cyto-keratin pattern similar to that of the lower part of the hair follicle, however distinct from that of the epidermal basal layer [16]. We have showed here that even in these conditions, an in-depth evaluation of the tumor volumes will reveal that these islands never remain completely independent in the dermis, keeping a permanent contact with the surrounding tumor volumes.

Lastly, we have showed that although inflammatory infiltrate occurs at different extents in different types of BCC, when not considering additional ulceration of the epidermis, most of this inflammatory infiltrate is located around the continuity points with the epidermal basal layer. Moreover, most often, these epithelial bridges do not exhibit a classical palisading pattern characteristic for the rest of the BCC areas, but become rather spindly and amphophilic, even slightly eosinophilic, an observation also made by other studies [24]. There is no other study

yet to address to the link between the epidermal-BCC bridges and the presence of inflammatory infiltrate, but it has been showed that systemic corticotherapy do not seem to increase the risk of BCC in young people, compared to long-term low-dose systemic corticotherapy that seem to be increasing the risk in older populations [36–39].

Powerful *in vivo* techniques like OCT, confocal microscopy, and volumetric histopathological evaluation using randomly selected sections stained with classical Hematoxylin and Eosin and corroborated with Cavalieri's principle volumetric estimations [40, 41], might compete as investigations of choice for both pre- and post-surgical evaluation of the patients with BCC and not only.

## Conclusions

Altogether, this study shows strong histopathological evidence that both mild and aggressive types of basal cell carcinoma still retain contacts with the epidermis and skin appendages, suggesting that this type of analysis can be further continued with immune three-dimensional investigations that might shed more light into the genesis of this and other related tumor entities.

## Conflict of interests

The authors declare that they have no conflict of interests.

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