Thesis

Characterization of the gut and skin microbiota in melanoma patients treated with immune checkpoint inhibitors: pilot study

submitted by

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Graz, 24th March 2023

Declaration of Academic Integrity

I hereby confirm that the present diploma thesis is the result of my own independent scholarly work. I also confirm that in all cases, where material from the work of others (in books, articles, essays, dissertations, and on the internet) is acknowledged, quotations and paraphrases are clearly indicated. No material other than that cited in the reference list has been used. I have read and understood the Medical University's regulations and procedures concerning plagiarism.

Graz, 24th March 2023 *Taissia Filippova* eh.

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List of Abbreviations

ALM	Acral lentiginous melanoma
BRAF	B-Raf Proto-Oncogene, Serine/Threonine Kinase
c-KIT	Receptor tyrosine kinase
CR	Complete remission/ response
CTLA-4	Cytotoxic T lymphocyte-associated antigen-4
ICIs	Immune checkpoint inhibitors
INF-α	Interferon alpha
Ipi	Ipilimumab
LDH	Lactate dehydrogenase
LMN	Lentigo maligna melanoma
MAPK	Mitogen-activated protein kinase
Nivo	Nivolumab
NNM	Nodular melanoma
NRAS	Neuroblastoma RAS viral oncogene homolog
OS	Overall survival
PD	Progressive disease
PD-(L)1	Programmed cell death (ligand) 1
Pembro	Pembrolizumab
PI3K	Phosphoinositide 3-kinase
PR	Partial response
RFS	Recurrence-free survival
SD	Stable disease
SLNB	Sentinel lymph node biopsy
SSM	Superficial Spreading Melanoma
TNM	Tumor-Lymph Nodes-Metastases
trAE	treatment-related Adverse Events
V600E	Valine (V) replaced by glutamic acid (E) at amino acid 600
W0	Week 0
W12	Week 12

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Zusammenfassung

Einleitung

Rezente vorklinische und klinische Studien haben den Einfluss des Darmmikrobioms auf die therapeutische Wirksamkeit von Immun-Checkpoint-Inhibitoren (ICIs) als palliative Therapie bei Patienten und Patientinnen mit fortgeschrittenen Melanom beschrieben. Wir untersuchten in einem ‚real world setting' (1) Veränderungen in der Mund-, Haut- und Darmmikrobiota unter adjuvanter und palliativer ICI-Therapie bei Patienten und Patientinnen mit fortgeschrittenem Melanom, (2) Unterschiede zwischen Mikrobengemeinschaften von ICI-Respondern und Nicht-Respondern, sowie (3) strukturelle Veränderungen der Darmmikrobiota von Patienten und Patientinnen, die höhergradigen immunvermittelte Nebenwirkungen entwickelten.

Methoden

Wir rekrutierten von November 2019 bis Dezember 2020 zwei verschiedene Kohorten: A) Patienten und Patientinnen mit neu diagnostiziertem Melanom (Stadien III/IV) vor Beginn einer adjuvanten/palliativen ICI-Therapie und B) Patienten und Patientinnen, die nach Abschluss einer adjuvanten/palliativen ICI-Therapie (Stadien III/IV) in klinischer Remission waren. Haut-, Mundabstrich- und Stuhlproben wurden vor und 12 Wochen nach ICI-Therapiebeginn gesammelt (Kohorte A), sowie bei einer Routine-Nachsorge (Kohorte B). Fragebögen zu Ernährung und Juckreiz wurden erhoben. 16SrRNA-Gensequenzierung wurde verwendet, um Diversität und taxonomische Charakterisierung des Mikrobioms durchzuführen. Wilcoxon-Rank-Tests und t-Tests wurden verwendet, um mikrobielle Diversität und Häufigkeit im Laufe der Zeit zu vergleichen.

Ergebnisse

In Kohorte A war die bakterielle Diversität von Darm- und Mundmikrobiom vor und 12 Wochen nach Einführung von ICI-Therapie vergleichbar. Die Zusammensetzung der Hautmikrobiota verschob sich unter ICI-Therapie, *Campylobacter* und *Faecalibacterium* waren 12 Wochen nach Therapiebeginn signifikant häufiger vertreten. Im Darmmikrobiom konnten wir mehrere Bakterientaxa (*Parabacteroides (Tannerellaceae), Odoribacter (Marinifilaceae)* und *Alphaproteobacteria)* identifizieren, die mit Therapieansprechen assoziiert sind. Non-Responder der Kohorte A hatten signifikant mehr *Enterococcaceae* und *Faecalitalea* in ihrem Darmmikrobiom als Patienten und Patientinnen der Kohorte B (langfristige Remission), die mehr *Dorea* aufwiesen.

Patienten und Patientinnen mit höhergradigen immunvermittelten Nebenwirkungen (≥Grad 3) wiesen signifikant mehr *Barnesiellaceae, Rikenellaceae, Parasutterella und Ruminococcaceae NK4A214* in ihren Stuhlproben auf.

Schlussfolgerungen

Diese Erkenntnisse könnten nützlich sein, um neue Biomarker zu ermitteln, mit denen sich das Ansprechen auf ICI vorhersagen oder verbessern und schwere Nebenwirkungen verhindern lassen. Zum ersten Mal konnten Veränderungen im Hautmikrobiom unter ICI-Therapie beschrieben werden. Weitere Forschung ist erforderlich, um die Rolle der Darm- und Hautmikrobiota und ihre Bedeutung für die Entwicklung von Nebenwirkungen nach der Einführung von ICIs zu verstehen.

Abstract

Introduction

Recent studies have shown that the gut microbiota can affect the efficacy of palliative immune checkpoint inhibitor (ICI) therapy in patients with advanced melanoma. In a real-world setting, we aimed to investigate (1) changes of the oral, skin and gut microbiota in response to adjuvant and palliative ICIs in melanoma patients, (2) differences in composition of the gut microbiota between responders versus non-responders to ICIs, and (3) alterations of the gut microbiota of patients with severe treatment-related adverse events (trAEs).

Methods

This prospective cohort study included patients A) with recently diagnosed melanoma (stages III/IV) receiving adjuvant/palliative ICI therapy, and B) in complete remission after adjuvant/palliative ICI therapy (stages III/IV). Skin, oral and stool samples were collected before and 12 weeks after ICI initiation (cohort A), and at routine follow-up exam (cohort B). Patient-reported questionnaires on nutrition and itch were collected at similar time points. 16SrRNA gene sequencing was used to perform diversity and taxonomic characterization of the microbiome. Wilcoxon signed-rank tests and t-tests were used to compare microbial diversity and abundance over time.

Results

In cohort A, bacterial diversity of oral and gut microbiota was comparable before and 12 weeks after start of ICI therapy. The skin microbiota composition changed during ICI therapy, and *Campylobacter* and *Faecalibacterium* were significantly more abundant 12 weeks after initiation of therapy. Several bacterial taxa of the gut microbiota (*Parabacteroides (Tannerellaceae), Odoribacter (Marinifilaceae)* and *Alphaproteobacteria)* were associated with therapy response. Non-responders (cohort A) had significantly more *Enterococcaceae* and *Faecalitalea* in their gut microbiome than patients from cohort B (long-term remission), which had more *Dorea*. Patients with high-grade trAE (≥grade 3) had significantly more *Barnesiellaceae, Rikenellaceae, Parasutterella,* and *Ruminococcaceae NK4A214* in their intestinal microbiome.

Conclusion

These findings could help to identify new biomarkers to predict or even improve the response to ICI therapy and prevent severe side effects. For the first time, changes in the skin microbiome during ICI therapy have been described. Further research is needed to understand the role of gut and skin microbiota and their importance in the development of trAEs.

1 Introduction

1.1 Melanoma

Melanoma is a malignant skin cancer that originates from melanocytes, which are pigment-producing cells. It usually develops in cutis but can also arise on the mucosa, uvea, and leptomeninges [1].

The first written description of melanoma dates back to the 5th century BC when Hippocrates of Cos described melanoma. The first archaeological evidence of this skin cancer was found in the skeletons of 2400 years old Pre-Colombian mummies in Peru [2]. The incidence of melanoma is increasing. Melanoma affects young individuals more often than other types of cancer [3]. The early detection and prevention of melanoma significantly reduces mortality. However, it remains difficult to manage if it is detected at an advanced stage. Immune therapy has revolutionized the therapeutic approach for advanced melanoma, but the survival period ranges from eight to 12 months [4]. The combination of checkpoint inhibitors and radiotherapy has increased the survival rate to several years, but research is urgently needed to improve the response rate and overall survival [5].

Bacteria and other microorganisms inhabit humans and contribute to human health and disease regulation. An imbalance in the gut microbiome causes dysbiosis, which is associated with inflammatory bowel disease, obesity, metabolic syndrome, and other diseases. Recent studies have shown an interaction between the microbiome and the immune system [6]. This study aimed to investigate the influence of the microbiome on the response to checkpoint inhibitor therapy in patients with melanoma.

1.1.1 Epidemiology

In 2020, it was estimated that 324,635 people worldwide were diagnosed with melanoma [7]. In Europe, the incidence of melanoma varies from state to state: 3-5/100,000 in Southern countries, 12-35/100,000 in Nordic countries, whereas worldwide, Australia has the highest incidence, 50/100,000 [8].

In Austria, more than 1,500 new melanoma cases were diagnosed in 2019 (17/100,000 cases). Moreover, this type of cancer accounts for 3.6% of all cancers and 1.8% of all deaths due to cancer [9].

Melanoma is more frequent in males than in females, and the median age at first diagnosis is 65 years; however, people of any age can be affected [8].

The overall mortality rate in Nordic Europe was higher than in the rest of Europe. The highest mortality is registered in Norway (3.5/100,000 world standard population in 2012), but the overall survival rate has increased. In Europe, the 5-year relative survival rate between 2002 and 2007 was almost 87%, with the highest rates in Nordic countries (90%) and the lowest in Eastern Europe (almost 70%) [10].

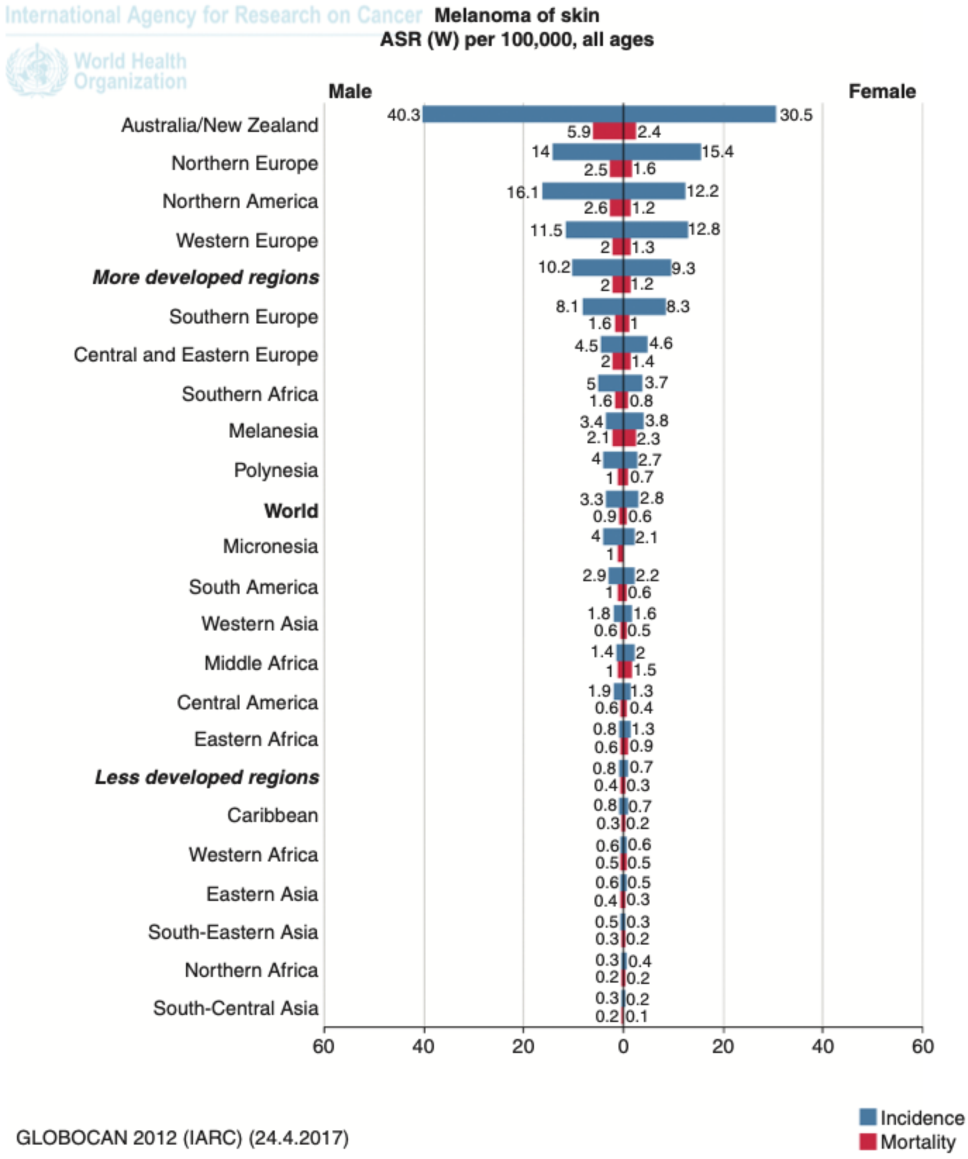


Figure 1 *Age-standardized annual incidence and mortality rates of melanoma by sex, world region, and development. ASR = Age-standardized rate (world) expressed per 100,000 persons. Image taken from Ref. [3]*

1.1.2 Risk factors and causes

Multiple risk factors can lead to melanoma development. There are constitutional or acquired risk factors. Constitutional risk factors for malignant melanoma include the skin type and (large) congenital nevus. The number of nevus cell nevi correlates with the risk of melanoma, and almost 25% of melanoma develops from nevi [11, 12]. Acquired risk factors include a previous history of melanoma, family history of melanoma, number of acquired nevi, and clinically atypical moles [13]. Ultraviolet (UV) irradiation is one of the most important risk factors associated with DNA damage in skin cells. Intermittent ultraviolet (UV) exposure and sunburns are associated with a higher risk of melanoma development [14]. Moreover, different mutations are associated with an increased risk of melanoma, for example, mutations in cyclin-dependent kinase inhibitor 2A (CDKN2A or p16) or cyclin-dependent kinase 4 (CDK4) [15]. Individuals with hereditary genetic mutations, such as familial retinoblastoma, Li-Fraumeni cancer syndrome, and Lynch syndrome type II, can develop melanoma at a younger age (<40 years) [16, 17].

1.1.3 Forms

The melanoma classification (**Table 1**) is based on clinical and histological characteristics and can be divided into four main subtypes: superficial spreading melanoma, nodular melanoma, lentigo malignant melanoma, and acral lentiginous melanoma [18].

Superficial Spreading Melanoma (SSM) is the most common type of melanoma associated with intermittent sun exposure. Typical localizations are on the backs of women's legs and the backs of men [19].

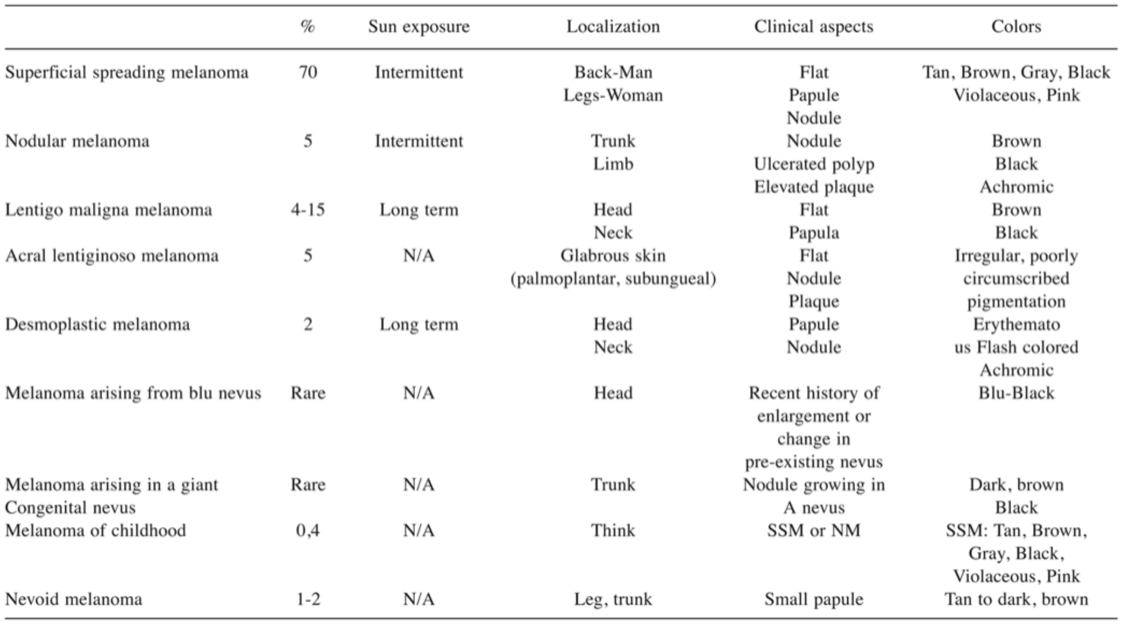
Nodular Melanoma (NM) is responsible for 16% of malignant melanomas. This melanoma usually occurs on the trunk and limbs and mainly occurs in patients in the fifth and sixth decades of life [19]. It shows vertical and exophytic growth and is characterized by ulcerative lesions [18]. The nodular structure originates from small nests and aggregates of cancer cells [20].

Lentigo maligna melanoma (LMM) typically occurs after long-term sun exposure in older adults [19]. Distinct localizations are neck and head [20]. Its main characteristic is the lentiginous proliferation of atypical melanocytes on the basal layers of the epidermis, and it shows different colors [18, 19].

Acral lentiginous melanoma (ALM) is typically to find under nails or palms and soles [18, 19]. It usually occurs in elderly Asians, Hispanics, and Africans [19].

Furthermore, depending on UV radiation (UVR) exposure and localization, specific gene mutations have been identified in melanomas. BRAF mutations are associated with low UVR exposure, and this type of melanoma typically arises on the trunk and extremities (SSM). NRAS mutations are associated with high UVR-exposure in LMM and dysplastic melanoma. Melanomas not associated with sun exposure, such as spitz melanoma, acral melanoma, and others, sometimes carry c-KIT mutations [18].

Table 1 *Melanoma clinical subtypes, typical localization, clinical aspects and colors. Table taken from Ref. [19]*



	%	Sun exposure	Localization	Clinical aspects	Colors
Superficial spreading melanoma	70	Intermittent	Back-Man Legs-Woman	Flat Papule Nodule	Tan, Brown, Gray, Black Violaceous, Pink
Nodular melanoma	5	Intermittent	Trunk Limb	Nodule Ulcerated polyp Elevated plaque	Brown Black Achromic
Lentigo maligna melanoma	4-15	Long term	Head Neck	Flat Papula	Brown Black
Acral lentiginoso melanoma	5	N/A	Glabrous skin (palmoplantar, subungueal)	Flat Nodule Plaque	Irregular, poorly circumscribed pigmentation
Desmoplastic melanoma	2	Long term	Head Neck	Papule Nodule	Erythemato us Flash colored Achromic
Melanoma arising from blu nevus	Rare	N/A	Head	Recent history of enlargement or change in pre-existing nevus	Blu-Black
Melanoma arising in a giant Congenital nevus	Rare	N/A	Trunk	Nodule growing in A nevus	Dark, brown Black
Melanoma of childhood	0,4	N/A	Think	SSM or NM	SSM: Tan, Brown, Gray, Black, Violaceous, Pink
Nevoid melanoma	1-2	N/A	Leg, trunk	Small papule	Tan to dark, brown

1.1.4 Diagnostic approach

Clinical evaluation of pigmented lesions involves visual analysis (ABCDE rule), intra-individual comparison of the lesions (ugly duckling sign), and chronologic analysis of changes [18]. The ABCD(E) stands for:

- **A – Asymmetry –** The form and pigmentation of a lesion are analyzed for symmetry. Typical melanomas are asymmetric.
- **B – Borders –** In melanoma, the borders are irregular and poorly demarcated.
- **C–Color-**lesions with only one color are generally benign. Malignant skin tumors often appear in many colors: black, brown, white, red, or blue.
- **D – Diameter –** Skin tumors < 5 mm are mostly benign but not all. If the diameter of a lesion is more extensive than 6 mm, it could be a sign of malignancy.
- **E – Evolution:** It is essential to ask the patient about changes in the lesion. In case of recent changes, further examination should be considered.

The sensitivity of self-skin examination using the ABCD(E) rule ranges from 57% to 90% [21]. These criteria are suitable for SSM, whereas papular or nodular lesions are evaluated using the EFG rule [18]:

- **E – Elevated –** nodular lesions typically show vertical growth.
- **F – Firm –**Tumors are firm to touch.
- **G – Growing –** the most crucial factor: Lesion growth**.**

Dermoscopy is the most important noninvasive method used to examine lesions and evaluate differential diagnoses **(Figure 2)**. The dermoscopic criteria used to identify melanoma are as follows: atypical pigment network, irregular dots/globules, irregular streaks, irregular pigmentation, regression structure, blue-whitish veil, and vascular pattern [19].

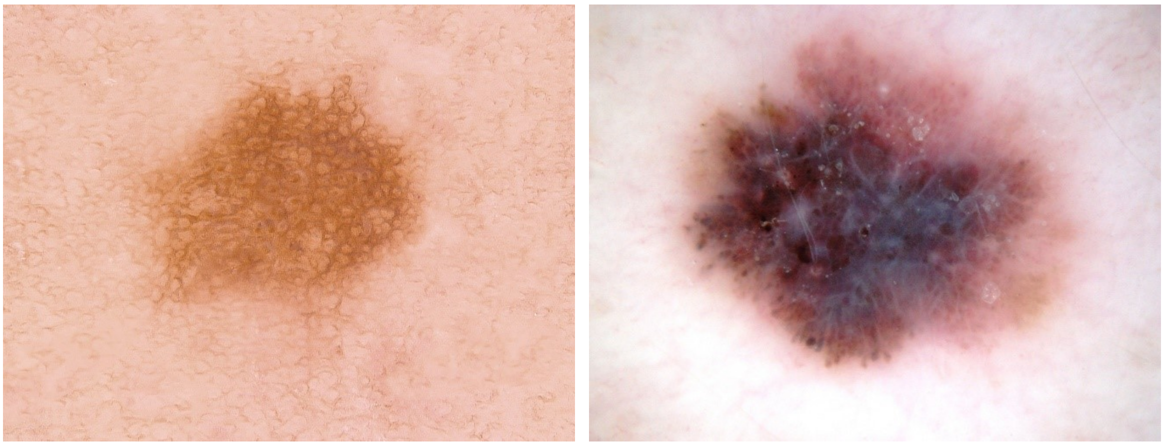

benign malignant

Figure 2 *Dermoscopy of benign and malignant lesions. The benign lesion shows a typical network, no white or blue color, and a symmetric form and pigmentation. Malignant lesion (melanoma) exhibits morphologically different networks, many different colors, and an asymmetrical distribution of colors and forms. Images taken from Ref:[22, 23]*

Total-body photographic images and digital dermatoscopy are helpful for the early detection of melanoma in high-risk individuals. This reduces the number of unnecessary excisions of atypical lesions [18].

Other non-invasive methods include **reflectance confocal microscopy**, which examines melanocytic lesions in vivo at histological resolution [19].

Excision and histological examination are crucial for confirming the diagnosis and clinicopathological type, tumor thickness in mm, presence or absence of ulceration, number of mitoses per mm2, and microsatellites [18]. Radical excision should be performed at safe distances to the tumor margin to avoid local recurrence of the tumor [24].

Moreover, **mutational analysis** is performed in patients with distant or non-resectable regional metastases. These patients may be treated with BRAF and MEK inhibitors if BRAFV600 mutation is confirmed. NRAS mutations and c-KIT mutation status have also been investigated [18].

1.1.5 Staging

Melanoma staging requires a tumor-node-metastasis (TNM) staging system. The definition of a primary tumor relies on the Breslow depth (thickness) and histopathological evidence of ulceration (**Table 2)**. The node classification focuses on the invasion of regional lymph nodes (**Table 3)**. Finally, metastasis classification reflects distal metastasis and serum lactate dehydrogenase (LDH) levels (**Table 4**) [1].

There are four stages of melanoma (0-IV). Stage 0 represents in-situ melanoma; the first two stages (I and II) include localized melanoma, whereas in stage III, local lymph nodes are affected. Stage IV indicates the presence of distal metastasis (**Table 5**) [1].

Table 2 *AJCC 8th edition: Primary tumor classification [25]*

Classification	Thickness	Ulceration status
Tis (melanoma in situ)	N/A	N/A
T1	≤ 1.00 mm	a: <0.8 mm without ulceration b: 0.8-1.00 mm with/ without ulceration and <0.8 mm with ulceration
T2	1.01-2.00 mm	a: without ulceration b: with ulceration
T3	2.01-4.00 mm	a: without ulceration b: with ulceration
T4	> 4.00 mm	a: without ulceration b: with ulceration

Table 3 *AJCC 8th edition: Classification of regional metastases [25]*

Classification	Lymph nodes with metastasis	Type of metastases
N0	none	
N1	1	a: micrometastases b: macrometastases
N2	2-3	a: micrometastases b: macrometastases c: metast. in lymph nodes and in-transit, satellite or microsatellite metastases
N3	4 or more	a: micrometastases b: at least one macrometast. c: metast. in lymph nodes and in-transit, satellite or microsatellite metastases

***Table 4** AJCC 8th edition: Classification of distant metastases [25]*

Classification	Location of distant metast.	LDH
M0	-	-
M1a	Skin, soft tissue, non-regional lymph nodes	(0) normal (1) elevated
M1b	Lungs ± 1a	(0) normal (1) elevated
M1c	Organs other than CNS ± 1a, 1b	(0) normal (1) elevated
M1d	CNS ± 1a, 1b, 1c	(0) normal (1) elevated

***Table 5** AJCC 8th edition: Clinical stage groups [25]*

	T	N	M
0	Tis	N0	M0
IA	T1a	N0	M0
IB	T1b T2a	N0	M0
IIA	T2b T3a	N0	M0
IIB	T3b T4a	N0	M0
IIC	T4b	N0	M0
III	Any T	≥ N1	M0
IV	Any T	Any N	M1

1.1.6 Prevention

UV exposure is the main acquired risk factor for malignant melanoma development. Therefore, the primary prevention focuses on the prevention of UV exposure. The main protective factors are avoiding exposure to intense solar radiation and wearing suitable clothing and sunscreens. Secondary prevention focuses on the early detection of skin cancer [13].

1.1.7 Prognosis

The prognosis of melanoma depends on its clinical stage. In the first stage, the 5-year survival rate is > 90%. Ulceration decreases the survival rate. The stage III survival rate varies from 70% to 40% in patients with ulcerative tumors. In stage IV, the prognosis is poorer and depends on the location of distant metastasis. Visceral metastases have poorer outcomes than non-visceral metastases. Other important prognostic factors include age, sex, and anatomic site [1].

1.1.8 Cellular Signaling Pathways in Melanoma

Tumor development depends on genetic predispositions, environmental factors, and immune responses. Oncogenes and suppressor genes can promote or inhibit tumor development. The origin of melanoma is sometimes associated with mutations in the mitogen-activated protein kinase (MAPK) and phosphoinositide 3-kinase (PI3K) signaling pathways, such as BRAF, NRAS, and KIT (receptor tyrosine kinase) **(Figure 3)**. Mutations in the MAPK pathway (e.g., BRAF and NRAS) are strongly associated with the origin of melanoma without chronic sun-induced damage. Mutations in KIT genes are responsible for 30-40% of acral and mucosal melanomas and melanomas resulting from chronic sun-induced damage (**Figure 4**) [1, 26].

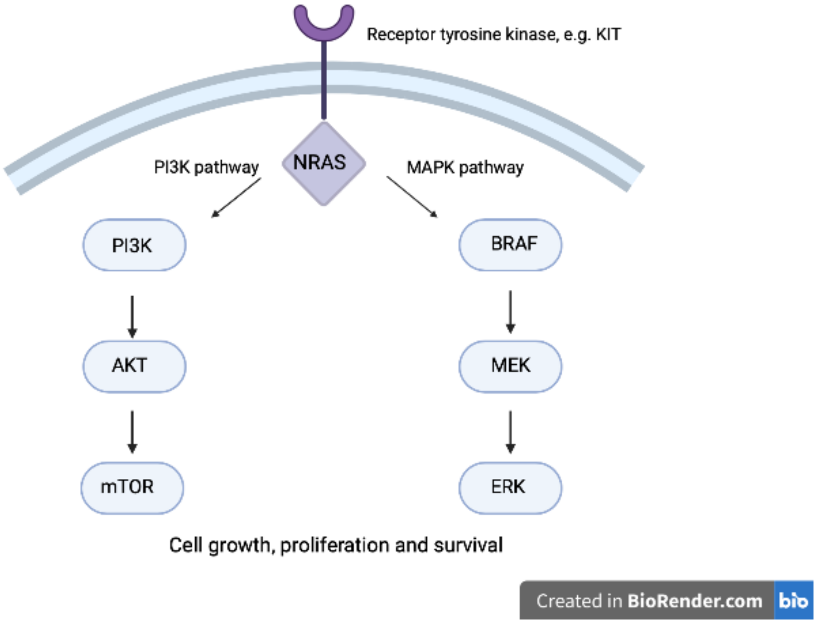


Figure 3 *MAPK and PI3K signaling pathways. MAPK and PI3K pathways regulate cell growth, proliferation and survival. An activating mutation in the BRAF and RAS genes leads to cell proliferation. Figure created in BioRender.com*

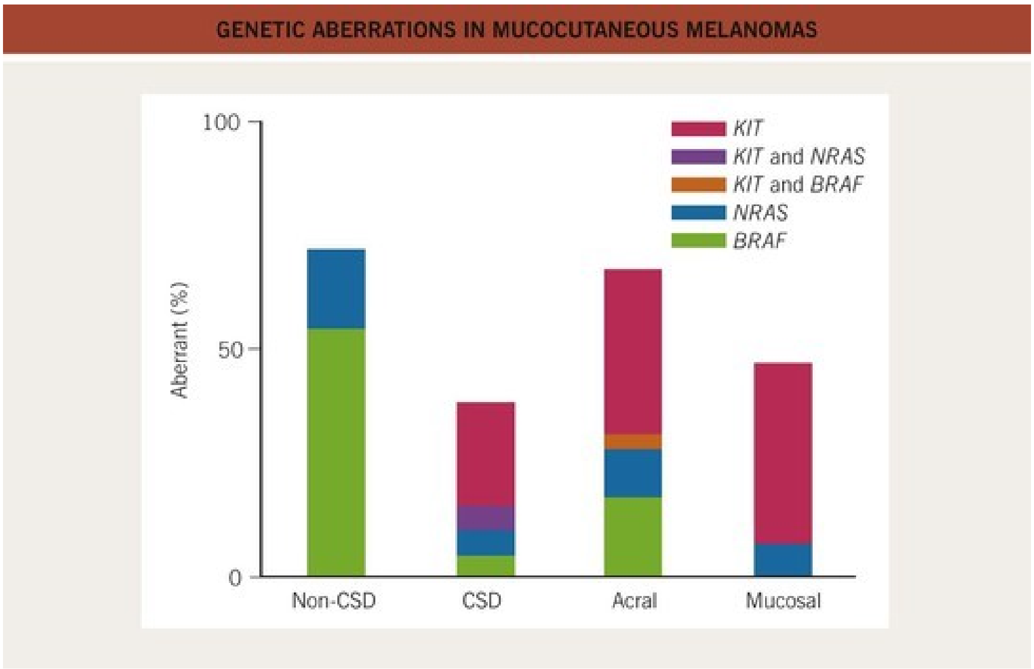


Figure 4 *Genetic aberrations in mucocutaneous melanomas. CSD: skin with chronic sun-induced damage; non-CSD: skin without chronic sun-induced damage. Figure taken from Ref. [26]*

1.1.9 Therapy

Diagnosis of genetic mutations has allowed the introduction of targeted therapies in 2011 [1, 27].

Stage I and II melanoma

The diagnosis of melanoma requires histological examination. After confirming the diagnosis, excisional margins should be adjusted depending on the Breslow depth: 1 cm margin for melanomas with Breslow depth of <2.0 mm and up to 2 cm margins when the depth is 2.01-4.0 mm. Excision to the subcutaneous fat tissue is sufficient. Facial or mucosal membranes are often surgical challenges because of their limited surface area. Patients with stage IIC melanoma should receive the same treatment as stage III melanoma because of the high risk of recurrence [24].

Stage III melanoma

At this stage, the local lymph nodes metastasize. Sentinel lymph node biopsy (SLNB) is recommended for melanomas with a depth >1 mm. It provides information about the presence of micrometastases in clinically unremarkable lymph nodes. Elective lymph node dissection after positive SLNB showed no evident survival benefit compared with the observation of lymph nodes with ultrasound [24, 28]. Therapeutic lymph node dissection is indicated in cases of lymph node metastasis without evidence of distant metastasis [24].

Adjuvant therapy

Adjuvant therapy aims to eliminate micrometastases and prolong patient survival. The indication for this therapy is high-risk stage II or III melanoma. Anti-PD-1-antibodies (stage IIIA-D or IV (no evidence of disease (NED)), BRAF/MEK inhibitors (BRAF V600E or V600K mutations), and IFN-α (stage IIB/C) are approved for this purpose [1, 24].

Stage IV melanoma

At this stage, the metastases had spread. Therapy with kinase inhibitors and immune checkpoint inhibitors may improve survival [29]. Resection of singular metastases in the palliative setting may improve overall survival and quality of life [30]. Moreover, radiotherapy can be used in palliative settings to reduce metastasis-associated symptoms such as bone pain and brain compression symptoms [1]. Adjuvant radiotherapy after lymph node excision does not improve overall survival [31].

1.1.9.1 Molecularly targeted therapy

Systemic therapy is key to treating melanomas with metastases. 40-60% of melanoma patients have BRAF mutation [28]. BRAF is a gene located on chromosome seven that encodes a protein called BRAF. It plays a vital role in cell growth. A specific mutation that occurs is V600E; valine (V) is replaced by glutamic acid (E) at amino acid 600 [32]. 15-20% of melanomas show NRAS mutations. NRAS is an oncogene that plays an essential role in MAPK activation and subsequent cell growth and proliferation [33].

The approved inhibitors of BRAF kinase are **vemurafenib**, **dabrafenib, and encorafenib** (**Figure 5**). The main problem with these therapies is the development of therapy resistance within six month [1]. The combination of MEK inhibitors, such as **trametinib, cobimetinib or binimetinib,** with BRAF inhibitors, increases the resistance-free interval to 10 months [34]. 25% of the patients with NRAS mutations responded positively to MEK inhibitors. Melanomas with KIT mutations may show a response to KIT receptor inhibitors (off-label use), such as **imatinib, sunitinib, dasatinib, or sorafenib** [1].

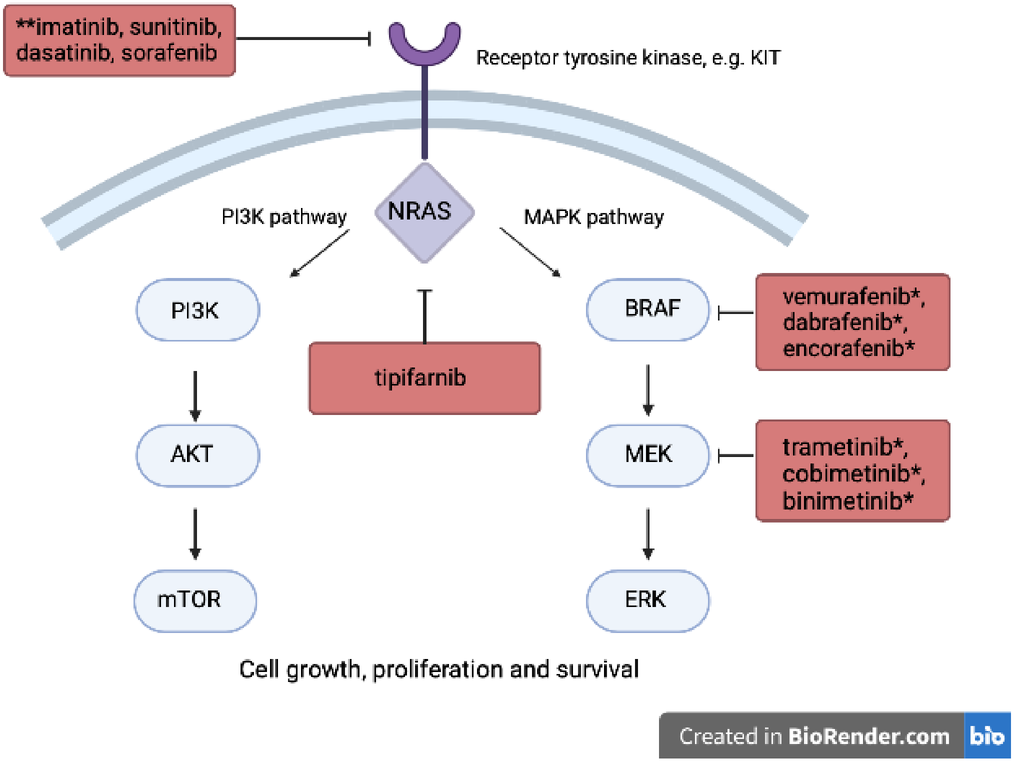


Figure 5 *MAPK and PI3K signaling pathways and targeted therapy. Figure created in BioRender.com*
**Drugs approved for melanoma treatment*
*** Off-label drugs used in melanoma treatment*

1.1.9.2 Immunotherapy

Immunotherapy is one of the most promising therapies for the advanced stages of melanoma.

A) Biological Immunotherapy. Cytokines, interferons, and granulocyte-monocyte colony-stimulating factors are considered biological. Biologicals are used in combination with stereotactic radiotherapy to treat melanoma.

B) Vaccination strategy: This therapy is currently being studied and is based on peptides, proteins, viruses, DNA, or dendritic cells.

C) Adoptive Cell Therapy. Lymphokine-activated killer cells, tumor-infiltrating lymphocytes, and other lymphocytes are processed to induce anticancer immune responses. This approach is still experimental and requires further study for its safety and efficiency.

D) Immune checkpoint inhibitors. Specific antibodies can block inhibitory checkpoints and allow immune cells to activate antitumor activity [35].

1.1.9.2.1 Immune checkpoint blockade

Immunotherapy attempts to activate the host immune system, which recognizes malignant cells and eliminates them. Malignant cells "hide" from the immune system cells so that they can grow without being destroyed.

Immune checkpoints are cell surface proteins that control the activation of immune cells. Antibodies that antagonize the inhibitory receptors on T cells are used as immune checkpoint inhibitors. There are two types of antibodies: cytotoxic T lymphocyte-associated antigen-4 (CTLA-4) blockade antibodies and programmed cell death 1 (PD-1) antibodies [1]. CTLA-4 and PD-1 are overexpressed in melanoma cells [35].

CTLA-4 is a protein found on T cells that binds to another protein called B7 and, as a result, prevents T cells from attacking other cells. CTLA-4 inhibits T cell activation; the antibodies block CTLA-4 and allow the activation of T cells that can recognize and kill malignant cells (**Figure 6**) [36]. **Ipilimumab** and **tremelimumab** are antibodies used to block CTLA-4. A response was observed in 15% of patients if used alone. Colitis, hepatitis, thyroiditis, and hypophysitis are side effects of this therapy [1]. The combination of ipilimumab and nivolumab (anti-PD-1 antibodies) increased the response rate by up to 61%. The side effects were significant (grades 3 and 4) [37].

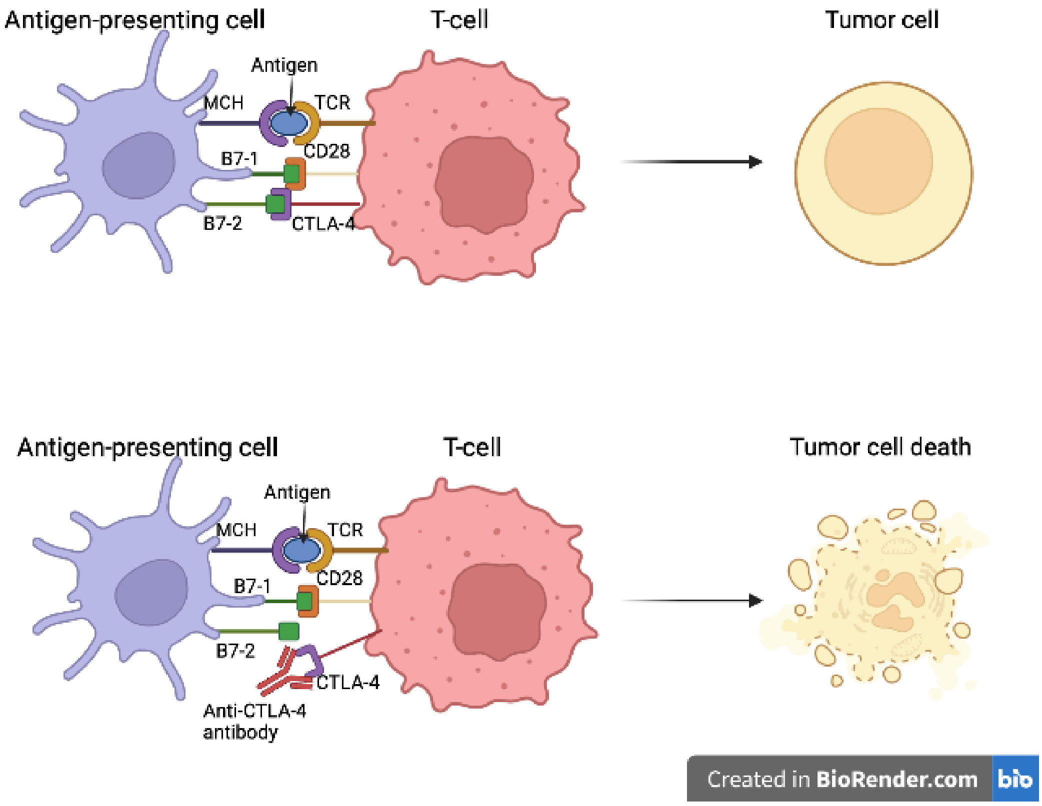


Figure 6 *Function of CTLA-4. CTLA-4 binds to the B7-2 protein and inhibits T-cell activation. Anti-CTLA-4 antibody inhibits the CTLA-4 receptor and activates t-cells. Figure created in BioRender.com*

Programmed death-ligand 1 (**PD-L1**) is a transmembrane protein expressed in many different cells, such as tumor cells, that can bind to **PD-1 proteins** on T cells and prevent T cells from killing other cells. This mechanism prevents autoimmune diseases, but in the same way it does not allow T cells to kill cancer cells. Anti-PD-1 antibodies block these receptors, resulting in T cell activation (**Figure** 7) [38]. Therapies approved as anti-PD-1 antibodies are nivolumab and pembrolizumab. Patients who received nivolumab showed a higher one-year survival rate (73%) than those who received chemotherapy with dacarbazine (42%) [39]. In addition, nivolumab and pembrolizumab were more effective in progression-free survival than ipilimumab [40]. Treatment-related adverse events of anti-PD-1 antibodies include gastrointestinal, endocrine, dermatological, pulmonary, and joint toxicities, in addition to cardiotoxicity and neurotoxicity [1]. The incidence of trAE is higher with ICI combination therapy than with ICI monotherapy [41].

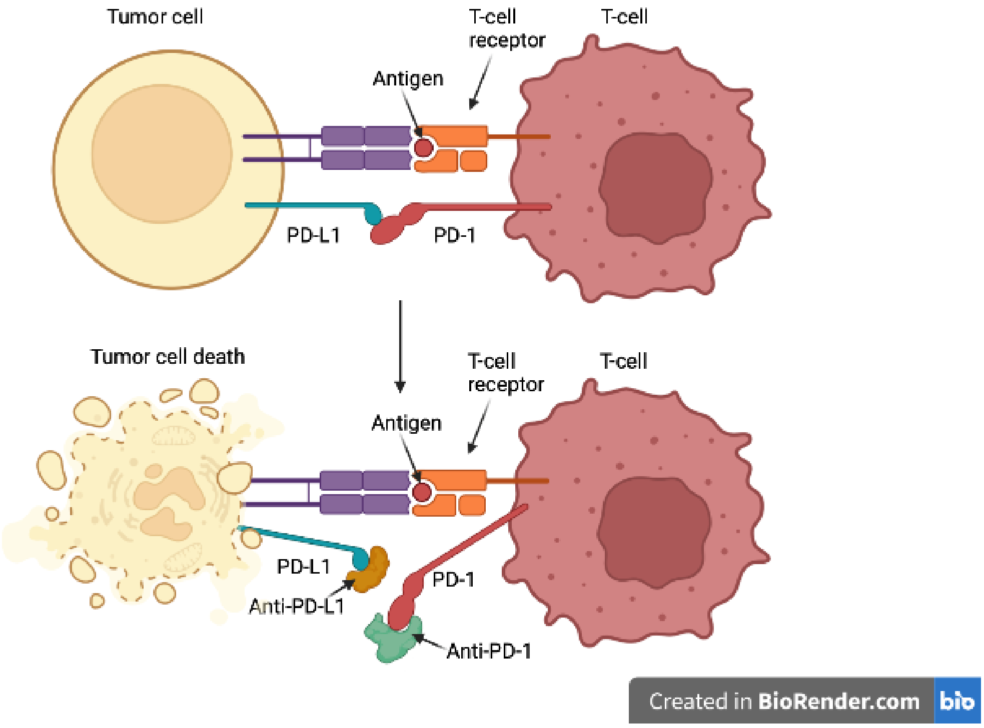


Figure *7 Function of anti-PD-(L) 1 antibodies. PD-L1 of the tumor cell binds to PD-1 of the t-cell and inhibits the killing of tumor cells. By inhibiting these proteins with anti-PD-(L) 1 antibodies, t-cells recognize tumor cells and kill them. Figure created in BioRender.com*

The combination of immunotherapy with other immune checkpoint inhibitors, chemotherapy, radiotherapy, or targeted molecular therapy showed the most successful results [35]. Ipilimumab alone showed only 2.9 months of progression-free survival (PFS) compared with 6.9 months of nivolumab-only therapy. The combination of ipilimumab and nivolumab showed 11.5 months a of PFS [42].

In 2018, anti-PD-1 antibodies were approved as adjuvant therapy for patients with melanoma stage III A-D and IV (NED) whereas first ICIs have been used in palliative setting since 2011 in Europe [28].

1.1.9.3 Chemotherapy

Chemotherapy is an alternative therapy when first-line therapy (BRAF/MEK-Inhibitors or PD-1-antibodies) has no effect. In lower-income countries, chemotherapy is often the first-line therapy because of the high cost of targeted or immune checkpoint therapy [1]. Monochemotherapy with **dacarbazine** (DTIC) is administered to patients with non-resectable malignant melanoma with metastasis [28].The response rate of DTIC is 10-15% if used as monotherapy [1]. **Temozolomide** has active metabolites similar to DTIC, and shows a similar response rate. The main adverse effects of these drugs are loss of appetite, nausea and vomiting, leukocytopenia, thrombocytopenia, and anemia [28].

In patients with a high tumor burden, polychemotherapy contributes to stabilization of progression. In a systematic review, polychemotherapy showed a better response rate than DTIC alone (18.5% vs. 10.2%, $p=0.09$); however, the survival period was not significantly longer. A commonly used therapy scheme is the CarboTax scheme: Carboplatin AUC6 i.v. and paclitaxel 225 mg/m2 i.v., after five days of dose reduction [24].

1.2 Microbiome

Humans host numerous microorganisms inside the body and skin. Bacteria are the main components of the microbiome, but there are also viruses, fungi, archaea, and protists [43]. Microbial symbionts produce proteins and other metabolic products that interact with the host and influence the immune system, drug metabolism, and other metabolic processes [44]. The interaction between humans and their microbiome may be key to understanding the development of various diseases.

The definition of a core microbiome is complex. The composition of the microbiome differs from person to person and depends on delivery mode, diet, age, geography, medications, and other factors. In infants under three years of age, *Bifidobacterium (Actinobacteria*) is dominant, whereas in adults, *Bacteroidetes* and *Firmicutes* are more prevalent [45]. Gut and vaginal microbiomes are more stable than the oral microbiome [46].

The main bacterial phyla in the human gut are *Firmicutes, Bacteroidetes, Actinobacteria* and *Proteobacteria* [47]. The gut microbiome plays a vital role in the metabolism of food components, synthesis of vitamins, and immune system regulation [6]. Bacteria use carbohydrates and other undigested foods to produce short-chain fatty acids (SCFAs) that can modulate the immune system and inflammatory responses [6, 48]. Alterations in the microbiome are associated with inflammatory bowel disease, obesity, metabolic syndrome, and other diseases (**Figure 8**) [6].

The human skin microbiome depends on the region of its localization. The skin microbiome is also involved in homeostasis. Species that inhabit sebaceous skin include *Cutibacterium*, *Staphylococcus*, and *Malassezia* yeasts. *Cutibacterium* acnes and *Staphylococcus* species are primarily present in dry skin. The moist skin is inhibited by *Corynebacterium*. *S. epidermis* can activate IL-17+ and CD8 T cells and enhance the innate barrier immunity of the skin [6, 49].

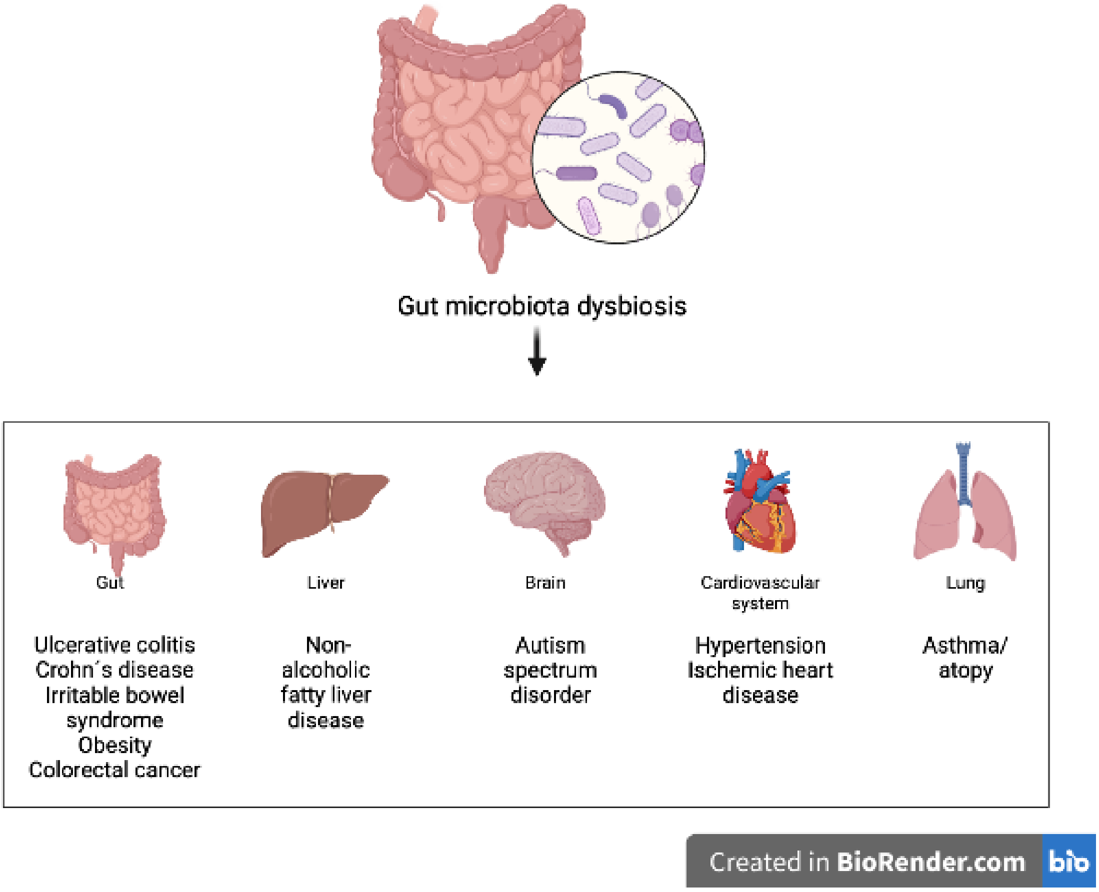


Figure 8 *Diseases influenced by gut microbial metabolism and dysbiosis***.** *Figure created in BioRender.com*

Proteomics (protein analysis) and metabolomics (metabolic analysis) are computational methods used to analyze the functional roles of the microbiome. Metagenomic analysis provides information on the composition of the microbiome [50]. Alterations in the microbiome composition are associated with various diseases and changes in drug tolerance [51].

Understanding the function of microbiota opens new possibilities for its therapeutic use. Prebiotics, probiotics, and synbiotics can modulate the gut microbiome [44]. The use of probiotics such as *lactobacilli* and *bifidobacteria* is well established for the treatment and prevention of antibiotic-associated diarrhea [6, 52]. Non-digestible oligosaccharides are prebiotics that can enhance bacterial growth and or activity [53]. Fecal transplantation showed promising results in patients with *Clostridium difficile*-associated diarrhea [54]. Moreover, 30% of patients with refractory anti-PD-1 immunotherapy responses showed a positive ICIs response after fecal microbiota transplantation and reintroduction of ICIs [55].

1.2.1 Melanoma-related changes in the microbiome

The skin microbiome of pigs is similar to that of humans and consists of the same phyla. Mrazek et al. investigated the differences in the skin and melanoma microbiomes of piglets. Microbiome samples were collected from different skin locations and compared with the melanoma microbiome. The results showed that bacterial diversity significantly differed between the skin and melanoma surfaces. *Fusobacterium* and *Trueperella* genera were more frequent in melanoma samples, suggesting a correlation between melanoma development and skin microbiome alterations [56]. Similar results were obtained in a study examining the association between melanoma growth and microbiome changes in the skin and gut of pigs [57].

1.2.2 Immunotherapy interaction with microbiome

As previously described, anti-CTLA-4 antibodies such as ipilimumab are used to treat advanced melanoma. Previous microbiome studies were conducted in palliative care settings. A study by Vetizou et al. showed that the antitumor effects of CTLA-4 inhibition depend on *Bacteroides* species. Initially, mice treated with antibiotics or germ-free mice did not respond to the CTLA-4 blockade. After the transfer of *B. fragilis*, therapy response increased [58].

Another study by Gopalakrishnan et al. suggested that the response to anti-PD-1-antibodies in humans affected by melanoma may depend on the gut microbiome. Patients with high levels of *Ruminococcaceae* and *Faecalibacterium* in their gut showed a better immune response to PD-1 inhibitors. Non-responders received fecal transplants from responders and showed higher therapy responses [59].

These findings suggest an essential interaction between the immune system and the microbiome. Studying the microbiome of patients affected by melanoma could help predict the probability of success of therapy and even help select a patient-targeted treatment.

2 Material and Methods

2.1 Study design

This prospective cohort study was conducted at the Dermatooncology Unit of the Department of Dermatology and Venereology at Medical University Graz. The ethical committee approved this study in 2017 (29-192 ex 16/17). We recruited two cohorts.

> A) 21 patients with recently diagnosed melanoma (stages III/IV) and planned to receive checkpoint inhibitor immunotherapy (anti-PD-1-mAB) planned to receive adjuvant/palliative ICI therapy
>
> B) 29 patients in complete remission after adjuvant/palliative ICI therapy (stages III/IV) who responded to checkpoint inhibitor immunotherapy

The patients included in the study signed informed consent forms, and the study was conducted in accordance with the principles of the Declaration of Helsinki. The recruitment of patients aged ≥ 18 years was initiated in November 2019 and concluded in December 2020. The patients were followed-up until October 2021. We excluded patients who had received molecularly targeted therapies (including BRAF and MEK inhibitors), antibiotics in the last three months before ICI therapy, patients with intake of probiotics within two weeks before inclusion, and a history of major surgery of the GI tract (five years).

2.2 Source data

Patients recruited at Dermatooncology completed a standard nutritional questionnaire (modified Cambridge food questionnaire) and answered general questions about their comorbidities and family history. We acquired data on clinical markers and melanoma related data from the OpenMEDOCS. De-identified clinical data were saved in an Excel document for further analysis. Therapy response was evaluated radiologically after 12 weeks, and every 12 weeks thereafter for 12 months, until disease progression or discontinuation of treatment and divided into four categories: complete response (CR), partial response (PR), stable disease (SD), and progressive disease (PD).

Adverse events labelled as possibly/probably related to ICI therapy were considered trAEs for this analysis.

2.3 Sample acquisition

Skin, oral and stool samples were collected before and 12 weeks after ICI initiation (cohort A), and at routine follow-up exam (cohort B).

Skin swabs were obtained from the sun unexposed upper inner arm. Oral mucosal swabs were obtained buccal and from the gingiva.

Cotton swabs were placed into cryovials, and all samples were stored at -80°C until further analysis.

2.4 DNA isolation, 16S rRNA library preparation and sequencing

Sequencing was performed at the core facility of Molecular Biology at the Center for Medical Research Graz.

DNA was isolated by mechanical and enzymatic lysis using a Magna Pure DNA Isolation Kit (Roche, Mannheim, Germany). The eluted DNA was stored at -20°C until PCR amplification. For target-specific PCR amplification of hypervariable regions, the primers 27f (AGAGTTTGATCCTGGCTCAG) and 357r (CTGCTGCCTYCCGTA) were used. Amplification was verified using 1% agarose gel, and the pooled PCR products were normalized on a SequalPrep normalization plate (Life Technologies, Vienna, Austria). 15µl of the normalized PCR product were used as the template for indexing PCR in a single reaction to introduce barcode sequences for each sample [60]. After indexing, five µl of each sample was pooled, 50µl of the unpurified library was loaded to a 1% agarose gel (Sigma–Aldrich, St. Louis, USA) and purified using a Qiaquick Gel Extraction Kit (Qiagen, Hilden, Germany). The pool was quantified using PicoGreen dsDNA reagent (Life Technologies, Vienna, Austria) and visualized for size validation using an Agilent 2100 Bioanalyzer (Agilent Technologies, Waldbronn, Germany). The sequencing library pool was diluted and run on a MiSeqII desktop sequencer (Illumina, Eindhoven, Netherlands) using version three 600 cycles of chemistry with 20% PhiX (Illumina, Eindhoven, Netherlands).

2.5 Data analysis

The analysis was performed with the Quantitative Insights Into Microbial Ecology QIIME 2 Version 2019.7 [61] next-generation microbiome bioinformatics platform integrated in an own Galaxy server using Medical University Graz MedBioNode HPC cluster. After the initial quality control of the raw sequence data with FastQC and MultiQC initial data preprocessing was performed with the DADA2 pipeline [62] which included the steps for quality filtering and adapter trimming, denoising and removal of chimeric artefacts. QIIME2 Naive Bayes classifier trained with 16S rRNA SILVA 132 Database [63] was used to provide taxonomic annotation on representative sequences from the Amplicon Sequence Variants ASVs discovered by the DADA2 workflow. Alpha diversity indexes, like richness, Shannon and faith pd, as well as beta diversity distances, weighted UniFrac, unweighted UniFrac, bray-curtis and jaccard were also calculated with QIIME2, whereas all further statistical downstream analysis and plotting was performed in R 4.0.5 program (R Core Team, 2021) for statistical computing and graphics. To detect significantly abundant taxa we used LEfSe (Linear discriminant analysis Effect Size) [60] tool from Huttenhower lab.

2.6 Literature research

Relevant sources were identified using the PubMed and Google Scholar online databases. In addition, the theoretical basics of dermatological textbooks have been supplemented.

3 Results

3.1 Cohort A

We enrolled 21 patients with advanced melanoma (stages III and IV) and collected stool, skin, and oral samples before adjuvant (6/28.6%) and palliative (15/71.4%) ICI therapy initiation (W0) and after 12 weeks (W12) of therapy (**Figure 9**). Before the start of treatment (W0), we collected six stool, six oral, and six skin samples from six patients before receiving adjuvant ICI therapy, and 13 stool, 14 oral, and 14 skin samples from 15 patients before receiving palliative ICI therapy. During the first 12 weeks of ICI treatment, four patients (19%) in the palliative therapy group died. After 12 weeks of treatment (W12), we collected six stool, six oral, and six skin samples from patients who received adjuvant therapy, and nine stool, 11 oral, and 11 skin samples from the remaining 11 patients who received palliative therapy.

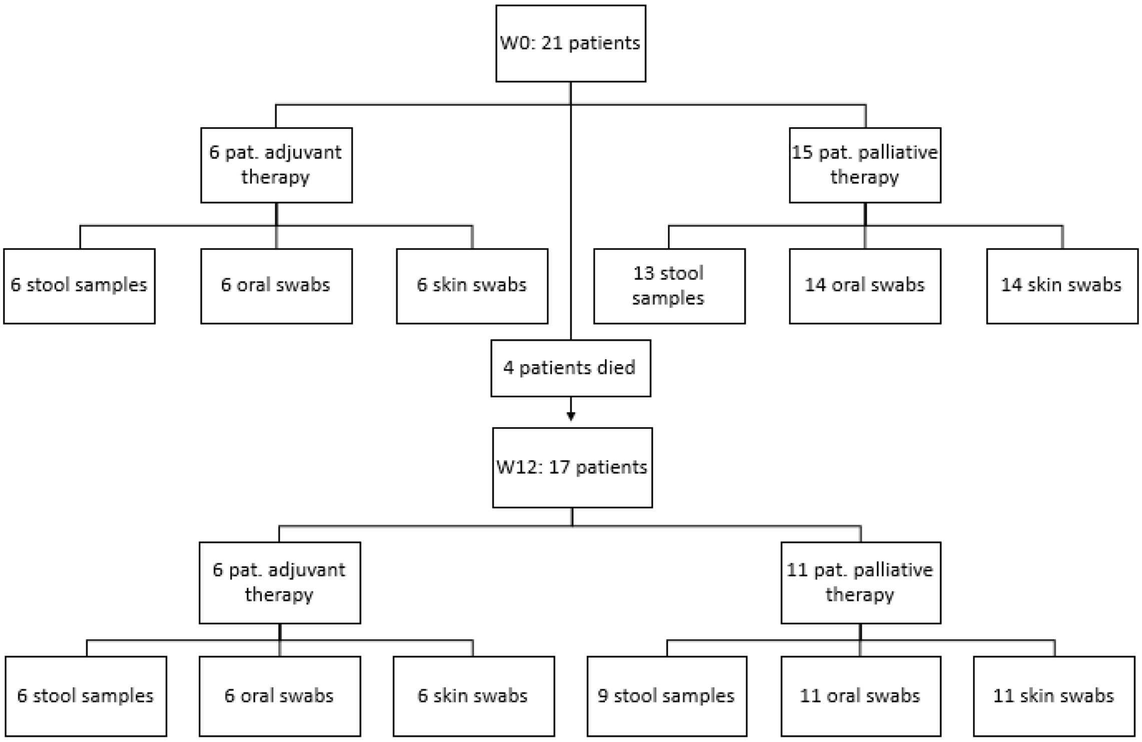


Figure 9 *Cohort A: Adjuvant and palliative patients, number of samples collected at week 0 (W0) and week 12 (W12)*

3.1.1 Cohort A: Adjuvant therapy group

The adjuvant therapy group included six patients (**Table 6**), four (66.7%) females and two (33.3%) males, with a median age of 61 years (range 35-86 years). Two (33.3%) patients had stage IIIC melanoma, and four (66.7%) had stage IV (NED) melanoma. Four (66.7%) patients had BRAF mutations. Three (50.0%) patients received nivolumab, two (33.3%)

received a combination of ipilimumab and nivolumab, and one (16.7%) received pembrolizumab. After 12 weeks of adjuvant therapy, two (33.3%) patients had PD, three (50.0%) patients had RFS, and one (16.7%) patient was in PR.

At the end of our study (February 2021), one patient (16.7 %) had PD, one (16.7%) patient was in PR, four (67.7%) patients were tumor free. All participants were still alive.

According to medical records (until February 2021), four (66.7%) patients experienced trAE of any grade. Three (50.0%) patients had colitis and four (66.7%) patients had skin involvement. Three patients (50.0 %) experienced high-grade trAEs (grade≥ 3).

Table 6 *Cohort A: Adjuvant therapy group*

Characteristics		**Adjuvant (n=6)**
Sex *n, (%)*	Female	4 (66.7)
	Male	2 (33.3)
Age (years), median (range)		61 (35-86)
Stage *n, (%)*	IIIC	2 (33.3)
	IV (NED)	4 (66.7)
BRAF mutation *n, (%)*	Yes	4 (66.7)
Type of ICI *n, (%)*	Ipilimumab/Nivolumab	2 (33.3)
	Nivolumab	3 (50.0)
	Pembrolizumab	1 (16.7)
Response Week 12 *n, (%)*	PD	2 (33.3)
	RFS	3 (50.0)
	PR	1 (16.7)
Response at the end of the study (February 2021) *n, (%)*	PD	1 (16.7)
	PR	1 (16.7)
	Tumor free	4 (66.7)
trAE *n, (%)*	Yes	4 (66.7)
trAE (grade≥ 3) *n, (%)*	Yes	3 (50.0)

3.1.2 Cohort A: Palliative therapy group

We included 15 melanoma patients (stage IV) to receive palliative ICI therapy (**Table 7**). There were nine (60.0%) females and six (40.0%) males, with a median age of 60 years (range 46-86 years). 11 (73.3%) patients had BRAF mutations. Seven (46.7%) patients received an ICI combination therapy (ipilimumab/nivolumab), and eight (53.3%) received an ICI monotherapy (6 nivolumab, 2 pembrolizumab). After 12 weeks of therapy, six (40.0%) patients had PD, five (33.3%) patients PR, and four (26.7%) patients had died. At the end of the study (February 2021), two (13.3%) patients had PD, three (20.0%) PR, two (13.3%) patients were in CR, and eight (53.3%) patients had died. According to medical records, nine (60.0%) patients experienced trAEs of any grade. Four (26.7%) patients had skin involvement, three (20.0%) patients had pneumonitis, one (6.7%) patient had colitis, one (6.7%) patient developed hypothyroidism, and one (6.7%) patient had hepatitis. Three (20.0%) patients experienced severe trAEs (grade$\geq$ 3).

***Table** 7 Cohort A: Palliative therapy group*

Characteristics		**Palliative (n=15)**
Sex *n, (%)*	Female	9 (60.0)
	Male	6 (40.0)
Age (years), median (range)		60 (46-86)
BRAF mutation *n, (%)*	Yes	11 (73.3)
Type of ICI *n, (%)*	Ipilimumab/Nivolumab	7 (46.7)
	Nivolumab	6 (40.0)
	Pembrolizumab	2 (13.3)
Response Week 12 *n, (%)*	PD	6 (40.0)
	PR	5 (33.3)
	Death	4 (26.7)
Response at the end of the study (February 2021) *n, (%)*	PD	2 (13.3)
	PR	3 (20.0)
	CR	2 (13.3)
	Death	8 (53.3)
trAE *n, (%)*	Yes	9 (60.0)
Type of adverse events *n, (%)*	Skin	4 (26.7)
	Pneumonitis	3 (20.0)
	Colitis	1 (6.7)
	Hypothyroidism	1 (6.7)
	Hepatitis	1 (6.7)
trAE (grade≥ 3) *n, (%)*	Yes	3 (20.0)

3.2 Cohort B

We recruited 29 patients with advanced melanoma (stages III/IV) in long-term remission after completion of ICI therapy (adjuvant/palliative). One palliative patient was finally excluded from the analysis due to disease progression (after three years in complete remission). Of the remaining 28 patients, 14 patients (50%) received adjuvant and 14 patients (50%) received palliative ICI therapy. In total, 27 stool, 27 oral, and 27 skin samples could be collected (**Figure 10**).

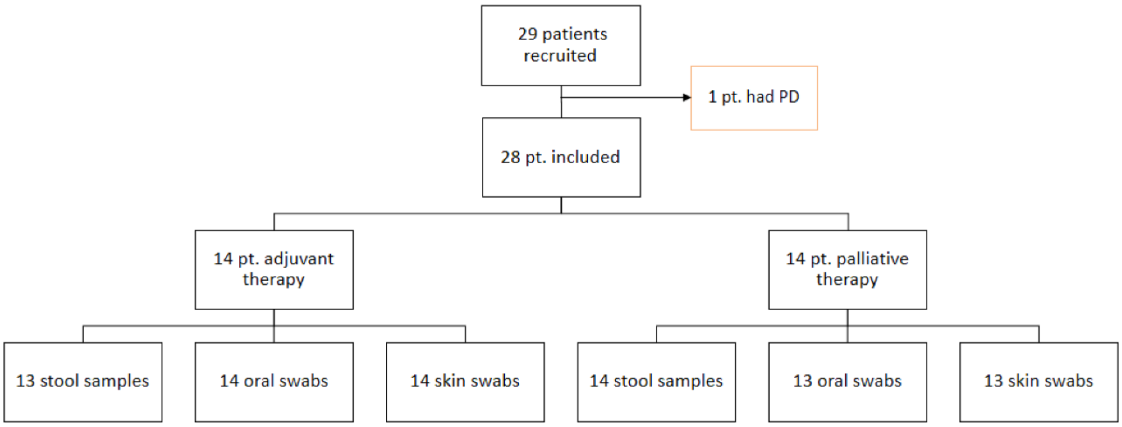


Figure 10 *Cohort B: samples collected, PD-progressive disease*

3.2.1 Cohort B: Adjuvant therapy group

Of 14 tumor-free patients after completion of adjuvant ICI therapy (**Table 8**), seven were males (50.0%) and seven were females (50.0%), with a median age of 63 years (range 21-85). Four (28.6%) patients had stage IIIB, nine (64.3%) patients had stage IIIC, and one (7.1%) patient had stage IIID melanoma. Three (21.4%) patients received pembrolizumab and 11 (78.6%) patients received nivolumab monotherapy. Adjuvant patients received a median of 15 cycles (range 4-43) of ICI, and the median duration of therapy was eight months (range 2-33). After the end of ICI therapy, the median follow-up duration was 30 months (range 2-33). Four (28.6%) patients had severe trAEs (grade ≥3), and in six (42.9%) patients therapy had to be discontinued due to trAEs.

Table 8 *Cohort B: Adjuvant therapy group*

Characteristics		**Adjuvant (n=14)**
Sex n, (%)	Male	7 (50.0)
	Female	7 (50.0)
Median Age in years (range)		63 (21-85)
Stage n, (%)	IIIB	4 (28.6)
	IIIC	9 (64.3)
	IIID	1 (7.1)
Type of ICI n, (%)	Pembrolizumab	3 (21.4)
	Nivolumab	11 (78.6)
Median ICI Cycles (range)		15 (4-43)
Median ICI Cycles in months (range)		8 (2-33)
Median Follow up in months		30 (9-54)
trAE (grade≥ 3) *n, (%)*	Yes	4 (28.6)
End of therapy because of trAE n, (%)	Yes	6 (42.9)

3.2.2 Cohort B: Palliative therapy group

Of 14 patients in long-term remission after completion of palliative ICI therapy (**Table 9**), eight (57.1%) were males and six (42.9%) were females, with a median age of 65 years (range 43-81 years). All patients had stage IV melanoma. Four (28.6%) patients received ipilimumab monotherapy, two (14.3%) patients received nivolumab monotherapy, four (28.6%) patients received pembrolizumab monotherapy, and four (28.6%) patients received the ICI combination therapy (ipilimumab/nivolumab). Palliative patients received a median of 25 cycles (range 2-52) of ICI therapy, and the median duration was 13 months (range 1-24 months). After the end of ICI therapy, the median follow-up duration was 39 months (range 14-110). According to medical records, one (7.1%) patient had a severe trAEs (grade ≥3), and in two (14.2%) patients therapy had to be discontinued due to trAEs.

Table 9 Cohort B: *Palliative therapy group*

Characteristics		**Palliative (n=14)**
Median Age in years (range)		65 (43-81)
Sex n, (%)	Male	8 (57.1)
	Female	6 (42.9)
Type of ICI n, (%)	Ipilimumab	4 (28.6)
	Nivolumab	2 (14.3)
	Pembrolizumab	4 (28.6)
	Ipilimumab/Nivolumab	4 (28.6)
Median ICI Cycles (range)		25 (2-52)
Median ICI Cycles in months (range)		13 (1-24)
Median Follow up in months		39 (14-110)
trAE (grade≥ 3) n, (%)	Yes	1 (7.1)
End of therapy because of trAE	Yes	2 (14.3)

3.3 Microbiome analysis

3.3.1 Cohort A

Cohort A included patients with recently diagnosed melanoma (stages III/IV) receiving adjuvant/palliative ICI therapy. We wanted to compare oral, skin, and gut microbiome diversity before (W0) and after 12 weeks (W12) of ICI therapy using Faith's phylogenetic diversity (PD), Richness (observed operational taxonomic units, *OTUs*) and Shannon's diversity indices. In summary, there was no statistically significant difference in oral and gut microbiome diversity between W0 and W12 samples.

Faith PD p-values were calculated using the Wilcoxon signed-rank and t-test. We compared the samples obtained before start of ICI therapy (W0) and after 12 weeks of therapy (W12). Skin samples showed differences between weeks zero and 12, although the results were not statistically significant. The stool and oral swab samples showed no significant changes after 12 weeks of ICI therapy (**Figure 11**, **Table 10**).

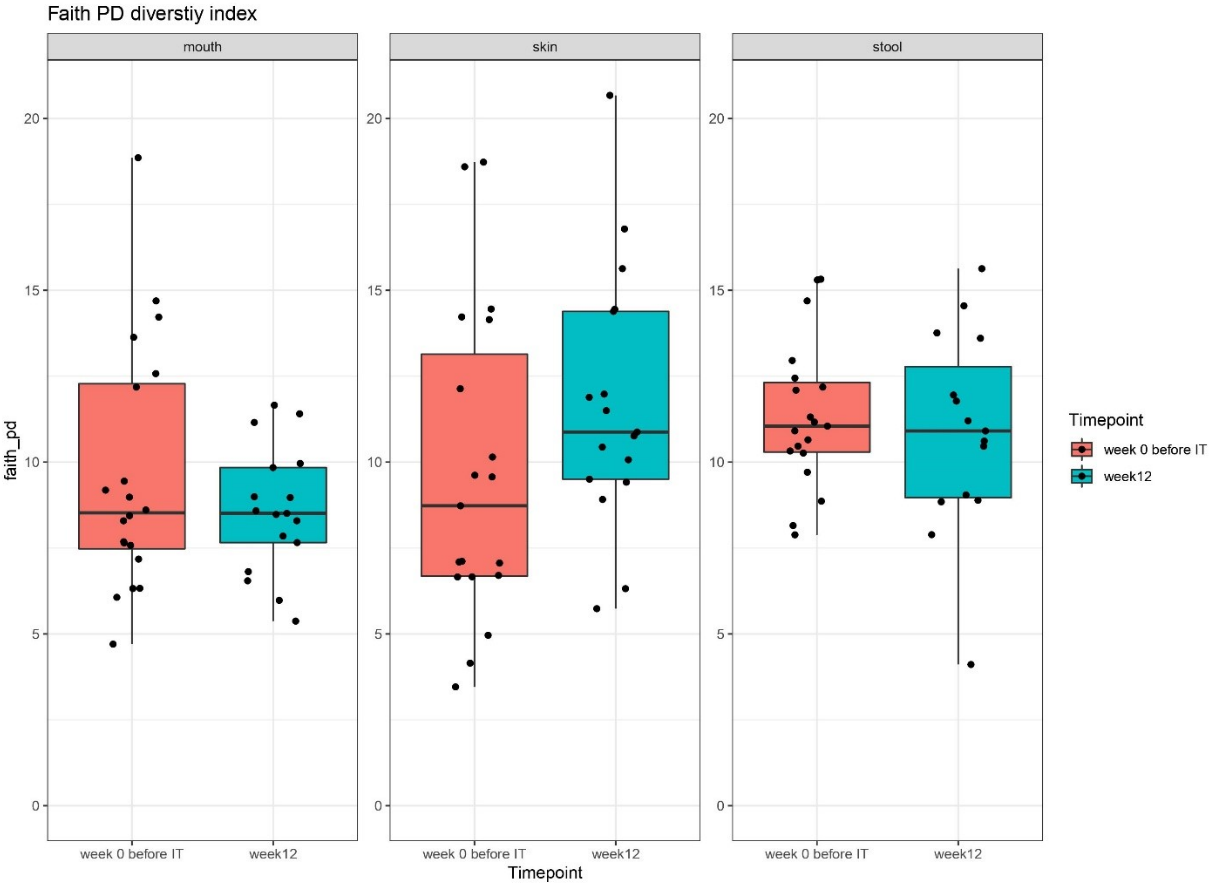


Figure 11 *Cohort A: Faith´s diversity of stool, oral and skin samples between week 0 and 12.*

Table 10 *Cohort A: comparison between week 0 and week 12 of oral, skin and stool samples. Faith Phylogenetic Diversity index was evaluated with Wilcoxon signed-rank test and t-test.*

p Values	oral	skin	stool
faith_pd.wilcox	0,597	0,065	0,626
faith_pd.ttest	0,389	0,089	0,656

Richness analysis was used to determine the total number of observed species in oral, skin, and stool samples. We compared the W0 samples to the samples from week 12 after initiation of ICI therapy (**Figure 12**). The skin samples showed a greater change in richness, but the results were not statistically significant (**Table 11**). Oral and stool samples did not change significantly after the introduction of the therapy.

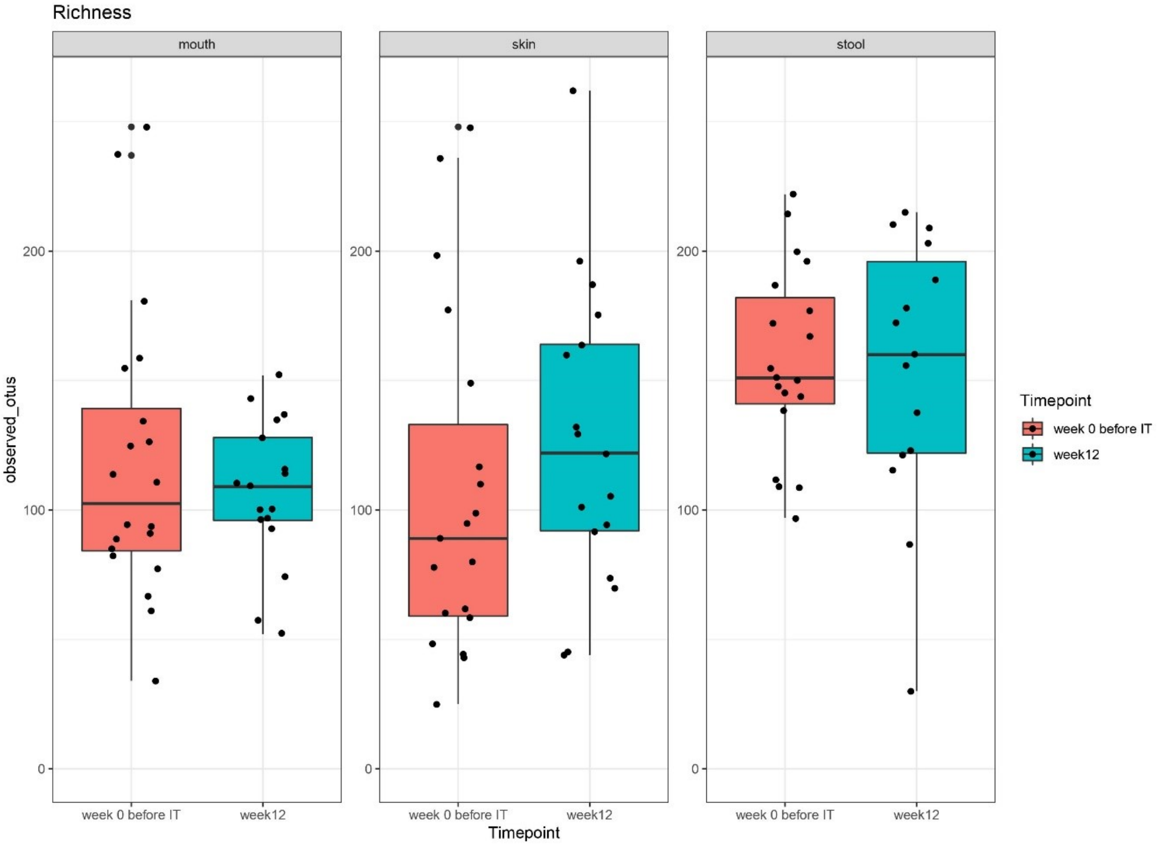


Figure 12 *Cohort A: Richness comparison of stool, oral and skin samples between week 0 and 12. Skin samples showed more richness in the samples taken after 12 weeks of the immune therapy.*

Table 11 *Cohort A: Richness of the oral, skin and stool samples evaluated with the Wilcoxon signed-rank test and t-test.*

p Values	oral	skin	stool
richness.wilcox	0,632	0,375	1
richness.ttest	0,506	0,298	0,799

Shannon's diversity analysis showed similar results to those of the other two analyses. Shannon index indicates the number of different bacteria present in a sample. The higher the Shannon diversity value, the lower the diversity. Oral and stool samples showed similar numbers of bacteria before and after the start of immunotherapy (**Figure 13**). After 12 weeks of therapy, the skin samples had fewer bacteria than at week zero. None of the groups showed significant changes; however, a trend was observed for the skin samples (**Table 12**).

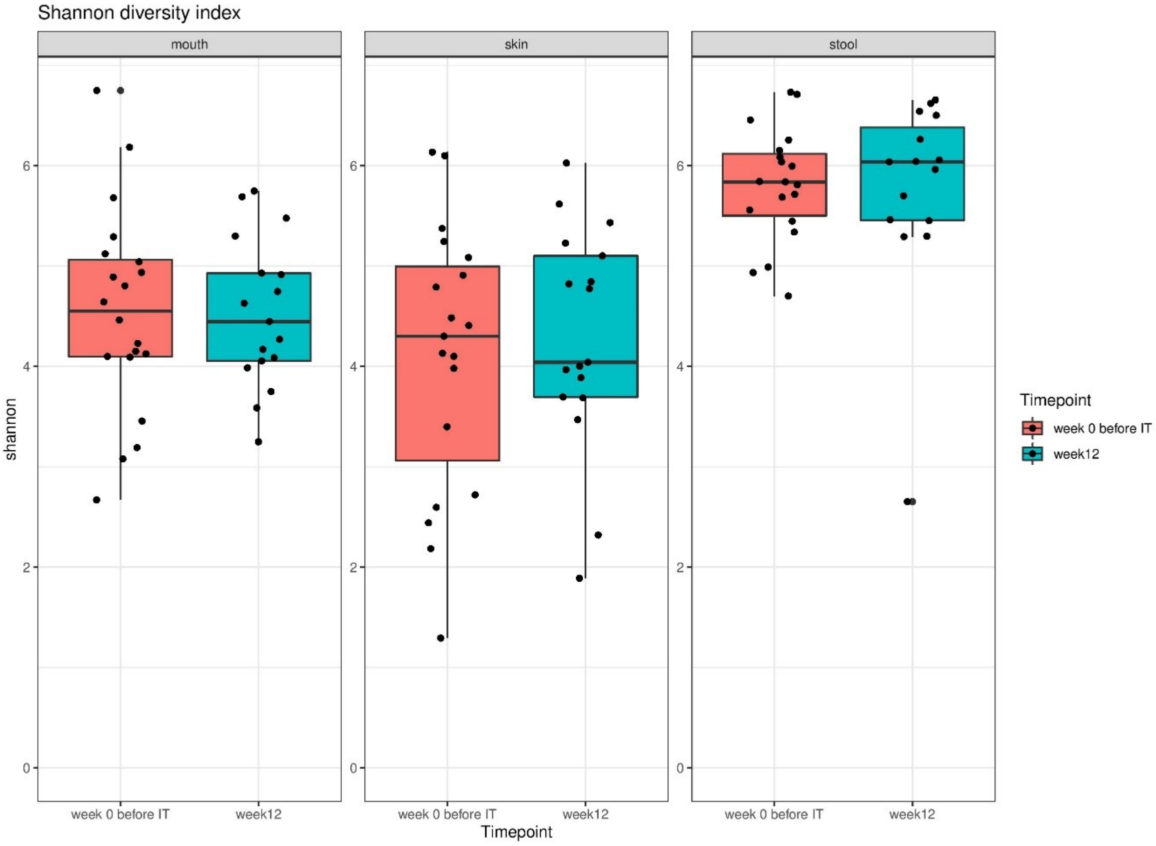


Figure 13 *Cohort A: Shannon diversity index of stool, oral and skin samples compared between week 0 and 12. Skin samples after 12 weeks showed lower Shannon index compared to week 0.*

Table 12 *Cohort A: Shannon diversity of the oral, skin and stool samples evaluated with the Wilcoxon signed-rank test and t-test.*

p Values	oral	skin	stool
shannon.wilcox	0,528	0,782	0,326
shannon.ttest	0,425	0,708	0,433

3.3.1.1 Cohort A: Linear discriminative analysis (LDA) effect size results of the skin samples

We analyzed changes in bacterial composition after the introduction of therapy. Analysis of the samples revealed evident changes in the microbiome of the skin samples after 12 weeks of immunotherapy. *Campylobacter* and *Faecalibacterium* were significantly more abundant after 12 weeks of therapy in skin microbiome samples (**Figure 14**, **Figure 15**).

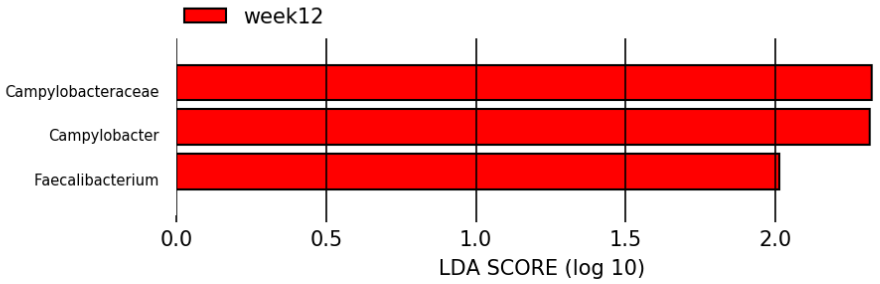


Figure 14 *Cohort A skin sample analysis: comparison of phyla composition between week 0 and 12.*

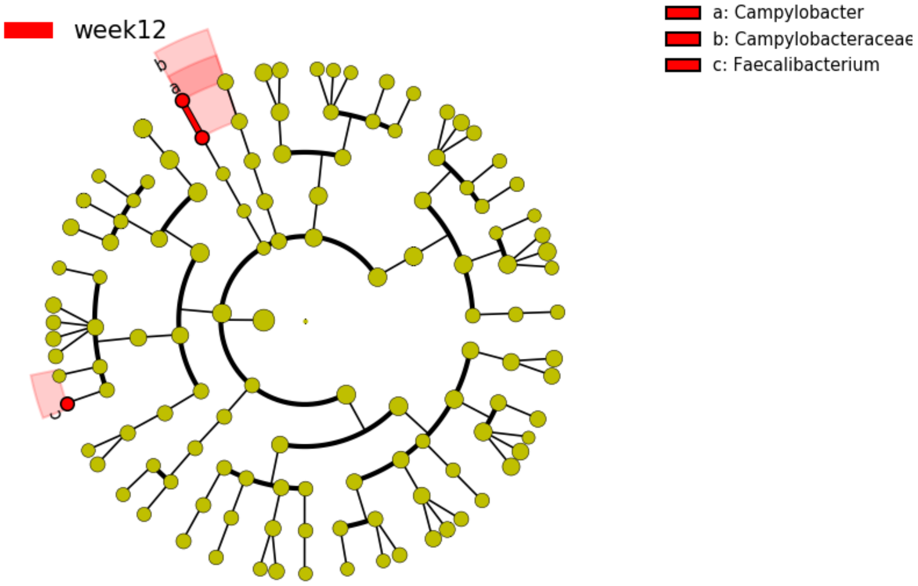


Figure 15 *Cohort A: Bacterial species more frequently found in skin samples at week 12 represented in a cladogram.*

3.3.2 Cohort B

Cohort B included patients with advanced melanoma (stages III/IV) in complete remission after completion of ICI therapy (adjuvant/palliative). Here, we wanted to compare oral, skin, and gut microbiome diversity of adjuvant and palliative patients using Faith's phylogenetic diversity (PD), Richness (observed operational taxonomic units, *OTUs*) and Shannon's diversity indices. Overall, there was no statistically significant difference in oral, skin and gut microbiome diversity between adjuvant and palliative patient samples.

Using Faith's PD, there was however a trend of higher phylogenetic diversity in the skin microbiome of palliative patients (**Figure 16**).

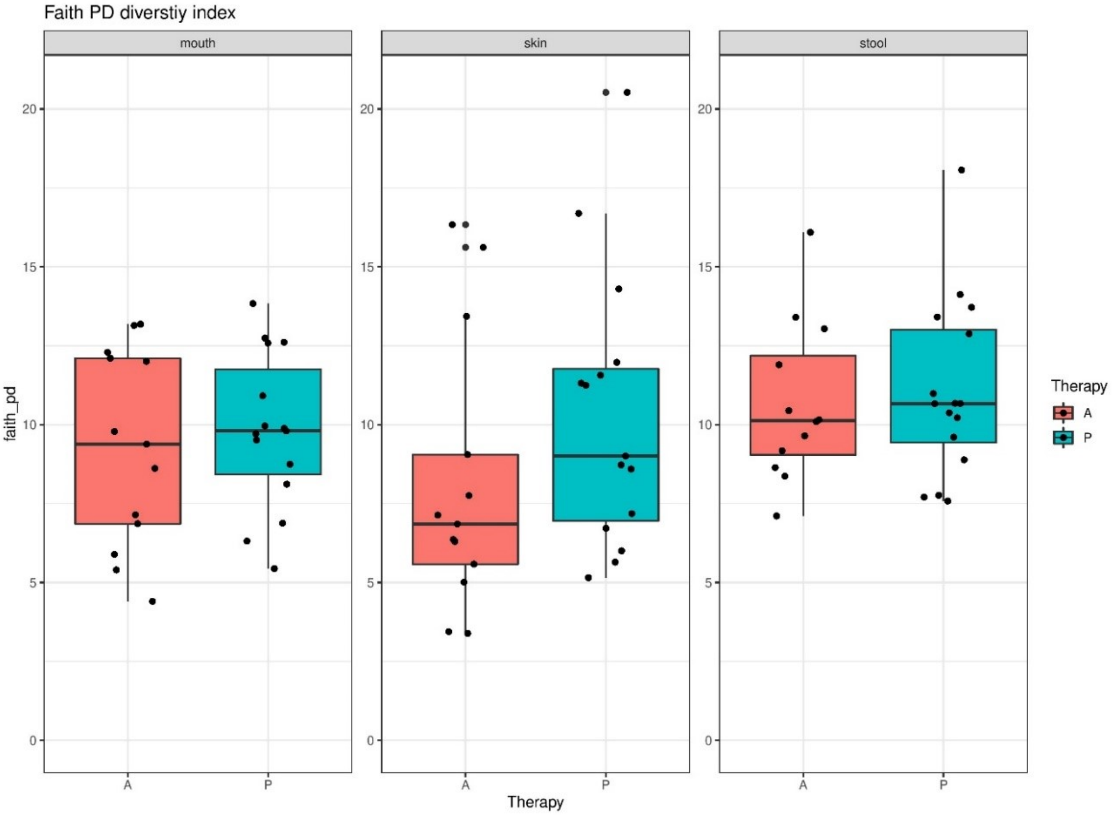


Figure 16 *Cohort B: Phylogenetic diversity of oral, skin and stool samples of adjuvant (A) and palliative (P) patients using Faith's phylogenetic diversity (PD) index*

Table 13 *Cohort B: Faith's phylogenetic diversity of oral, skin and stool samples, comparing adjuvant and palliative patients using Wilcoxon signed-rank test and t-test.*

p Values	oral	skin	stool
faith_pd.wilcox	0,525	0,156	0,537
faith_pd.ttest	0,609	0,204	0,687

Richness index was used to investigate the total number of bacteria between adjuvant and palliative patients. There was a trend of higher microbiome richness (oral, skin and gut) in palliative patients (**Figure 17**), however, the p-values of Wilcoxon signed-rank test and T-test were not statistically significant (**Table 14**).

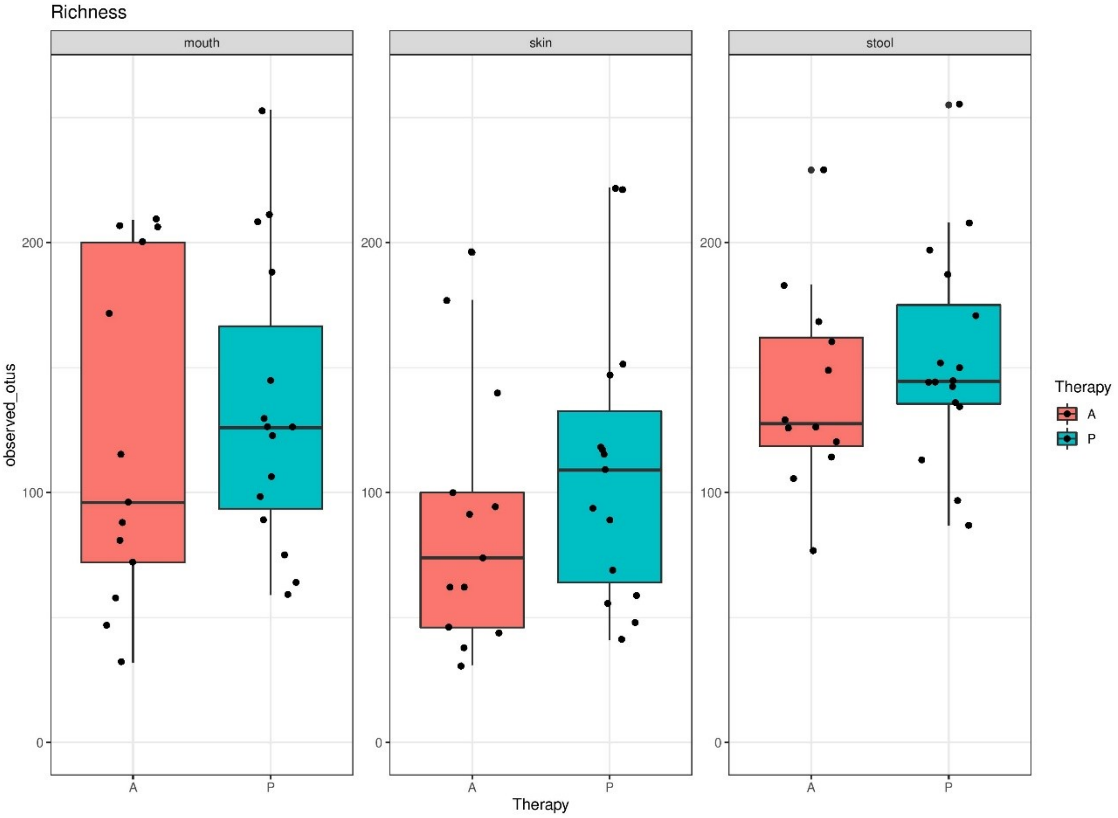


Figure 17 *Cohort B: Richness of stool, oral and skin samples compared between palliative and adjuvant therapy groups.*

Table 14 *Cohort B: Richness of the stool, skin and oral samples evaluated with the Wilcoxon signed-rank test and t-test. Palliative and adjuvant therapy groups were compared.*

p Values	oral	skin	stool
richness.wilcox	0,381	0,240	0,341
richness.ttest	0,631	0,306	0,406

Shannon´s diversity analysis showed that the number of bacteria in the samples of adjuvant and palliative patients was similar (**Figure 18**). There was no statistically significant difference in oral, skin and gut microbiome diversity between adjuvant and palliative patient samples (**Table 15**).

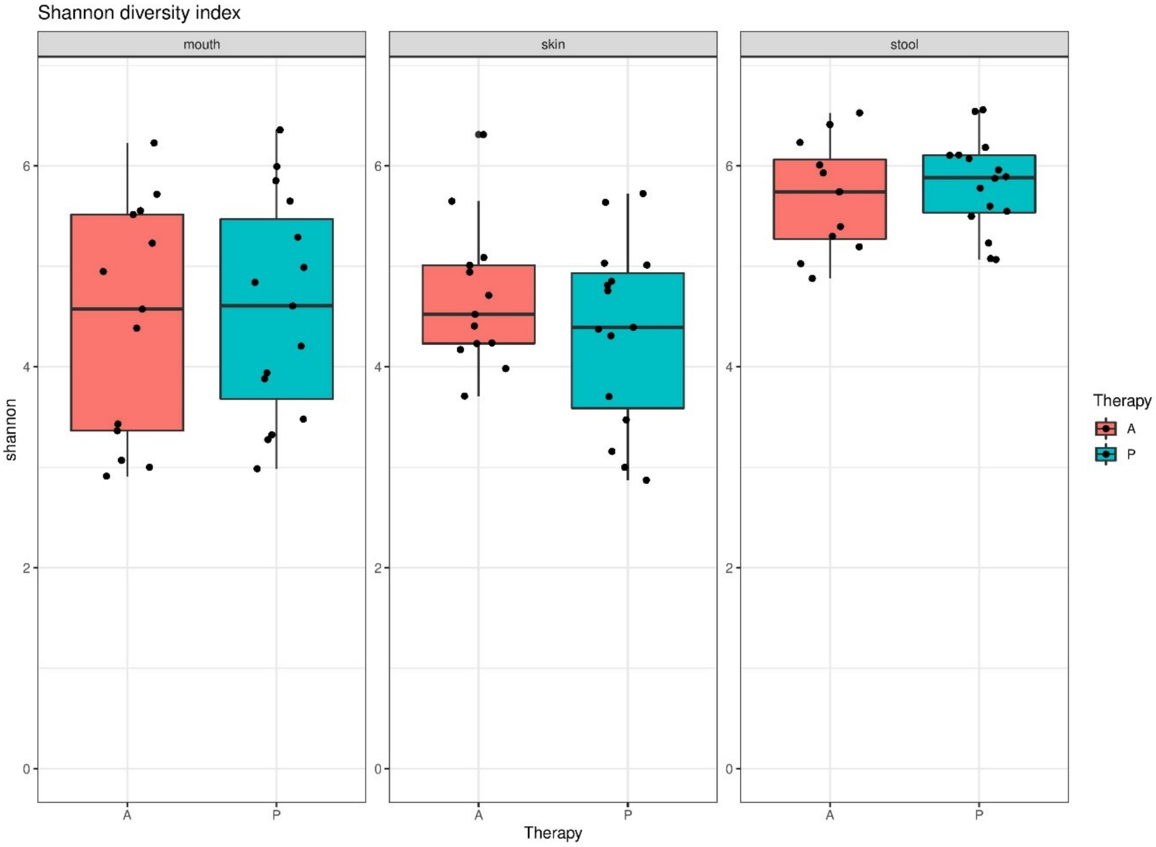


***Figure 18** Cohort B: Shannon's diversity of oral, skin and stool samples of adjuvant (A) and palliative (P) patients.*

***Table 15** Cohort B: Shannon's diversity of oral, skin and stool samples, comparing adjuvant and palliative patients using Wilcoxon signed-rank test and t-test..*

p Values	oral	skin	stool
shannon.wilcox	0,717	0,496	0,507
shannon.ttest	0,780	0,267	0,545

3.3.3 Linear discriminant analysis (LDA) effect size

Next, we wanted to describe differences in gut microbiome composition of responders and non-responders after 12 weeks of palliative ICI therapy. *Parabacteroides* (*Tannerellaceae*), *Odoribacter* (*Marinifilaceae*) and *Alphaproteobacteria* were significantly more abundant in responders compared to non-responders. Whereas, *Erysipelotrichaceae*, *Dorea*, and *Blautia* were significantly more abundant in non-responders compared to responders (**Figure 19**, **Figure 20**).

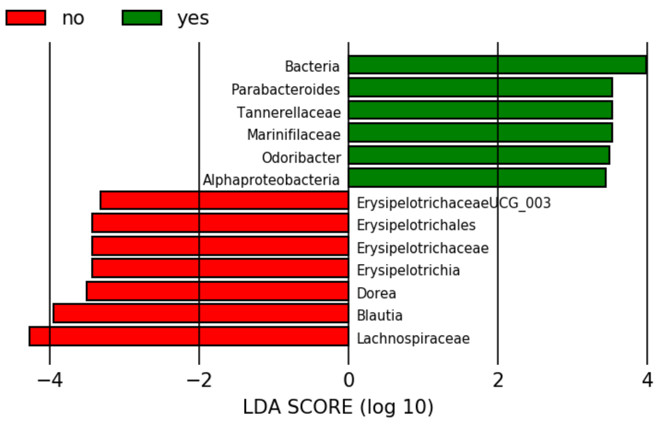


Figure 19 *Cohort A, palliative ICI therapy, stool analysis: Differences in microbiome composition between responders (yes) and non-responders (no).*

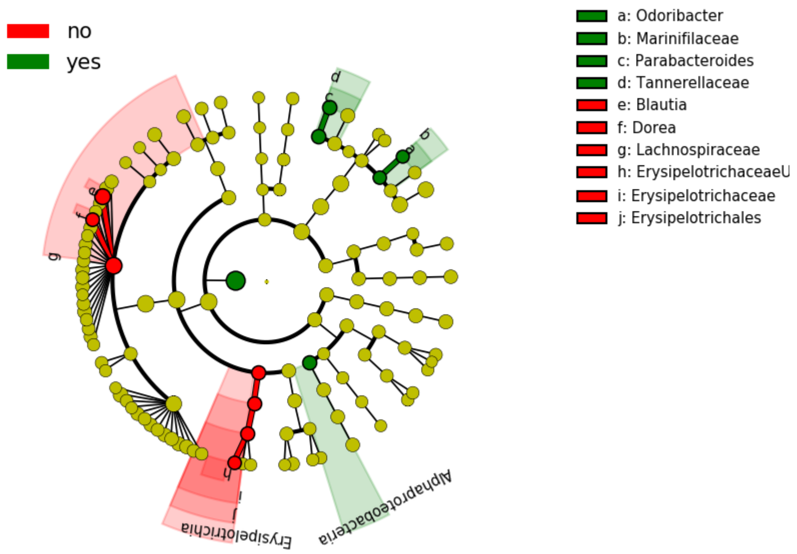


Figure 20 *Cohort A cladogram, palliative ICI therapy, stool analysis: Differences in microbiome composition between responders (yes) and non-responders (no).*

Next, we compared the gut microbiome of non-responders after 12 weeks of ICI therapy with the gut microbiome of patients in long-term remission (cohort B). Patients in long-term remission (cohort B) had more *Dorea* in their gut microbiome. Whereas, non-responders from cohort A had more *Faecalitalea* and *Enterococcus* in their gut microbiome (**Figure 21**, **Figure 22**).

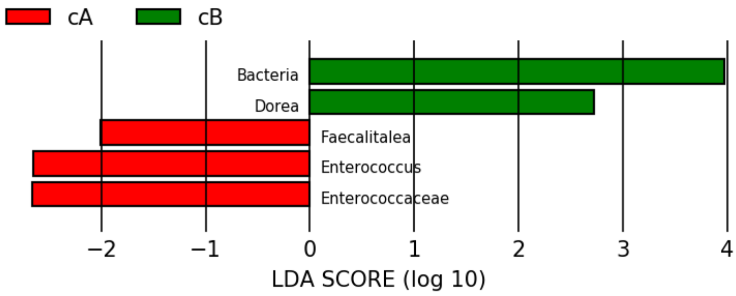


Figure 21 *Gut microbiome from cohort A (cA) and B (cB). Microbiome of non-responders from cA compared to responders from cB.*

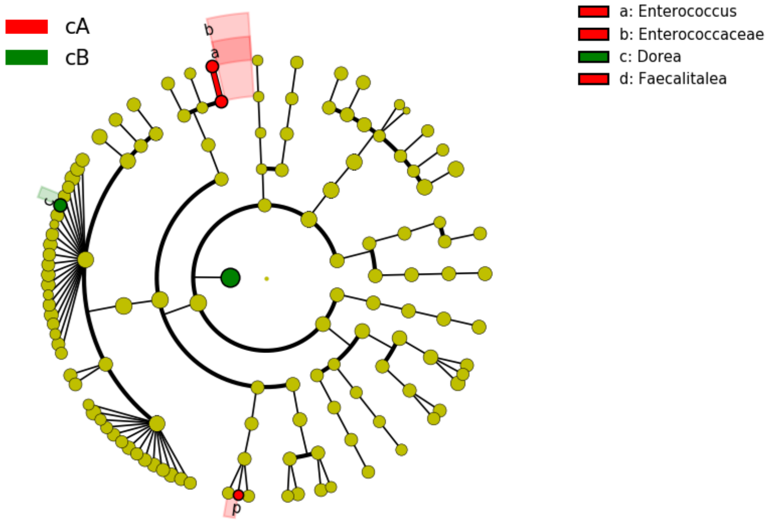


Figure 22 *Cladogram gut microbiome from cohorts A (cA) and B (cB). Gut microbiome of non-responders from cA compared to responders from cB.*

Furthermore, we wanted to investigate the differences of the gut microbiome in patients with and without brain metastases. Six patients (40%) from cohort A had brain metastases before palliative ICI therapy start. Patients with brain metastasis had significantly more *Actinobacteria* and *Subdoligranulum* in their gut microbiome compared with patients without brain metastases (**Figure 23**).

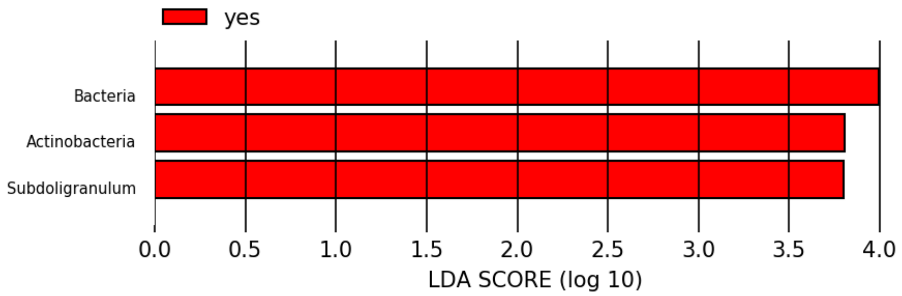


Figure 23 *Cohort A: Stool samples from the palliative ICI therapy group at week 0. Patients with brain metastases had significantly more Actinobacteria and Subdoligranulum in their gut microbiome than patients without brain metastases.*

Finally, we wanted to determine whether it was possible to predict the onset of high-grade (≥ grade 3) treatment-related adverse events analyzing the composition of the gut microbiome. Both, adjuvant and palliative patients of cohort A were included in the analysis.

Patients with high-grade trAEs had significantly more *Barnesiella (Barnesiellaceae), Rikenellaceae, Parasutterella*, and *Ruminococcaceae NK4A214* group compared to patients without severe trAEs. By contrast, patients without high-grade trAEs had significantly more *Lachnospiraceae* in their stool samples (**Figure 24**, **Figure 25**).

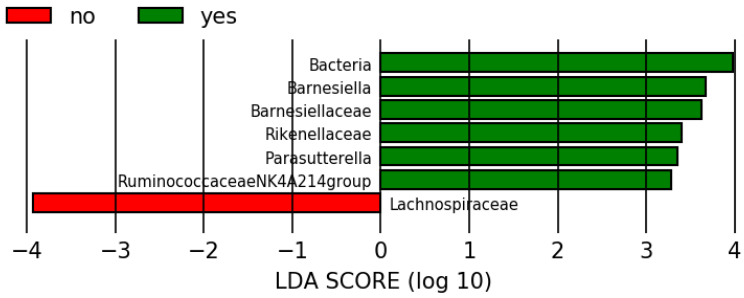


Figure 24 *Cohort A stool samples at week 0: Microbiome of patients with adjuvant and palliative ICI therapy with at least grade 3 trAEs (yes) compared to patients without grade 3 trAEs (no).*

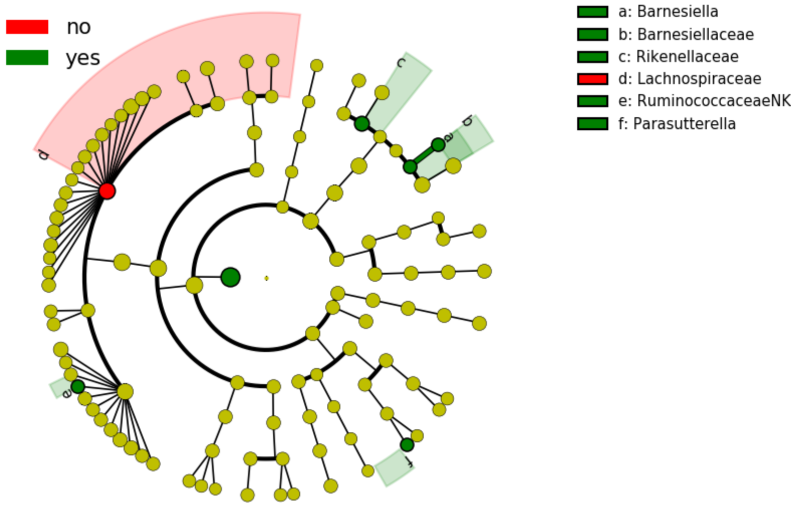


Figure 25 *Cladogram Cohort A stool samples: Microbiome of patients with adjuvant and palliative ICI therapy with at least grade 3 trAEs (yes) compared to patients without grade 3 trAEs (no).*

4 Discussion

Here, we investigated changes in the diversity of the gut, oral, and skin microbiota in relation to ICI therapy in patients with advanced melanoma, and aimed to identify differences in the relative abundance of specific bacterial taxa between treatment responders and non-responders. Additionally, we compared the overall structural changes in the gut, oral, and skin microbiota of melanoma patients at risk for treatment-related adverse events.

In cohort A, we observed notable differences in the relative abundance of specific bacterial taxa in skin samples for the first time. Specifically, we found an increase in *Campylobacter* and *Faecalibacterium* in skin samples after 12 weeks of ICI treatment. Both bacteria are usually known as members of the intestinal microbiota. To date, no prior study has investigated changes in the skin microbiota composition of patients with melanoma after the introduction of ICIs. However, numerous trAEs involve the skin, for example, itching or dry skin. The potential of skin microbiota as biomarker has been explored in atopic dermatitis (AD), acne vulgaris (AV), psoriasis vulgaris (PV), hidradentis suppurativa (HS), seborrheic dermatitis/pityriasis capitis (SD/PC), and ulcus cruris (UC). A systematic review by Niemeyer-van der Kolk et al. suggested that skin microbiota may be a suitable biomarker for clinical trials of AD. In fact, *Staphylococcus* abundance was correlated with disease severity. *Propionibacterium* may also be used as a biomarker for AV because of its correlation with acne severity grade [64]. In our samples, *Campylobacter* and *Faecalibacterium* were significantly more abundant after the introduction of ICIs. A case study by Roberts et al. suggested that immunocompromised patients had a higher susceptibility to *C. jejuni*, and some had *Campylobacter*-induced cellulitis. The pathogenesis remains unclear, but a lack of secretory IgA in the intestinal mucosa and serum IgM may play a role. Reduced humoral immunity may allow *Campylobacter* to enter the bloodstream [65]. Moreover, HIV-positive patients with *Campylobacter jejuni* had more severe bacteremia than non-infected individuals, including cellulitis cases [66]. *Faecalibacterium* also plays a crucial role in immune checkpoint-related colitis and can produce short-chain fatty acids. Furthermore, *Faecalibacterium prausnitzii* is associated with dermatological diseases such as psoriasis and atopic dermatitis [67, 68]. It has been shown that *F. prausnitzii* was more abundant in the stool of patients with malignant melanoma who responded to the ICIs [59, 69]. To date, the role of *Campylobacter* and *Faecalibacterium* in skin homeostasis remains unclear. They may contribute to skin barrier dysfunction, development of itch etc., and further research is needed to investigate their role in cutaneous treatment-related adverse events.

In addition to changes of the skin microbiota, we observed changes in the gut microbiota in response to ICI treatment. *Parabacteroides (Tannerellaceae), Odoribacteria (Marinifilaceae)*, and *Alphaproteobacteria* were significantly increased in the stool of responders compared to non-responders. In contrast, *Erysipelotrichaceae, Dorea,* and *Blautia* were more abundant in non-responders than in the responders at week 12. *Erysipelotrichaceae* belong to the phylum Firmicutes. Recent studies have suggested that *Erysipelotrichaceae* correlates with colorectal cancer and is significantly higher in the tumor group than in the control group. Moreover, specific taxa of *Erysipelotrichaceae* may be correlated with inflammation [70].

Dorea and *Blautia* belong to the *Lachnospiraceae* family. These genera are associated with diseases, such as metabolic disease, multiple sclerosis syndrome, major depressive disorder, and Crohn's disease. Moreover, the growth of *Blautia* may be enhanced by the use of gases produced by *Dorea* [71].

Furthermore, we discovered some interesting differences in the gut microbiome composition of patients in long-term remission (cohort B) compared to non-responders from cohort A. Patients in long-term remission showed an increase in *Dorea* compared to non-responders. Non-responders showed an increase in *Faecalitalea (Erysipelotrichaceae)* and *Enteroccaceae* compared to patients in long-term remission. In contrast to our results, in Matson´s study the microbiome of responders to ICIs showed more *Enterococcus faecium*, *Bifidobacterium longum*, and *Collinsella aerofaciens* [72]. In a study by Frankel et al., pembrolizumab (anti-PD-1) was administered to patients. Similar to our findings, the intestinal flora of responders was rich in *Dorea formicignerans* [73]. Gopalakrishnan et al. examined gut microbiome of melanoma patients receiving anti-PD-1 immunotherapy (n=112) and found that *Ruminococcaceae* and *Faecalibacterium* were significantly more abundant in responders to ICIs, whereas non-responders were more enriched with *Bacteroidales* [59]. In another study, oral supplementation with *Akkermansia muciniphila* restored the efficacy of anti-PD-1 immunotherapy in germ-free mice that received fecal microbiota transplantation (FMT) from non-responders to ICIs [74].

In a subgroup analysis of cohort A, we wanted to investigate for the first time the differences of the gut microbiome in patients with and without brain metastases. The results showed a higher prevalence of *Subdoligranulum* and *Actinobacteria* in samples from patients with brain metastases. Recent studies have suggested that *Subdoligranulum* can produce butyrate as a health promoter [73]. Moreover, immunocompromised patients with HIV have a significantly lower abundance of *Subdoligranulum* [75]. McCulloch et al. reported that *Actinobacteria* and *Lachnospiraceae* in gut microbiome were associated with partial response or stable disease in 94 ICI-treated melanoma patients [76]. Further research with more participants is needed to investigate the importance of a gut-brain interaction in melanoma patients with brain metastases.

Finally, we investigated the intestinal microbiome of patients with severe trAEs (≥grade 3) compared to patients without severe trAEs. Patients with severe trAEs had more *Barnesiellaceae, Rikenellaceae, Parasutterella,* and *Ruminococcaceae NK4A214*. Whereas patients without severe trAEs had more *Lachnospiraceae*. Chaput et al. analyzed the gut microbiome of 26 patients with metastatic melanoma before and after four cycles of ipilimumab. The results showed that patients with a higher presence of *Lachnospiraceae* and *Ruminococcus* had longer overall survival. Fecal samples enriched with *Firmicutes* phylum (*Lachnospiraceae, Ruminococcus, Blautia, Clostridium IV*) were more likely to develop colitis [77].

In summary, our findings might be useful for identifying new biomarkers to predict or enhance ICI response and predict or prevent severe trAEs.

Our study was limited by the small sample size, especially in subgroup analyses including melanoma patients with brain metastases. Additional studies examining the microbial communities in these subgroups would shed further light on this important area. Because our study focused only on the bacterial microbiota, additional studies examining viruses, fungi, and archaea are needed to fully characterize the microbiome of melanoma patients receiving ICI treatment. Finally, the pathogenicity of different strains of the same bacterial species may vary. Thus, different sequencing techniques are needed in future studies to confirm our findings and analyze the relative abundance distribution at the strain level.

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