Thieme

The Microbiome – The Unscheduled Parameter for Future Therapies

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ABSTRACT

The microbiome is defined as the total of cellular microorganisms of bacterial, viral, or e. g. parasite origin living on the surface of a body. Within the anatomical areas of otorhinolaryngology, a significant diversity and variance can be demonstrated. For ear, nose, throat, larynx, and cutis different interactions of microbiome and common factors like age, diet, and lifestyle factors (e. g. smoking) have been identified in recent years. Besides, new insights indicate a possible pathognomonic role of the microbiome towards diseases in the ENT area. This review article summarizes the present findings of this rapidly developing scientific area.

Contents

ABBREVATIONS

CRS	chronic rhinosinusitis
OUT	operational taxonomic unit
MALDI-TOF	Matrix assisted laser ionization mass spectrometry – time of flight
Teff	effector T cells
Treg	regulatory T cells

1. Introduction and Definition

The microbiome is defined as the total of all microorganisms living on humans or other creatures (e. g. earthworms, reptiles, cows). The composition of the microorganisms is locally very different. It includes bacteria (planktonic and as biofilm), viruses, fungi, and all other types of microorganisms (archaea, amoebas, flagellates, bacteriophages etc.). Organisms where the interaction with the microbiome is inhibited artificially, remain physiologically immature regarding important regulatory mechanisms such as immune defense and are very prone to pathogens [1]. Beside systematic effects of this kind, the microbiome influences the epithelial function of the organism on all body surfaces, also in the field of otorhinolaryngology.

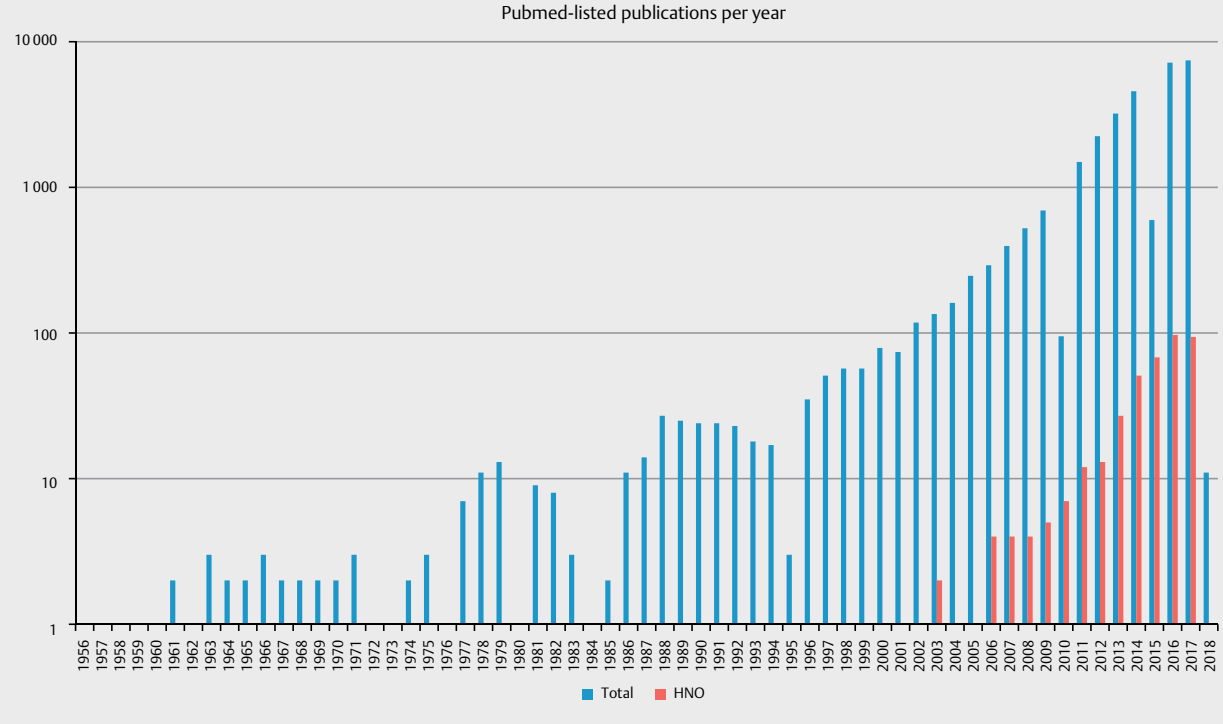
Because of different local environments, the microbiome varies on skin and mucosa but also in different areas of the head and neck. Furthermore, the composition of the microorganisms modifies reactively due to aging process, diseases, and also depending on therapies. It may cause several diseases or favor their development. Those diseases may also include malignant diseases.

The human microbiome is identified by advanced sequencing of the DNA and includes pathogen as well as commensal microbes. Individual differences are considered as being responsible for the susceptibility of patients and their risk to develop diseases. The susceptibility is influenced by various factors such as nutrition, metabolism, detoxification, hormone status, immune tolerance, and in particular inflammation processes [2–5].

The human microbiome is in the focus of intensive research and it is still not fully understood. Since December 2007, the US American Human Microbiome Project (https://hmpdacc.org/), initiated by the National Institute of Health, investigates the sequencing of all genomes of microorganisms living on humans. The investigation is based on specimens from mouth, pharynx and nose, skin, gastrointestinal tract, and female urogenital tract. A specific registry was established to facilitate the cooperation between the single groups [3].

Since 2008, the National Institute of Dental and Craniofacial Research (NIDCR) examines separately the oral microbiome. It already includes more than 600 microorganisms. At the same time, also the microbiomes of other defined areas of the body are evaluated. With the background of those intensive scientific investigations, the specific literature on the significance of the microbiome has increased exponentially during the last years. Currently more than 30,000 publications are found in this field, among those 400 specifically for the discipline of otorhinolaryngology. Even if many aspects regarding the microbiome are still not clarified, relevant basic knowledge is now available on all sub-specialties of otorhinolaryngology.

Peer-reviewed publications on "microbiome" increased enormously. ▶ **Fig. 1** describes the number of publications listed under www.pubmed.com in comparison with publications that are rele-



▶ **Fig. 1** Number of publications listed in pubmed with the key words of "microbiome" (total) or (microbiome AND (Rhinology or otology or otitis or nose or sinus or (head and neck) or laryngology); classified here as "ENT" (last retrieved on October 1, 2017).

- How do the microbiome and the health state interact?
- Which inter- and intraindividual differences exist?
- How relevant are the differences observed up to now?
- What is the influence that age, gender, lifestyle, climate, time of the day, and other physiological influencing factors have?
- How stable is the microbiome and how, how fast, and how long does it react on disturbances (resilience) such as acute infections?
- Can the microbiome be influenced therapeutically?
- How do the microbiome and the host respond to antibiotic therapy, immunosuppression, or immunotherapy?
- Are there international differences in the microbiome that can be traced back to the healthcare system (prescription behavior), genetic differences, or lifestyle?

▶ **Fig. 2** Open questions on the significance of the microbiome.

Latin	English
Regnum	Kingdom
Phylum	Phylum
Classis	Class
Ordo	Order
Familia	Family
Genus	Genus
Spezies	Species

▶ **Fig. 3** Taxonomic classification in the international use.

vant for the field of otorhinolaryngology. This aspect was defined by entering the key words of "microbiome AND (rhinology or otology or otitis or nose or sinus or (head and neck) or laryngology)". Of course this list cannot be complete due to the heterogeneity of the discipline. Already the number of the references of this review article illustrates this fact. ▶ **Fig. 1**, however, demonstrates a similar, dynamically increasing development in otorhinolaryngology with 274 publications in the field of rhinology, 153 in the field of laryngology/oncology, and 124 in the field of otology. Even an intensive research of the literature makes obvious that currently the practical consequences of those articles for the clinic are very limited, because of the low rate of comparability due to rapid technical developments as well as numerous influencing factors. However, for basic research and the development of new therapeutic approaches they might be highly interesting.

Working on the microbiome, several questions have been defined after the development of first technical standards, based on the knowledge of the interactive networking influence of microbiome and host. ▶ **Fig. 2** summarizes the aspects that will be discussed in the following.

The human microbiome data portal, available under https://hmpdacc.org/ contains the current state of research on the microbiome including data of healthy individuals. In detail, the buccal mucosa ranks second regarding most hits, followed by gingiva (ranking 5th), nasal cavity (7th), dorsum of the tongue (9th), nares (10th), palatal tonsils (11th), right (12th) or left (15th) retroauricular fold, hard palate (13th), pharynx (14th), saliva (16th), nasopharynx (23rd), and oral cavity (34th). Hereby the available reference data are also classified according to technical aspects.

2. Terminology

For better understanding of the following paragraphs, but also the international literature, some terms will be introduced here as technical terms:

Taxon/taxa is the umbrella term for one/several groups of living beings that can be differentiated from other organisms due to common characteristics. With regard to the microbiome, this term is used for the level of microorganisms (▶ **Fig. 3**).

Modifications of the microbiome are generally reported as alpha and beta diversity. Alpha diversity describes the level of different types of microorganisms that are found in an individual or an examined area of this individual. Alpha diversity represents a measure for the biodiversity of a habitat. This expression was introduced by the ecologist Robert Whittaker in 1960.

For example, the oral cavity disposes of the highest alpha diversity of the gastrointestinal tract with more than 1,000 different bacterial species including aerobe and anaerobe species.

The beta diversity is the variability between individuals of the same habitat with regard to the identified microorganisms. This term was well introduced by Robert Whittaker and characterizes the measure of the difference in the biodiversity.

Gamma diversity is a measure of the species diversity in a landscape, beginning with about 1,000 ha up to about 1,000,000 ha; together with the epsilon diversity describing the biodiversity of several landscapes in one geographic region, they play a role in biological literature but not in the field of medicine.

The relationship between a modified microbiome and a specific disease is called dysbiosis, probably originating from a bacterium that uses an ecological niche as "alpha bug" [2]. Dysbiosis-related inflammations cause carcinogenesis via different metabolic pathways in the same way as chemical carcinogens like acetaldehyde and N nitrate compounds.

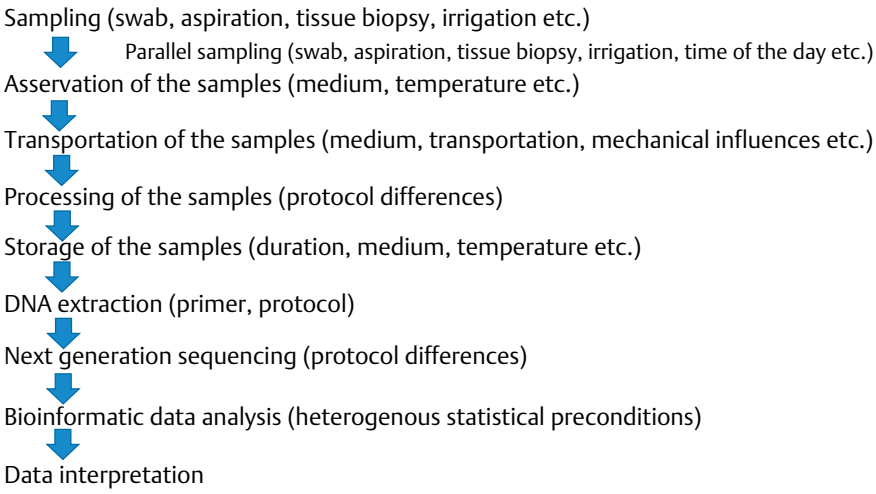
2.1 Taxomomy of bacteria

In the context of microbiome examinations, the classification of bacteria is performed based on the appearance, physiology, and phylogenetics. For description of the bacteria, their names were defined according to the requirements of the International Code of Nomenclature of Bacteria (ICNB) revised in 1980. Each term is based on stored type material that is the basis for classifying a bacterium to a taxon.

A microorganism is clearly defined based on its stored type material as identifiable taxon. The term and classification are subject to scientific modifications. The current taxa are published in the respective version of Bergley's Manual of Systemic Bacteriology [6].

3. General Factors Influencing the Microbiome

Traditional culture procedures only allow isolating and characterizing a very low percentage of the microorganisms of a microbiome. Those procedures have been replaced by culture-independent DNA-based sequencing methods.



▶ **Fig. 4** Procedure of a microbiome study with possible influencing factors (modified according to [7]).

3.1 Procedure of a microbiome study

In the context of microbiome trials, those techniques are applied nearly exclusively. They amplify and sequence the genetic information of small subunits of ribosomal RNA (16 S-rRNA) for taxonomic characterization. The ribosomal nucleic acids are part of the bacterial ribosomes that build proteins of the according genetic information. They are sequenced in order to identify and clearly differentiate different types of bacteria. 16 S-rRNA is highly conserved with regard to genetic information and thus appropriate for taxonomic classification.

The general procedure of a microbiome trial should be standardized (▶ **Fig. 4**).

The collected tissue specimens are frozen and stabilized without contaminations, the DNA is extracted and sequenced by means of amplification to 10^5 sequences rRNA or replicated to 10^7 sequences as metagenomics by means of a shotgun PCR, also called shotgun metagenomics. On the one hand, the diversity of the specimen is evaluated with the detection of rare or excessive quantities (abundance) of specific microbes, also comparing the structures, as well as the matrix of the microorganisms that were found.

The sequencing methods with broad spectrum (chain-termination method/Sanger sequencing, pyrosequencing, sequencing-by-synthesis) vary according to technical features such as the maximum read length of the sequence, number of sequences, time per run, and throughput volume. Hereby, conventional sequencing by means of Sanger sequencing that is relatively time-consuming and allows the analysis of smaller DNA molecules plays the role of confirmation technique. Nowadays, methods of the second generation are applied for time-effective analysis because they replicate much faster. In the context of the references included in this article, the majority used the pyrosequencing technique.

3.2 Sampling technique and technical aspects

For sample collection, several procedures were and are applied, sometimes even parallel. Many published data are based on smears, tissue biopsies, aspirates, irrigation etc. As gold standard of sampling and post-processing, the protocols published in 2013 by the Human Microbiome Project were applied. Meanwhile, those protocols have been regionally developed as well as the biostatistical and bioinformatic evaluation so that the comparability of the presented results is limited and contradictions have to be questioned methodically. The complexity of interactions between microbiome and host, but also the microorganisms involved in the microbiome, has complicated research significantly. The improved availability of next-generation sequencing techniques, also based on research of proteomics and genomics, allows more and more research groups assessing parameters of the microbiome. The evaluation, however, is so complex that the results are reduced to the diversity of the microbiome. In this context, bioinformatics have to develop in order to report data gained by cooperation of patient/physician/microbiologist in a way that is free of false-negative errors.

All the technical steps described in ▶ **Fig. 4** for determination of the microbiome have an impact on the outcome.

Each step in the procedure may change the quantity and the quality of the extracted DNA. Contamination is a relevant problem because of the sensitivity of subsequent procedures. However, investigations on the technical impact of different influencing factors provide contradictory statements: Storage at room temperature significantly modified the microbiome of stool in one study [8], in another it did not relevantly change [9], while storage of the specimens at −80 °C for different durations showed lower effects on the diversity [10, 11]. On the other hand, the vaginal microbiome seems to be more stable [12]. ENT-specific trials are rare. For the laryngeal microbiome a comparability for swab- and biopsy-based studies could be revealed in an animal model of pigs [13]. The same findings appeared in patients with chronic rhinosinusitis (CRS) with 36 identified bacterial species in the tissue and 30.6 specimens taken by a swab [14].

In order to meet those diverse influencing factors, several efforts have been undertaken to standardize the procedure of microbiome trials. Available online under http://microbiome-standards.org/#SOPS, international experts provided standard operating pro-

cedures (SOP). Those SOPs give valuable hints for a standardized procedure regarding sampling (e. g. of saliva or buccal swabs). This problem has already been discussed in several journals [15], even in the internal guideline on the publication of microbiome data [16]. There are different software packages that can evaluate biostatistically the complex data structure of microbiome data (low number of detected bacteria with at the same time high number of different species, high homology of the evaluated bacteria with 97 % matches and same phyla), such as QIIME [17–21], MOTHUR [22, 23], RDP tools [24–27], and VAMPS [28]. Based on the matches with the selected primers, operational taxonomic units (OTU) are identified. Comparing 16 s RNA gene amplification – the current gold standard – and MALDI-TOF (matrix assisted laser ionization mass spectrometry – time of flight), the examination of a microbiome regarding Streptococcus viridans by means of MALDI-TOF achieved a sensitivity of 80 % and a specificity of 100 %. The authors recommended to apply different assessment procedures in parallel, which, however, would eliminate the advantage of time efficiency of the sequencing methods of the second generation [29]. In contrast, MALDI-TOF supports the identification of Gram-positive bacteria (here: Corynebacteria) when conventional 16 s RNA sequencing failed [30]. So MALDI-TOF increased the detection rate to 92.49 % compared to 85.89 % by means of conventional microbiological examination [31].

A comparative evaluation of the same specimens on 3 different industrial sequencing platforms could identify further relevant deviations in the data analysis [32]. While the profiles of the microbiome composition were similar, the average abundance of the species depending on the platform, the used database, and the bioinformatics analysis seemed different. Detailed assessment of the bioinformatic analysis criticized especially the high number of false-positive detections [33]. Even the reference literature has an impact on the presented final result, but less than the one of the above-mentioned parameters [34].

In summary, the comparability of microbiome data is generally considered as being limited which also applies to the data presented in the following [35]. With this background, efforts have to be supported unconditionally regarding a standardized reporting of the original articles under methodical aspects.

3.3 General influencing factors

Gender, age, geographic location, climate, culture, and lifestyle are general influencing factors on the diversity of the microbiome that are discussed in the literature. But also different percentage distributions of various bacteria at different times of the day were reported [36]. Alternatively, microbiome-associations are explained by host-related factors such as smoking, alcohol, diet, obesity, physical (in)activity, and polymorphism in important human oncogenes. Some factors with significance for the interpretation of the “normal” microbiome will be discussed more in detail.

3.1.1 Age

During the development of an adolescent to an adult animal, the nasal microbiome seems to mature. In an animal model of pigs, it was possible to identify that in the comparison of newborns to animals of 2–3 weeks of age the alpha diversity increased and characteristic taxa could be detected [37].

In a murine model, the comparison of young, middle-aged, and old mice revealed significant changes of the microbiome, even after contact with Streptococcus pneumoniae (added by local rinsing of the upper respiratory tract). Resident Staphylococci and Haemophilus were sensible against Streptococcus. Furthermore, the colonization with Streptococcus pneumoniae increased with age and the mucociliary clearance seemed to be less effective [38].

Regarding chronic rhinosinusitis (CRS), an age-related reduction of S100 proteins was considered as being the origin for a modified microbiome with development of CRS at higher age [39]. This probably provides an approach for a specific endotype of CRS in higher ages.

Age-related effects can also be found in the oropharynx. The microbiome in older people is characterized by an increased abundance of Streptococci, especially Streptococcus salivarius, but not Streptococcus pneumoniae [40].

In comparison to younger people of the same gender, the microbiome of the stomach in 100-year-old people revealed another composition with consecutively increased plasma levels of IL6 and IL8. Generally, the biodiversity reduces with higher ages with a tendency to increase optionally pathogenic bacteria. Bacteria rather decrease, that are relevant for the metabolism of enterocytes of the gut because of their production of short-chain fatty acids (e. g. butyrate) [41].

Meanwhile, age effects have become a therapeutic objective via intervention of the microbiome: a tryptophan-reduced diet was applied in mice in order to delay premature aging by increasing the diversity of the microbiome [42]. The positive effect of the tryptophan diet is expected to be influencing the B cell differentiation. In the microbiome, an accumulation of Akkermansia was achieved, which is a species that is often detected in healthy individuals and that is particularly negatively influenced by the aging process of the host [43].

3.3.2 Gender

Data on gender-specific differences in the context of the microbiome are currently considered as being less reliable although some studies could identify gender-specific differences in nutrition known for the gastrointestinal tract because of the role assignment. Bacteroides, Ruminococci, Eubacteria, and Blautiae were found more often in males and Treponemen in females [44, 45]. It is assumed that the observed gender differences are a consequence of different lifestyles and nutrition.

3.3.3 Smoking

Cigarette smoke is supposed to increase the permeability of the epithelial barrier against microorganisms and thus contribute to proneness to infection. The origin might be a dysbiotic microbiome that triggers for example carcinogenesis in the area of the larynx and lung.

Investigations on the effect of smoking show, independently from the selected technique, that smoke modifies the composition of oral bacteria [46, 47], especially of favorable aerobic species [48]. Furthermore, bacteria may activate the carcinogen nitrosamine [49, 50]. Smoking makes the oral cavity more susceptible to proliferation of pathologic bacterial species [51]. Accordingly, the alpha diversity of the subgingival microbiome was significantly reduced in smokers. The analysis of the beta diversity also revealed differences of smokers compared to patients with chronic periodontitis of other genesis [52]. Similar changes that, however, were only detected on one side, were obvious in the nasopharynx [47] and the saliva [47]. In contrast, effects on the nasal microbiome could not be revealed [53].

Analysis of the exhaled air [54] showed 3 relevant modifications: increased pro-inflammatory markers were detected as a hint to increased free oxygen radicals. They revealed changes in the endogenous metabolism. Second, exogenous components were found [55]. Finally, also here an interaction between the microbiome and the host was obvious. While 12 metabolites could help differentiating smokers from absolute non-smokers, only the metabolites of eucalyptol and benzyl alcohol even revealed differences in the exhaled air between active and former smokers [54].

Regarding biopsies of the lung, 2 taxa with disproportionate relative abundance, i. e. Variovorax and Streptococcus, were found [56]. Specific for the occurrence of squamous cell carcinomas, more Acidovorax could be revealed in comparison to control tissue.

Also passive smoking changes the microbiome [57]. The microbiome of the nasopharynx and oropharynx of children depends on the smoking behavior of the mother. The detection rate of Streptococcus pneumoniae is significantly increased in active and passive smokers while Haemophilus influenzae seems to be unchanged.

3.4 Nutrition

3.4.1 Probiotics

Probiotics are preparations that contain viable microorganisms. Even if general understanding mostly focuses on oral, systemic application – e. g. eating yoghurt cultures to strengthen the intestinal flora – probiotics are not limited to this application but they can also be applied locally in the field of otorhinolaryngology or be relevant for it. For example a reduction of respiratory infections due to probiotics is discussed.

The application as food supplement is best evaluated. Via food, the composition of the intestinal microbiome of humans can be modulated effectively and in a reproducible way within 24–48 h [58]. This also represents an approach for application of probiotics [59] to e. g. stimulate the immune defense. Probiotics include unusable carbohydrates, among them fibers, resistant starch and non-starch polysaccharides, that are not enzymatically digested. Those substances are fermented by the commensal microbiome in the area of the colon/terminal ileum to propionate, butyrate, and acetate [59]. Probiotics influence the composition and activity of the intestinal microbiome and can improve well-being and health of the host [60]. The highest evidence for probiotic effects is available for fructans of the inulin type (fructo-oligosaccharides, inulin, and oligo-fructose) as well as for galacto-oligosaccharides [61]. Those probiotics shall promote the growth of Lactobacilli and Bifidobacteria [62]. In an animal model a modified composition of the intestinal microbiome could be achieved as well as a reduction of the body weight by feeding short-chain fatty acids [63].

ENT-specifically, an exemplary investigation was performed topically by inoculating Staphylococcus epidermidis with and without Staphylococcus aureus in a mouse model with sinusitis to find out whether the nasal microbiome can be influenced positively [64]. After 3 days of application, more goblet cells were found under inoculation with Staphylococcus aureus alone. Additional inoculation with Staphylococcus epidermidis attenuated this effect significantly while inoculation with Staphylococcus epidermidis alone achieved similar and lower detection rates than control. The concept is based on the assumption that Staphylococcus epidermidis may competitively inhibit the biofilm development by Staphylococcus aureus, for example via inhibitory serine protease EPS. In a pilot study, it could also be demonstrated that Staphylococcus aureus in human carriers can be suppressed by additional inoculation with Staphylococcus epidermidis [65]. This pilot study shows interesting technological approaches, for example also in the context for MRSA eradication by means of antibiotics.

For the oropharynx, it could be revealed that an earlier exposition to Streptococcus salivarius may impede in vitro the cell adherence of Pneumococci [66]. Further probiotic therapeutic approaches are described below in the context of the respective microbiome that should be influenced.

3.4.2 Alcohol

Tobacco and alcohol abuse are significant risk factor for developing head and neck cancer [67], and it is assumed that microbes mediate those risk factors. So the bacterium Neisseria that is often found on the oral mucosa disposes of alcohol dehydrogenase that transforms ethanol to the carcinogenic acetaldehyde [68]. However, the respective studies for the field of malignant diseases of the oral cavity are based mainly on tissue based examinations with older technologies [50]. Alcohol addiction seems to be associated with determined alterations of the gastrointestinal microbiome that can be found in the stool [69]. The quantity of Klebsiella increases while Coprococcus, Faecalibacterium praunitzii, and Clostridiales decrease. Additionally, alterations are found that can also be observed in liver cirrhosis. They include the reduction of Aciaminococcus and an increase of various Lactobacilli and Bifidobacteria.

3.5 Antibiotic therapy

A short-term effect of antibiotic therapy on the microbiome can be expected. After 5 days of oral application of amoxicillin with clavulanic acid a significantly reduced bacterial concentration could be revealed [70]. In particular, also the Bifidobacteria concentration in the stool was reduced. While this effect could be expected at that time, a follow-up examination 2 months after antibiotic therapy revealed a persisting alteration of the microbiome. So otherwise healthy individuals still had an abundance of Bifidobacteria that was reduced to 60 % of the original value. An older investigation showed an increased resilience of the gastrointestinal microbiome compared to amoxicillin alone [71], but it confirms changes more than 2 months after antibiotic therapy.

Antibiotic treatment in early childhood is associated with a higher risk to develop asthma later. So an antibiotic therapy at the ages of 0–2 years increases the risk to develop asthma at the age of 7.5 significantly (odds ratio 1.75; 95 % confidence interval of 1.40–2.17) while multiple antibiotic therapies increase this risk even further (e. g. 4 or more therapies: odds radio 2.82, 95 % confidence interval of 2.19–3.63). With the background that children suffering from atopic disease currently receive about 1.9 times as frequently antibiotic than children without atopy [72], the prescribing behavior of ENT specialists should be questioned critically.

3.6 Vaccination

Vaccination against Haemophilus influenzae does not relevantly modify the microbiome of the nasopharynx. This seems to indicate a directed elimination of the target [73]. In the context of a prospective, placebo-controlled study [74] before and in parallel to vaccina-

tion against influenza, the oral application of Lactobacillus casei 431 showed no changes in the response rate by serum conversion, while the duration of respiratory complaints was shorter when the probiotic was applied (average ± standard deviation: 6.4 ± 6.1 vs. 7.3 ± 9.7 d, P = 0.0059). Since the influence of vaccination on the microbiome has been evaluated clearly less frequently, methodical weaknesses cannot be excluded.

4. Microbiome in Otorhinolaryngology

4.1 Ear

Despite the common pathophysiology of adenoids and chronic otitis media with effusion, the microbiomes are totally different. In otitis media with effusion, Alloiococcus otitidis (23 % average relative abundance), Haemophilus (22 %), Moraxella (5 %), and Streptococcus (5 %) were found while the detection of Alloiococcus and Haemophilus correlated inversely and Haemophilus occurred more frequently in bilateral otitis media with effusion [75]. As bacterial pathogens, in addition Turicella and Pseudomonas were found increasingly in the age group older than 24 months [76]. Whereas Turicella and Actinobacteria were more rarely associated with severe conductive hearing loss, Haemophilus seems to be clearly more often causal [76]. Similar microbiomes were detected in Australian children originating from aborigines [77]. In contrast, significant differences could be found between the microbiome of otitis media with effusion and the one of the palatine tonsil [78]. According to other investigations, pseudomonas dominated the microbiome of the middle ear with a detection rate of 82.7 % [78]. Genetic differences could be described as possible causal influencing factor for different characteristic microbiomes [79].

4.2 Nasopharynx

Hyperplasia of the adenoids is one of the most frequent reasons to present a child to an ENT-specialist. The colonization of the nasopharynx in children was already described above with regard to the pathophysiological correlation with chronic otitis media with effusion. Pseudomonas, Streptococci, Fusobacteria, and Pasteurellaceae dominate the microbiome of adenoids [78].

Adenoids are frequently associated with acute rhinosinusitis [80]. Accordingly, adenotomy was approved as possible therapy of chronic rhinosinusitis in children [80]. An explanation for the interactive influencing of adenoids and paranasal sinuses in children is the detection of biofilm on the adenoid surface [81, 82]. A prospective observational study in children between the ages of 1 and 12 years revealed a high association between the microbiome on adenoids, their center, as well as the middle nasal meatus. This shows that recurrent infections of the paranasal sinuses and the nasopharynx in our pediatric patients may be explained on a bacteriological basis by the re-distribution of certain microbiomes. Furthermore, the clinical success of adenotomy in patients with concomitant acute rhinosinusitis can be explained [83]. In the area of adenoids, mainly Haemophilus, Staphylococcus, and Streptococcus are found [80].

However, no significant correlation between the colonization of the adenoid surface and the detection of microbes in the center of adenoids could be revealed in other studies [83, 84] so that the association of the superficial microbiome and the microbiome of adenoid tissue cannot be confirmed.

Former premature children have a stronger heterogeneity of the nasopharyngeal microbiome than normal children of the same age. Hereby, Proteobacteria were increased and Firmicutes were reduced. These differences persisted despite infection with a rhinovirus which was interpreted as a hint to persisting immune modulation regarding inflammations of the respiratory tract after premature delivery [85].

In the nasopharyngeal microbiome of children with asthma between 6 and 18 years Moraxella, Staphylococcus, Dolosigranulum, Corynebacterium, Prevotella, Streptococcus, Haemophilus, Fusobacterium, and Neisseriaceae were found in 86 % of all microbiome examinations. Different seasons could not reveal relevant differences of the alpha and beta diversity. But the relative percentage of Haemophilus, Moraxella, Staphylococcus, and Corynebacterium varied between summer and fall as well as within the evaluated age groups [86].

Finally, an acute viral infection with human rhinovirus or respiratory syncytial virus changed the profile of the nasopharyngeal microbiome in an evaluation of n = 123 healthy children regarding the bacterial composition [87]. So in summary, the microbiome of the nasopharynx has to be considered as highly variable.

Because of possible pathophysiological correlations between acute viral infection of the upper airways with the nasopharynx as possible reservoir and the risk to develop pediatric bronchial asthma [88], further investigations on the microbiome of the nasopharynx were conducted. Prospectively, an initial colonization with Staphylococcus or Corynebacterium before stable colonization with Alloiococcus or Moraxella could be detected in 234 children. Virus associated changes could be found due to the transient detection of Streptococcus, Moraxella, or Haemophilus. An early asymptomatic colonization with Streptococcus turned out to be a significant predictor for later development of bronchial asthma [89].

In cases of pediatric pneumonia acquired in the population, investigations revealed bacterial genesis in 95.13 % and only in 0.72 % viral genesis based on the microbiome. Most frequently, Paramyxoviridae, Herpesviridae, Anelloviridae, and Polyomaviridae were detected [90]. An extensive assessment of the viruses in the nasopharynx revealed a viral origin of about 1/7 of all microbiomes in more than 700,000 microbiome data of 210 patients. Paramyxoviridae, Picornaviridae, and Orthomyxoviridae were detected and additionally a new rhinovirus C was found [91]. These evaluations on the viral components of the microbiome indicate a high and nearly unknown percentage that interacts closely with the bacterial microbiome.

Regarding therapy of the nasopharyngeal microbiome, another publication is available. According to this study, Pneumococci were found in the microbiome of about 25 % of the examined adults. An intranasal application of Pneumococci in adults with high diversity of the nasopharynx led more often to subsequent pneumococcal colonization [92], which then favored an increased diversity of the microbiome.

4.3 Nose and paranasal sinuses

The endonasal microbiome is highly variable [93]. Therefore the nasal microbiome is significantly different from the less diversified microbiome of the lower airways. However, reports exist about significant cohort differences [93]. In this context, also intraindividual differences of the microbiome of the middle nasal passage, the midd-

le turbinate, and the inferior turbinate were found [93]. Aerobic bacteria are observed more frequently in the nasal cavity with about 80 % of the microorganisms compared to anaerobes [94].

In all patients who underwent surgery for control or for CRS, also fungi could be found in the nose [95]. The alpha diversity of the fungi was slightly lower in the controls compared to CRS (8.18 vs. 12.14, respectively). After surgery of the nasal cavity, the alpha diversity decreased, which was mainly associated with a reduction of Fusarium and Neocosmospora.

With regard to therapeutic change of the nasal microbiome, a double-blind cross-over study was performed: a mixture of Lactobacilli and Bifidobacteria was applied once in healthy individuals without detecting side effects or changes of the commensal bacterial as well as selected cytokines (including IL8 and IL15) [96].

4.3.1 Allergic rhinitis

In addition to the traditional hypothesis that hygiene promotes allergic sensitization, the microbiome/microflora hypothesis was established [97]. Disturbance of the gastrointestinal microbiome interferes with immune mechanisms of the tolerance development. In this way, the increased incidence of allergic diseases [98, 99] and bronchial asthma [100] might be explained. It is based on investigations according to which a reduced diversity of the gastrointestinal microbiome is associated with a higher prevalence of allergic diseases in schoolchildren [98, 99].

However, the exact mechanism is currently not clear. The hypothesis is supported by the detection of pathophysiological relations between a disturbed gastrointestinal microbiome and the occurrence of asthma [101–103]. One possibility of influencing the local and systemic inflammation of the respiratory tract [104] is the formation of short-chain fatty acids that are built by fermentation of fibers by intestinal bacteria [105, 106]. The increased risk of developing bronchial asthma after antibiotic therapy in early childhood was already mentioned.

4.3.2 Microbiome and chronic rhinosinusitis

The microbiome of patients with CRS varies enormously. There are probably significant differences in the composition of CRS without nasal polyposis (CRSsNP) and with nasal polyposis (CRSwNP) [93]. CRSsNP seems to be characterized by a microbiome with reduced diversification as well as anaerobic enhancement [93]. Streptococcus, Haemophilus, and Fusobacterium are measured in increased quantities. CRSwNP, however, is characterized by increased percentages of Staphylococcus, Alloiococcus, and Corynebacterium. Hereby, the detected variations are significantly different from the microbiome of patients with allergic rhinitis.

In the middle nasal meatus of patients with rhinosinusitis mainly Staphylococcus aureus, Staphylococcus epidermidis, and Propionibacterium acnes were found [107]. Also in the maxillary sinus predominantly aerobic bacteria (about 60 %) were detected. Most frequently, Streptococci (28.8 %) and Prevotella (17.8 %) were found. Streptococcus pneumoniae, Haemophilus influenzae, and Staphylococcus aureus, however, were identified in less than 10 % of the specimens [94]. The variance between the patients seems to be higher than in the different nasal regions. In particular, the middle nasal meatus reflects representatively the microbiome of the entire nose and paranasal sinuses (compared to nostrils, maxillary sinus, frontal sinus, sphenoid sinus). However, it overestimates the incidence of Corynebacterium [108].

4.4 Oral cavity

The subgingival microbiome of Chinese twins is exemplary for the high variety characterized by 18 phyla and 179 genu [109]. Caries was associated with a high percentage of Actinobacteria and the reduced detection of Fusobacteria. In adults, more often Treponemen were found, but these seem to be typical for adult periodontitis. Further marker of periodontitis were Spirochetes, Synergistetes, Firmicutes, and Chloroflexi whereas Actinobacteria, especially Actinomyces, was attributed a rather protective value [110]. Since very recent data consider reduction of alpha diversity as hint for periodontitis without mentioning specific microorganisms, the scientific discussion seems to be controversial [111]. Twin studies indicate that the genetic influence on the oral microbiome is subordinate to the environment, in particular nutrition [109]. Pregnancy also has a subordinate impact on the composition of the subgingival microbiome [112]. In contrast, a genetic disposition for caries seems to favor this disease in a higher measure than the microbiome of the dental plaque [113]. Nonetheless, Streptococcus, Veillonella, Actinomyces, Granulicatella, Leptotrichia, and Thiomonas [114], Streptococcus, Granulicatella, and Actinomyces [115], and Streptococcus and Veillonella (in children younger than 30 months) [116] were frequently found with simultaneously present caries. More favorable and without caries detection is probably a microbiome containing Leptotrichia, Selenomonas, Fusobacterium, Capnocytophaga, or Porphyromonas [116].

4.4.1 Microbiome of saliva

Saliva contains an extremely high number of microorganisms [117, 118] including Streptococcus, Dialister, and Veillonella [119]. Comparing the saliva of different ages, the alpha diversity in children seems to be higher while the absolute abundance in adults is higher with similar composition of the taxa [120]. The central healthy microbiome of saliva encompassed the taxa of Streptococcus, Prevotella, Neisseria, Haemophilus, Porphyromonas, Gemella, Rothia, Granulicatella, Fusobacterium, Actinomyces, Veillonella, and Aggregatibacter [120] or Streptococcus, Prevotella, Haemophilus, Lactobacillus, and Veillonella [121], respectively. Lower percentages of Neisseria, Aggregatibacter (Proteobacteria), Haemophilus (Firmicutes), and Leptotrichia (Fusobacteria) could be detected in patients with squamous cell carcinomas of the oral cavity or the oropharynx [121]. Higher rates of Neisseria, Aggregatibacter, Haemophilus, or Leptotrichia, however, indicated a possible tumor development. A higher sugar percentage in the mouth, e. g. in the context of diabetes mellitus type II, reduces the absolute abundance of microorganisms in the saliva and shifts the relative abundance in adolescents [122]. Only some studies could confirm a correlation with caries [123], other could not [124]. However, there seems to be an association with poor oral hygiene [125].

Technically, the circadian rhythms of immunoglobulin A production in the saliva is important. Regarding sampling, aspiration turned out to be superior to swabs [126]. Furthermore, also the detection of different bacteria such as Firmicutes including Streptococcus and Gemella, and Bacteroidetes including Prevotella [127] is subject to variation. Accordingly, the times of the day when sampling is performed should be reported in studies on the microbiome of saliva.

From a technical point of view, it is of fundamental importance to perform an investigation on the re-test-reliability of the microbiome data of saliva [128]. Sampling performed every 2 months over one year revealed significantly different absolute frequencies of the detected taxa, even on the level of phyla, and interindividual differences regarding the composition of the microbiome with significantly different alpha diversity. Also the pH value of the saliva varied in the course of the year [128]. Those data relativize the interpretations of differences in the microbiome (also of other areas), while the authors allot the observed effects to the seasons. Specimens that were taken in shorter intervals of e. g. one week, seem to be more stable with regard to their reliability [129]. Because of the stronger influence of the environment of the individual compared to genetics [130], the suggestion was made to take this fact into account for recruiting control groups.

More than 70 % of the DNA in the saliva can be allotted to bacteria, only less than 1 % belongs to viruses [131]. The salivary microbiome is increasingly examined in the context of systemic diseases, e. g. in order to diagnose more easily autoimmune diseases [132] or for early cancer diagnosis [133]. So the microbiome in M. Behcet patients seems to be less diverse with a high abundance of Haemophilus parainfluenzae, but a clear reduction of Alloprevotella rava and genu Leptotrichia [134].

The application of amoxicillin for 5 days increased the relative abundance of Veillonellaceae, Actinomycetaceae, Neisseriaceae, Prevotellaceae, and Porphyromonadaceae while Streptococcaceae and Gemellaceae decreased. In contrast, the application of azithromycin led to an increase of Bifidobacteriales and Veillonellaceae while Clostridiales, Neisseriaceae, and Erysipelotrichaceae were reduced [119].

For stimulation of the immune defense, possibly the intake of Lactobacillus kunkeei YB38 is useful because it increased the immunoglobulin A secretion in the saliva in a mouse model [135]. In contrast, the oral intake of Lactobacillus paracasei F19 had no influence on the incidence of caries in children between 4 and 13 months [136]. The regular application of commercially available probiotics reduced the detection of fungi significantly, especially Candida albicans [137], but the clinical relevance is not yet confirmed. At the same time, the alpha diversity of the salivary microbiome seems to increase when probiotics are applied [138]. Another aspect of the interventional study investigated the influence of xylitol or sorbitol containing chewing gum on the microbiome. Children were asked to eat about 6 g per day as chewing gum for 5 weeks. While xylitol reduced Streptococci detectable by means of culture, sorbitol led to a significant decrease of Veillonella atypica in the salivary microbiome [139].

4.5 Pharyngeal space

Streptococci dominate the microbiome of healthy tonsils with a relative abundance of almost 70 % [78]. In the pharynx, this dominance is not so high with about 50 %, followed by Fusobacteria (about 8 %) and Prevotella (about 7 %) [140–142]. Within Waldeyer's tonsillar ring, sampling reveals a high variance only due to the exact location (e. g. posterior pharyngeal wall versus palatine tonsil) [143].

Tonsillar hyperplasia in children leads to the detection of Streptococcus (21.5 %), Neisseria (13.5 %), Prevotella (12.0 %), Haemophilus (10.2 %), Porphyromonas (9.0 %), Gemella (8.6 %), and Fusobacteria (6.4 %) [144, 145]. Children suffering from PFAPA syndrome (periodic fever, aphthous stomatitis, pharyngitis, and adenitis) have a different microbiome on their palatine tonsil. It is characterized by an increased detection and increased relative abundance of Cyanobacteria to the detriment of the relative abundance of Streptococci [146].

In cases of chronic tonsillitis, the culture-based identification of pathogens is successful only in about 60 % [147, 148]. Anaerobes are found in about 40–60 % of the patients at the surface and in nearly 50 % within the palatine tonsil [143, 147]. Most frequently, Porphyromonas is found. Chronic tonsillitis in adults seems to be associated with Fusobacterium necrophorum, Streptococcus intermedius, and Prevotella melaninogenica/histicola [144, 145].

An interventional study on the influence of gargling with benzethonium chloride in patients with halitosis could not reveal any changes of the tonsillar microbiome [149].

4.5.1 Excursion: Microbiome and immune system

The basis of the functional significance of the microbiome in the pathogenesis of different immune mediated diseases is the modulation of the innate as well as adaptive immunity due to the microbiome and vice versa the influence of immune cells on the microbiome [150]. The microbiome influences the immunity especially via interleukin 18 and 22 mediated signaling pathways [151, 152]. In addition, microbiome and B and T cells may influence each other and thus the microbiome can have an influence on the adaptive immune system [150].

In this context it should be mentioned as an example, that women show a higher correlation between tonsillectomy and the occurrence of sarcoidosis (odds ratio: 3.30; 95 % confidence interval 0.88–12.39) compared to males (odds ratio: 1.26; 95 % confidence interval 0.10–16.52) [153]. This indicates a possible influence of the pharyngeal microbiome on the development of autoimmune diseases in a similar way as the data on the effectiveness of chemotherapies with different microbiomes (see below).

4.6 Larynx

The laryngeal microbiome is significantly different from the one of the pharynx [140]. Primarily, consistent Firmicutes, Proteobacteria, and Bacteroidetes are reported [154]. More detailed investigations also state incidences: the detected phyla encompass Firmicutes (54 %), Fusobacteria (17 %), Bacteroidetes (15 %), Proteobacteria (11 %), and Actinobacteria (3 %). The identified genu include Streptococcus (36 %), Fusobacterium (15 %), Prevotella (12 %), Neisseria (6 %), and Gemella (4 %) [140].

Another investigation of the same group revealed a broad variance of the taxa with different percentages [155]. The phyla Firmicutes (46.4 %), Bacteroidetes (18.7 %), Fusobacteria (16.9 %), Proteobacteria (13.0 %), and Actinobacteria (2.4 %) could be found which confirmed earlier results. The genu Streptococcus (41.7 %), Helicobacter (2.6 %), and Haemophilus (2.3 %) showed a similar dominance. In comparison to the location of the vocal folds, the microbiome of the false vocal folds is not significantly different [13]. Technically, the results of biopsies and swabs were similar so that less invasive techniques for studies and control tissues are justified [13].

4.7 Trachea

In newborns, Acinetobacter can be reliably detected as part of the tracheal microbiome [156]. So the general assumption that the airways of newborns and especially the trachea are sterile seems to be

disproved. A reduced alpha diversity of the tracheal microbiome indicates an increased risk to develop a specific chronic pulmonary disease, i. e. bronchopulmonary dysplasia. Likewise, the oral and tracheal diversity in intubated patients was reduced. Often the taxa detected by means of sequencing could not be identified by means of culturing [157]. Intubated patients with pneumonia had a better prognosis with a relative abundance of < 4.6 % of Pseudomonas and < 70.8 % of Staphylococci [158]. Tracheostomized patients often showed Haemophilus in the microbiome in cases of infection. This increase occurred to the detriment of Acinetobacter, Corynebacterium, and Pseudomonas. In the context of infection, alpha and beta diversity decrease significantly [159].

4.8 Esophagus

4.8.1 Gastro-esophageal reflux

An investigation revealed that reflux has no impact on the laryngeal microbiome [160]. The application of proton pump inhibitors in newborns with gastro-esophageal reflux [161] neither changed significantly the alpha nor the beta diversity but representatives of the genu Lactobacillus and Stenotrophomonas decreased to the detriment of Haemophilus. After therapy interruption, the alpha and beta diversity re-increased together with the relative abundance of the phyla Firmicutes, Bacteroidetes, and Proteobacteria. In this way, the microbiome reflects the age and the diet.

4.8.2Neoplasms

Squamous cell carcinomas and adenocarcinomas are the most frequently occurring malignant neoplasm in the area of the esophagus. The development of an adenocarcinoma seems to be favored by gastro-esophageal reflux that also influences the complex microbiome of the esophagus and is possibly a co-factor of the pathophysiology of Barrett's esophagus [162]. The relative risk to develop an adenocarcinoma amounts to 30–400 in gastro-esophageal reflux patients [163].

The normal microbiome of the esophagus seems to be characterized by Gram-positive bacteria (phylum Firmicutes, especially with genus Streptococcus) [164]. Reflux as well as Barrett's esophagus changed this image in favor of more Gram-negative anaerobes of the phyla Bacteroidetes, Proteobacteria, Fusobacteria, and Spirochetes). In addition, the relative incidence of taxa seems to be more important for the pathophysiology than the absolute quantity of bacteria. So more frequently, Veillonellae (19 %), Prevotellae (12 %), Neisseriae (4 %), and Fusobacteria (9 %) were detected in the context of reflux disease or Barrett's esophagus.

In order to better examine the impact of gastro-esophageal reflux for example also on the risk to develop ENT-specific cancer, data material from the NordASCo cohort [165] is currently evaluated. A total of 945,153 patients with gastro-esophageal reflux from Scandinavian countries were assessed, 48,433 (5.1 %) of them underwent surgical intervention for reflux control.

5. Head and Neck Cancer and its Treatment

5.1 Carcinogenesis

Currently it is assumed that about 20 % of all cancer diseases are caused by microbial pathogens [166]. In otorhinolaryngology, e.g.the role of human papillomavirus is acknowledged. A shift within the microbiome may additionally favor carcinogenesis via chronic inflammations because protecting factors such as protective microbial peptides are missing, toxins accumulate or pathogens proliferate. Even after the development of malignant neoplasms, the microbiome plays an important role. Microorganisms or their metabolites may have an oncogenetic effect, favor tumor growth, provide growth factors, and develop pro-inflammatory and immunosuppressive effects that weaken the endogenous mechanisms of tumor defense.

However, it is a problem to differentiate tumor-associated, rather accompanying changes from those with causal relation. So an increased risk of cancer was proven in dependence of antibiotic application [167]. The risk to develop malignant neoplasms in the area of the oral cavity and the pharynx increases to the relative risk of 1.38 (1.17–1.64; age and gender adapted)in cases of 6 or more prescriptions of antibiotics. In the larynx, the risk to develop neoplasms was even higher with 1.45 (1.08–1.94).

Changes of the oral microbiome are strongly associated with the occurrence of tumors of the oral cavity [168]. A meta-analysis of 8 studies revealed an increased risk of 2.63 (95 % confidence interval: 1.68–4.14) to develop malignant neoplasm of the head and neck in cases of periodontitis [169]. Since – as described above – it is associated with changes of the oral microbiome, also differences in the occurrence of head and neck tumors can be expected.

Accordingly, a case control study using specimens of the pharynx, larynx, and also metastases of head and neck tumors showed a lower alpha diversity in tumors compared to normal mucosa of the same location [170].

Comparing the normal mucosa between the location of the primary tumor as well as a metastasis, the beta diversity reveals significant differences but also between the primary tumor and the metastasis, the beta diversity was clearly different. In comparison to the physiological microbiome of the oral cavity, the tumor microbiome is characterized by the increased incidence of Bacteroidetes, Proteobacteria, Spirochetes, and Fusobacteria with decrease of Firmicutes and Actinobacteria. Primary tumor of the larynx and pharynx revealed an increased colonization with Fusobacteria and decrease of Firmicutes. Finally, Fusobacteria also increased in metastases and especially the percentage of the species Streptococci from the phylum of Firmicutes decreased. Additionally, the detection rate of Proteobacteria was higher.

Furthermore, the risk to develop a lymphoma is explained based on microbiome-assisted approaches. Scandinavian investigations indicate a seropositivity with Borrelia burgdorferi [171, 172]. MALT lymphomas in different non-gastro-intestinal organs revealed Chlamydophila psittaci [173, 174].

5.2 Microbiome and checkpoint inhibitors

Approved for treatment of head and neck cancer or in the testing phase are currently antibodies that inhibit PD1 (programmed cell death protein 1, also CD279) or CTLA4 (cytotoxic T lymphocyte associated antigen 4) [175]. PD1 or checkpoint inhibitors were approved for Germany only in the last months (such as Nivolumab). Others are expecting approval here and are already approved by the FDA for treatment of squamous cell carcinomas of the head and neck (such as Pembrolizumab) [176]. PD1 is expressed by activated T lymphocytes, B cells, natural killer T cells (NKT), and Treg cells [177]. They belong to the family of CD28 co-receptors [178]. Hereby, the ligan-

ds PD1 and PD2 bind to those receptors; apparently only PD1 is expressed by tumor cells beside antigen presenting cells (e. g. macrophages [179, 180]). Furthermore, the expression of PDL1 in some squamous cell carcinomas (skin [181]) is associated with the tumor stage while the expression of PDL2 is rather determined by the tumor size and the differentiation [181].

The ligands of PD1 and PDL1 are located in the tumor environment and help that the tumor cells escape from the immune reaction of the host [182, 183]. The blockade of this binding causes a clear increase of interferon gamma (IFN-gamma) [184] and thus a significant change of the microenvironment around the tumor. The production of IFN-gamma can be influenced by the intestinal microbiome [185]. Ruminococcus (Gram-negative) as well as Alistipes (Gram-positive) are associated with IFN-gamma production. In contrast, a microbiome enhanced with Lactobacillus can nearly inhibit this IFN-gamma production. Investigations in an animal model of mice indicate that the microbiome may strengthen the effectiveness of anti-PDL1 therapy. Hereby, a microbiome with a high percentage of Bifidobacter shows an improved response.

CTLA4, however, is a global immune defense (checkpoint) to modulate immune responses by down-regulation of CD4 + T effector (Teff) cells and enhanced Treg cell activity [182, 183]. Even via this mechanism, the microbiome seems to influence the response of cancer therapies. Mice that are without bacterial colonization or after antibiotic therapy, only show low effects of anti-CTLA4 therapy [186]. Reversely, the CTLA4 treatment modifies the microbiome [186]. Finally, an immune-triggered colitis under CTLA4 therapy with Ipilimumab can be avoided by Bacteroides phlilym enhancement [187]. Furthermore, oral therapy with the antibiotic vancomycin improves CTLA4 immunotherapy [186].

In an animal model of genetically identical mice with melanoma induction, clearly different response rates on the different microbiome of animals of 2 different breeders could be explained and traced back to the positive effect of Bifidobacteria. The application of Bifidobacteria in animals with poorer response could improve tumor control and IFN-gamma production [188].

So the microbiome may be considered as biomarker for the therapy response or an approach to positively influence the effectiveness of a therapy.

5.3 Outlook

The treatment of the microbiome opens a completely new field of therapeutic options depending on the material used. Stool transplantation for treatment of Clostridium difficile was classified as treatment with a pharmaceutic by the Federal Institute for Pharmaceutics and Medical Products (Bundesinsitut für Arzneimittel und Medizinprodukte, BfArM) even if a reproducible production of stool according to current pharmacological understanding is currently not possible. So here, a grey area exists that has to be considered critically with regard to probiotic therapies – also in the head and neck. The discussion of modifying the microbiome even by “only” one single antibiotic application helps questioning the prescription behavior under this aspect. Based on the current German guidelines for tonisllectomy, 5-6 antibiotic therapies are previously required, however, in the future data assessment evaluating secondary damage of the microbiome with their impact on the host are reasonable and may help to verify a possibly earlier intervention. Because of the interactions between host and microbiome, also independent from a disease and currently limited knowledge, the microbiome turned out to be an unscheduled parameter of future therapies.

Conflict of Interest

The author states that he received remunerations for lectures paid by Pohl-Boskamp Company, Essex Pharma Company, and Happersberger Otopront Company. He was and is involved in studies financed by third parties in accordance with the German Drug Law for Allakos, Sanofi, and GlaxoSmithKline companies.

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