Dissertation

Cardiorespiratory Performance Capacity and Pulmonary Microbiome in Patients Following Surgical Repair of Esophageal Atresia

submitted by

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Statutory declaration

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Abbreviations and Definitions

ΔVO_2 / ΔWR	Aerobic Capacity
ALTE	Acute Life-threatening Events
ASD	Atrial Septal Defect
BMI	Body Mass Index
BMP	Bone Morphogenetic Proteins
BPD	Bronchopulmonary Dysplasia
BR	Breathing Reserve
BW	Body Weight
C	Celsius
CF	Cystic Fibrosis
COPD	Chronic Obstructive Pulmonary Disease
CPAM	Congenital Pulmonary Airway Malformation
CPET	Cardiopulmonary Exercise Performance Testing
CTRL	Control Group
DNA	Deoxyribonucleic Acid
dsDNA	Double Stranded Deoxyribonucleic Acid
EA	Esophageal Atresia
ECG	Electrocardiography
EIA	Exercise-Induced Asthma
ELBW	Extreme Low Birthweight
EoE	Eosinophilic Esophagitis
EQO_2	Respiratory Equivalent for Oxygen
EUROCAT	European Database for Congenital Anomalies
FDR	False Discovery Rate
FeO_2	Expiratory Fraction of Oxygen
FEV 1	Forced Expiratory Volume in 1 Second
FiO_2	Inspiratory Fraction of Oxygen
GALT	Gut-Associated Lymphoid Tissue
GER	Gastroesophageal Reflux
GERD	Gastroesophageal Reflux Disease
HAEC	Hirschsprung’s Associated Enterocolitis

HMP	Human Microbiome Project
HR	Heart Rate
HRM	High Resolution Manometry
ID	Identification
IgE	Immunoglobulin E
IQR	Interquartile Range
kg	Kilogram
L	Liter
LDA	Linear Discriminant Analysis
LEfSe	Linear Discriminant Effect Size
LGEA	Long Gap Esophageal Atresia
min	Minutes
ml	Milliliter
MRI	Magnetic Resonance Imaging
MVV	Maximum Voluntary Ventilation
n	Number
NEC	Necrotizing Enterocolitis
NIPB	Non-invasive Blood Pressure
NK	Not Known
O_2/HR	Oxygen Pulse
OTU	Operational Taxonomic Unit
PAL	Physical Activity lLvel
PCoA	Principal Component Analysis
PCR	Polymerase Chain Reaction
PPI	Proton Pump Inhibitor
RDA	Redundancy Analysis
RER	Respiratory Exchange Ratio
RNA	Ribonucleic Acid
rpm	Revolutions per Minute
rRNA	Ribosomal Ribonucleic Acid
RT	Room Temperature
SBS	Short Bowel Syndrome
SD	Standard Deviation

Shh	Sonic Hedgehog
spp	Several Species
TEF	Tracheo-Esophageal Fistula
TM	Tracheomalacia
TNF	Tumor Necrosis Factor
VC	Vital Capacity
VC_{max}	Maximum Vital Capacity
VE	Minute Ventilation
VLBW	Very Low Birthweight
VO_2	Oxygen Uptake
$VO_{2\ max}$	Maximum Oxygen Uptake
VSD	Ventricular Septal Defect
VT	Tidal Volume
WR	Work Rate
µg	Microgram
µl	Microliter

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Abstract in German

Einleitung: Patienten nach Korrektur einer Ösophagusatresie (EA) leiden häufiger unter respiratorischen Symptomen und kardiopulmonaler Beeinträchtigung. Es ist unklar, ob Veränderungen der mikrobiellen Besiedelung der Luftwege eine eventuelle Ursache dafür darstellen. Ziel dieser prospektiven Kohortenstudie war es, die kardiopulmonale Leistungsfähigkeit und das Atemwegsmikrobiom von jugendlichen und erwachsenen Patienten nach korrigierter EA zu untersuchen und die Ergebnisse mit einer gesunden, alters- und geschlechtsangepassten Kontrollgruppe zu vergleichen.

Methoden: Alle zwischen 1980 und 2010 behandelten EA-Patienten wurden zu einer prospektiven Studie eingeladen, die aus klinischer Untersuchung, konventioneller Spirometrie und Fahrradspiroergometrie bestand. Das Mikrobiom der Luftwege wurde aus tief induziertem Sputum durch 16S-rRNA-Gensequenzierung bestimmt. Die Ergebnisse wurden mit einer gesunden, alters- und geschlechtsangepassten Kontrollgruppe verglichen.

Ergebnisse: Neunzehn EA-Patienten mit einem Durchschnittsalter von 24,7 Jahren und 19 alters- und geschlechtsangepasste Kontroll-Probanden wurden eingeschlossen. EA-Patienten zeigten im Vergleich zur Kontrollgruppe eine signifikant geringere Muskelmasse. Die Spirometrie ergab eine signifikant niedrigere maximale Vitalkapazität und eine höhere Rate restriktiver Ventilationsstörungen bei EA-Patienten; in der Spiroergometrie wurden signifikant niedrigere relative Leistungskapazitäten und eine signifikant niedrigere maximale Sauerstoffaufnahme gefunden. Die Alpha- und Beta-Diversität des Mikrobioms der Atemwege unterschied sich zwischen den beiden Gruppen nicht signifikant. Die LEfSe-Analyse ergab eine signifikant vermehrte Zahl von *Prevotella uncultered, Streptococcus anginosus, Prevotella 7 Prevotella enoeca* und *Mogibacterium timidum* bei EA-Patienten.

Schlussfolgerung: Langfristig leiden EA-Patienten häufig an restriktiven Ventilationsstörungen und einer beeinträchtigten kardiopulmonalen Funktion, die mit diskreten Veränderungen des Mikrobioms der Atemwege verbunden sind.

Langzeituntersuchungen von EA-Patienten, die aus routinemäßiger Spirometrie und Spiroergometrie bestehen, scheinen notwendig zu sein, um eine beeinträchtigte kardiopulmonale Funktion frühzeitig zu erkennen und das Fortschreiten der damit verbundenen möglichen Komplikationen zu verhindern.

Abstract in English

Introduction: Patients following esophageal atresia (EA) repair more frequently suffer from respiratory symptoms and impairment of cardiopulmonary function. Possible underlying reasons may be alterations of the airway microbiome. The aim of this prospective cohort study was to investigate the cardiopulmonary performance capacity and the airway microbiome of adolescent and adult patients after corrected EA and to compare the findings to healthy age- and sex-matched controls.

Methods: All EA patients treated between 1980 and 2010 were invited to a prospective study consisting of clinical examination, conventional spirometry and exhausting bicycle spiroergometry. The airway microbiome was determined from deep induced sputum by 16S rRNA gene sequencing. The results were compared to a healthy age- and sex matched control group.

Results: Nineteen EA patients with a mean age of 24.7 years and 19 age- and sex-matched controls were included. EA patients showed a significantly lower muscle mass compared to the control group. Spirometry revealed a significantly lower VC_{max} and higher rates of restrictive ventilation disorders of EA patients; significantly lower relative performance capacity and a significantly lower peak VO_2 were found in spiroergometry. Alpha- and beta-diversity of the airway microbiome did not differ significantly between the two groups**.** LEfSe analysis revealed significantly enriched species of *Prevotella uncultured, Streptococcus anginosus, Prevotella 7 Prevotella enoeca and Mogibacterium timidum* in the EA patients.

Conclusion: In the long-term outcome, EA patients frequently suffer from restrictive ventilation disorders and impaired cardiopulmonary function associated with discreet alterations of the airway microbiome. Long-term examinations of EA patients consisting of routine spirometry and spiroergometry seem to be necessary in order to detect impaired cardiopulmonary function and to prevent the progression of associated complications.

1. Introduction

The majority of the clinical research on esophageal atresia (EA) focuses on the upper gastrointestinal tract. However, the respiratory tract may also be affected in many of these children resulting in a lifelong pulmonary impairment. Consequently, the importance of respiratory function in the follow-up of these patients has been increasingly recognized in recent years.[3–7] In this regard, it has been shown that patients following EA repair develop respiratory symptoms more frequently than the healthy population.[3–5,7,8] While the negative impact of those health conditions on the quality of life in adolescence and adulthood has been demonstrated in several studies, there is no exact idea about the relationship between early childhood disease progression and later pulmonary impairment.[5,7,9] Early identification of reduced cardiopulmonary performance capacity, therefore, is important in order to provide targeted physiotherapy and prevent physical or social impairment of these patients.

The determinants of reduced exercise capacity are still unclear, but comorbidities like tracheomalacia or ventilation disorders may be discussed as underlying causes.[3,6–8] Furthermore, recurrent respiratory tract infections may contribute to impaired pulmonary function. The common opinion that lungs are sterile, has been refuted in several studies, which could identify up to 2,000 bacterial genomes per cm^2 lung tissue.[10–12] In detail, an airway microbiome is already soon after birth and further develops during growth.[11,13,14] The importance of the human microbiome and its possible role in pulmonary health and immune response has been increasingly recognized in recent years and plays a substantial role in pulmonal health and immune response.[12,15–17] Comparisons between infants with chronic lung diseases and healthy controls have shown marked differences in their airway microbiome.[14,16–18] The scientific work of airway microbiota composition, however, mainly focuses on chronic pulmonary diseases whereas microbiome studies for EA patients are currently lacking. Additionally, it remains unknown whether there is an association between cardiopulmonary performance capacity and alterations of the airway microbiome in EA patients.

The aim of this prospective study was therefore to investigate the cardiopulmonary performance capacity and airway microbiome of adolescent and adult patients after corrected EA.

1.1 Historical Background

Esophageal atresia (EA) was first described in 1670 by Durston, who diagnosed a blind ending upper esophageal pouch in one infant of female thoracopagus conjoined twins.[19] The first classic esophageal atresia with a distal trachea-esophageal fistula (EA-TEF) was reported by Gibson in 1697. He described a a newborn, which would not swallow, even though it seemed very desirous of food; the diagnosis could be confirmed in a subsequent autopsy.[20] During the 19th century more and more case reports described the presence of an esophageal atresia. 1931 Rosenthal already collected the data of 255 EA patients.[21,22]

The possibility of an operative treatment of an EA-TEF was first suggested by Timothy Holmes in 1869.[23] In 1888 in London, Charles Steele did the first surgical attempt to repair an esophageal atresia without TEF.[24] Steele suspected an esophageal membrane and tried to perforate it with a bougie through a gastrostomy; unfortunately this attempt was unsuccessful and the subsequent autopsy revealed a blind-ending proximal and distal esophageal pouch.[24,25] In 1938, Robert Shawn in Dallas, Texas, first described a fistula ligation and a primary esophageal anastomosis in a case of EA-TEF.[26] This child died 12 days after the operation and until the late 1930s several attempts of surgical treatment of esophageal atresia failed and ended up into a 100% mortality.[25]

The first successful primary repair of an EA-TEF was accomplished by Cameron Haight at the University of Michigan on March 15, 1941. [25,27] After five subsequent failed attempts he successfully operated a 12-day-old female neonate with EA-TEF by using a left extra-pleural approach with fistula ligation and a primary single-layer anastomosis of the esophageal ends, but revised his procedure two years later to a right thoracotomy approach, because of a better exposure of the esophageal structures.[25,27] Following Dr. Haight's publication, reports of successful EA operations came up more frequently and by 1944 one third of infants with EA survived primary repair.[28] Over the following decades the rapid progress in pre- and

postoperative management, antibiotic therapy and operative technique led to a growing surgical success.[29]

1.1. Epidemiology

The estimated incidence of EA is 1:2,500 births, but seems to vary from 1:4,500 birth in the United States of America up to 1:2,440 in Finland with a slight male preponderance.[22,25] A large European database for congenital anomalies (EUROCAT) revealed an overall prevalence of 2.43 cases per 10,000 births for two decades (1987-2006) with regional differences ranging from 1.27 to 4.55.[30]

White populations show a higher prevalence rate and first pregnancy as well as maternal age below 20 years are known as risk factors for EA.[25] EA seems to be associated with prematurity and very-low-birth weight (VLBW), though this could also be related to concurrent obstetric complications such as polyhydramnios.[29] An increased rate of EA is also known in twins (7% vs. 2.3%).[29]

The vast majority of all EA cases occurs sporadic and is non-syndromic.[22] Chromosomal abnormalities are found in 4 - 7.8% and mainly include trisomy 21, trisomy 18 and trisomy 13.[29,31] Familial cases can occur in different genetic syndromes, like Pierre-Robin syndrome, Holt-Oram syndrome or Di-George syndrome. However, the recurrence risk in parents with an EA child is less than 1%.[29]

The recently published overall mortality rate in the newborn period is 11% and is described significantly higher in infants with additional congenital anomalies (13%), VACTERL (19%) and worse risk classification group (Spitz classification II or III) (18%).[32] Isolated EA with or without TEF show a mortality rate of 4%.

1.2. Associated Anomalies

Recent data show that up to 80% of EA patients have an additional congenital anomaly.[32] Congenital cardiac malformations represent the most frequent associated anomalies occurring in 22 – 35% of all EA cases.[22,25,29] Atrial and septal defects are the most common cardiac malformations, but also complex defects with a high mortality rate can occur.[29]

Gastrointestinal anomalies occur in 21 – 27% and are dominated by anorectal malformations, duodenal atresia, malrotation and pyloric stenosis.[29]

Urinary tract abnormalities are present in 14 – 24% of all EA cases.[22,25,29] Vesico-ureteric reflux may be the most common congenital genitourinary anomaly, but the majority of the congenital genitourinary anomalies, such as horseshoe kidney, unilateral agenesis or ureteric duplication may not require surgical treatment.[29]

Ten to twenty percent of all infants with EA show musculoskeletal anomalies, mainly vertebral defects commonly accompanied by rib anomalies.[22,25,29]

Concurrence of combined anomalies are summarized as VACTERL or also VATER association, which is typically defined by the presence of at least three congenital malformations such as vertebral defects, anal atresia, cardiac defects, tracheo-esophageal fistula, renal anomalies or limb abnormalities **(Table 1).**[33] A VACTERL association occurs in 1 of 10,000 up to 1 in 40,000 live-borns; in case of an esophageal atresia the incidence of VACTERL is estimated between 20% and 35%.[25,32,33]

VACTERL association			
V	Vertebral	60-80%	Hemivertrebrae, butterfly vertebrae, wedge vertebrae, vertebral fusion, supernumerary or absent vertebrae and other forms of vertebral dysplasia
A	Anal atresia	55-90%	Spectrum of anorectal malformations
C	Cardiac defects	40-80%	Structural heart anomalies
T E	Tracheo-esophageal fistula (TEF)	50-80%	Different subtypes of TEF with or without EA
R	Renal anomalies	50-80%	Unilateral (or bilateral) renal agenesis, horseshoe kidney, cystic and/or dysplastic kidneys
L	Limb abnormalities	40-50%	Radial anomalies, including thumb aplasia or hypoplasia, polydactyly, lower limb anomalies

Table 1: Prevalence of different components of the VACTERL association, modified from Solomon 2011.[33]

Numerous studies report incidence rates of associated anomalies in EA infants, reaching an overall incidence of one or more components of the VACTERL association of up to 70%.[22,25,31,34] Multiple congenital anomalies can occur in more than 20%, but interestingly, patients with a VACTERL association do not tend to have a neurocognitive impairment.[31,33] Genitourinary malformations can occur in up to 25% of patients with VACTERL association.[33]

Brown et al. could show in their family study that infants with EA-TEF frequently have associated VACTERL anomalies but also midline defects like cleft lip and palate and sacral dysgenesis as well as urogenital anomalies.[34] First-degree relatives of EA children seem to have a higher risk to have one or more components of the VACTERL association when compared with a healthy control group ($p < 0.01$).[34]

In 2% the esophageal atresia is one component of the CHARGE Association, consisting of coloboma, cardiac defects, choanal atresia, retarded growth and development, genital hypoplasia and ear malformations.[22,29] It is caused by an

autosomal dominant inherited mutation of the CHD7 gene.[35] These patients show a mortality rate up to 70% mostly due to major heart anomalies.[22]

The combined presence of an esophageal atresia and a cleft lip and palate leads to a higher mortality rate, mainly related to complex cardiac defects or the presence of multiple associated malformations.[22,29]

1.3. Long-Term Sequelae of EA and its Associated Malformations

Despite excellent survival rates in infants with esophageal atresia, severe and long-term complications can occur, including gastroesophageal reflux, esophageal dysmotility, eosinophilic esophagitis, tracheomalacia and disorders of the respiratory function.

1.3.1. Gastroesophageal Reflux

Gastroesophageal reflux disease (GERD) is the most important long term sequalae of esophageal atresia and can be seen as a part of the malformation.[36] Too much tension at the esophageal anastomosis and extensive mobilization of the esophagus lead to an insufficiency of the gastroesophageal junction and may weaken the lower esophageal sphincter, because of traction and partial denervation on the cardia.[36] This leads to an abnormal and ineffective peristalsis, deficient sphincter function and dysmotility, which does not improve by age.[37,38] Furthermore, the acid reflux seems to increase the risk of anastomotic insufficiency or formation of anastomotic strictures.[36] Acute life-threatening events (ALTE) may also be associated with proximal extension of the gastroesophageal reflux.[37] The gastroesophageal reflux in EA patients may also be related to coexisting abnormalities of the vagal nerve innervation or be secondary to iatrogenic vagal injury.[36]

The incidence of symptomatic gastroesophageal reflux (GER) in infants following EA repair ranges between 25% and 70% and most patients require a fundoplication within the first year of life.[37] Up to 20% of EA patients with GER show already Barrett's metaplasia.[38] The first line medical therapy for pure or long-gap EA patients with severe GER consists of proton pump inhibitors (PPI), but a significant

number of patients needs a fundoplication, which leads to a fundoplication rate between 10% and 45%.[37,38]

The surgical repair of long-gap EA is still challenging and delayed primary anastomosis of long-gap EA significantly leads to a higher rate of stricture formation (61.9%) and GER (40.8%).[39] However, conserving the natural esophagus is always superior to any esophageal substitution techniques, knowing that even delayed primary anastomosis under tension has better long term results than gastric or colonic interposition strategies.[36]

The type of fundoplication in EA patients is still discussed controversially; Nissen fundoplication may be associated with gas-bloat and dysphagia while partial wraps, like Toupet or Thal, show less adverse effects despite a higher failure rate.[37] In EA patients the fundoplication failure rate is significantly higher than in patients without underlying structural anomalies, ranging between 20% and 45%.[37,38,40] The wrap failure typically occurs 1.5 to 2.5 years after the fundoplication.[40]

The causes of fundoplication failure seem to be related to the short length and poor propulsive activity of the repaired esophagus as well as delayed gastric emptying and a smaller stomach, which may impair the formation of a suitable wrap.[37,38] Because redo fundoplication in EA patients can be demanding and can result even in a higher failure rate than the primary intervention, long-term PPI therapy should be considered as an alternative to repeat fundoplication.

1.3.2. Esophageal Dysmotility

Esophageal dysmotility occurs in all children born with EA; it can lead to severe dysphagia or feeding difficulties and is related to a higher aspiration risk.[35] The etiology of esophageal dysmotility remains unclear, but may have a congenital origin: hypoplasia and abnormal interganglionic connections in the myenteric plexus of the proximal esophageal segment as well as reduced density and immaturity of interstitial cells of Cajal were found in autopsy studies or animal models.[35] On the other hand, esophageal dysmotility may be acquired from extensive intraoperative mobilization and denervation of the esophageal segments.[29]

In children with esophageal dysmotility an impaired bolus transit and a reduced clearance of GER may cause progressive feeding disorders.[35] High-resolution manometry (HRM) can detect disorders of the esophageal peristalsis and is feasible also in small children.

1.3.3. Eosinophilic Esophagitis

Eosinophilic esophagitis (EoE) has a significantly altered prevalence rate compared to a normal population (up to 17% vs. 0.03%).[35] Many different hypotheses were suggested to explain the high prevalence of EoE in EA patients, including GERD related decreased mucosal barrier, esophageal dysmotility, food impaction and long-term PPI therapy.[35]

EoE is a result of non-immunoglobulin E (IgE) mediated antigen-related reactions leading to an eosinophilic inflammation of the esophagus[35]. Symptoms like dysphagia, impaction of food in the esophagus or regurgitation are caused by impaired esophageal wall compliance.[35]

1.3.4. Tracheomalacia

Tracheomalacia is a common concurrent anomaly and may be present in up to 75% of EA patients, but becomes symptomatic in only 10% to 25% of the cases.[25] It is defined as atrophy of the longitudinal elastic fibers of the pars membranacea and incomplete cartilaginous components of the trachea, leading to a tracheal collapse during expiration producing the typical hoarse cough.[37,41] The lack of tracheal stiffness results in an abnormal occlusion of the tracheal lumen, which can be seen in bronchoscopy under spontaneous ventilation. In severe cases these clinical symptoms can progress into acute life-threatening episodes of cyanosis or apnea.[37] Symptoms commonly occur shortly after feeding, because of an increased esophageal size with swallowing and GER, which may lead to a posterior tracheal compression.[41] The affected area of the trachea is always at or just slightly above the entry of the distal TEF.[37]

Tracheomalacia can eventually be seen in pulmonary function testing in older children with a reduced peak expiratory flow, but with a predictive value of 74% this is not a specific finding.[41]

The association between tracheomalacia and EA-TEF is explained by faulty division of the primitive foregut with inclusion of esophageal muscle and squamous epithelium in the membranous part of the trachea.[41] Furthermore, the tracheal compression of the dilated proximal esophageal pouch may lead to an impaired tracheal development in utero.[41]

Most of the EA children with tracheomalacia do not need a surgical intervention, because symptoms remain mild and can resolve spontaneously within 2 years of age.[41] In case of a severe tracheomalacia aortopexy is considered the current best treatment.[41] However, the indication for surgery must be made with caution, since tracheomalacia, recurrent TEF and severe GER can present with similar symptoms and may coexist in one patient.[41] Aortopexy as well as posterior tracheopexy can be performed safely thoracoscopically.[42]

1.3.5. Respiratory Function

Spitz postulated a demonstrable increase of the frequency and the duration of respiratory tract infections in children during the first 3 years of life.[22] The majority of EA patients has respiratory comorbidities and the lung function seems to worsen progressively by age.[43] The birth weight, age at follow-up, and episodes of general anesthesia were identified as risk factors for pulmonary function impairment.[44]

Chronic or recurrent respiratory symptoms can occur in up to 74% of EA patients.[37] These respiratory tract infections can be linked to GER and recurrent micro-aspirations or occur independently as primary respiratory abnormalities.[22] However, several studies have reported that surgical therapy of GERD does not prevent and might even worsen the pulmonal problems.[35] Patients with recurrent respiratory infections may benefit from prophylactic antibiotic therapy after confirmatory sputum cultures.[35]

Hospital admissions for pulmonary problems are reported in almost half of all patients in all age groups, 58% of them are readmitted to the hospital more than once and 11% readmitted more than five times.[45]

Pulmonary lung function test in EA infants, performed soon after the EA repair, showed an increased airway resistance and abnormal patterns of airflow.[46] Respiratory function tests have revealed that up to 90% of EA patients have an impaired ventilation, mainly restrictive but also obstructive ventilation disorders or both.[5] Bronchial hyperresponsiveness can affect up to 78% of patients.[47] High airway obstruction correlated significantly with recurrent pneumonias as well as overall respiratory symptoms. EA patients also showed an impaired respiratory symptoms related quality of life.[5] However, a recently published study investigated the pulmonary function following EA patients, including body plethysmography, dynamic spirometry, impulse oscillometry, and diffusing capacity of the lungs and found no significant correlation to respiratory morbidity.[44]

1.3.6. Performance Capacity

The maximum oxygen uptake ($VO_{2\,max}$) or peak VO_2 is known as one of the most important parameters representing aerobic capacity and thus basic endurance. Furthermore, the performances at the aerobic and anaerobic thresholds also represent significant parameters of the aerobic performance capacity.[48] Several studies investigated the cardiopulmonary performance capacity in patients following repair of EA.[3,7,8,49,50]

However, standardized assessment of cardiopulmonary performance capacity of patients is rare and many studies are based on small case numbers, inconclusive stress tests, and yield divergent results **(Table 2).** While some authors found no differences in cardiopulmonary adaption to exercise with a normal $VO_{2\,max}$[4,6], others reported a significant earlier exhaustion with a correlation to reduced lung function in EA patients.[7]

Author	Year	n	Age	Test	Results	p-value
Zaccara et al.[49]	1995	8	7 -14	Bruce - treadmill	Significantly lower exercise duration	**<0.01**
					No differences in mean $VO_{2\ max}$ at rest and on exertion	
					Significant different VO_2/kg at the end of exercise	**0.005**
					Mean Minute Ventilation (V_E) did not differ	
Gischler et al.[3]	2008	22	5	Bruce - treadmill	Maximal exercise tolerance significantly below the norm	**0.02**
Cammen-van Zijp et al.[50]	2010	29	5	Bruce - treadmill	Significantly reduced mean SDS endurance time	**0.001**
Peetsold et al.[6]	2011	25	11 -16	Bruce - treadmill	No significant differences in z-score VO_{2max} and $VO_{2\ max}$/kg	0.05 / 0.96
Dittrich et al.[7]	2017	22	5 - 20	Bruce - treadmill	Significant earlier exhaustion	**<0.01**
Toussaint-Duyster et al.[8]	2016	63	8	Bruce - treadmill	Exercise capacity significantly below normal	**<0.001**
Montgomery et al.[51]	1995	18	8 - 21	Bicycle ergometer	No difference in maximal working capacity between children with minor and severe respiratory complications	
Beucher et al.[4]	2013	31	6.5 - 12.5	Bicycle ergometer	Normal $VO_{2\ max}$ in all cases except one, who did not reach maximal effort	0.5
Olbers et al.[52]	2015	24	7	Bicycle ergometer	21% did not reach the limit of 2.5 W/kg	

Table 2: Literature review of cardiopulmonary exercise performance tests in EA patients.

The largest study cohort was published by Toussaint-Duyster at al. 2016, who included 63 EA patients and evaluated their cardiopulmonary performance capacity 8 years following surgical EA repair; the exercise capacity was significantly below normal and was positively associated with total lung capacity.[8]

1.4. Microbiology

Microbiological studies of esophageal atresia are rare and were already published more than 30 years ago.[53,54] The bacterial flora of the upper esophageal pouch before anastomosis showed no isolated organisms in 55% and normal oropharyngeal organisms in 45%.[54] The rate of intestinal colonization in infants with esophageal atresia is prolonged, especially anaerobic colonization was delayed and the onset of intestinal colonization seems to be related to the beginning of enteral feeding.[53] However, investigations of bacterial species including microbiome analysis based on sequencing of 16S ribosomal ribonucleic acid (rRNA) gene are lacking.

1.5. Etiology

The etiology of EA remains unclear and its pathogenesis seems to be heterogeneous and multifactorial with complex signaling pathways.[25] Esophageal defects are commonly associated with concurrent defects of the trachea and lung because esophageal epithelium as well as mucosal glands of the esophagus arise from the same component of the foregut that will also give rise to the respiratory cells.[55]

The early embryo consists of three different layers, known as outer ectoderm, middle mesoderm, and an inner layer endoderm.[55] The development of the gut starts in gestational week four out of the endoderm, which is divided into foregut, midgut and hindgut.[55] These steps are associated with numerous cellular expressions of different genes, including *Foxa 1, Foxa 2, Gata 4* and *Gata 6*.[29]

The esophageal organogenesis begins with the differentiation of the foregut cells into the trachea, lung, and esophagus triggered by the cellular expression of the *Nkx2-1 gene* with Bone Morphogenetic Proteins (BMP) signaling in the ventral foregut wall and *Nkx2-1* as a specific marker of respiratory tract cells; the differentiation of the dorsal foregut toward esophageal tissue begins with the expression of Sox2 in the endodermal cells.[55] BMP mediates further differentiation of the respiratory system from the foregut and gets antagonized by Noggin activity.[55] Disruption of the notochord, producing Noggin, resulted in tracheal-esophageal

fistulas and esophageal atresia in mouse models.[55] Following separation of lateral mesoderm and the ventral foregut, both structures experience a rapid elongation triggered by Wnt signaling.[55] Deletion of wnt5a has shown truncated esophagus development in mice.[55]

Hedgehog signaling, especially Sonic hedgehog (Shh), is another key player in the esophagus and tracheal organogenesis, thus its aberrant activation correlates with numerous developmental disorders.[35,55]

The longitudinal muscular layers are formed in week six, followed by immigration of the first ganglion cells of the myenteric plexus and proliferation of mesodermal cells into the submucosal layer to build the later blood supply to the esophagus in week seven.[55] The upper esophageal sphincter is derived from mesenchymal tissue, the lower esophageal sphincter derives from mesenchymal cells; the muscular layers are completed in week nine.[55]

Three different possible explanations were published to elucidate outstanding questions of foregut morphogenesis: the septation model suggests a septum formation that divides the foregut into trachea and esophagus; the watershed model describes that growing foregut tissue becomes either trachea or esophagus and the outgrowth model postulates that the trachea develops by an outgrowth process while the remaining foregut creates the esophagus.[35]

Adriamycin, a cytostatic drug that induces EA and VACTERL association in mice, is frequently used to investigate the esophageal organogenesis: Adriamycin-treated mouse embryos showed a failure of tracheo-esophageal separation, despite a normal foregut lengthening, indicating a primary role for the septation model.[56]

1.6. Prenatal Diagnosis

Polyhydramnios can be the first sign in obstetrical ultrasound in case of a fetus with an esophageal atresia and may result from the inability of the embryo to swallow and absorb the amnion fluid.[29] It occurs in 33% of EA with distal TEF and in approximately 100% in EA without fistula.[29] Furthermore, small or absent stomach bubble can be related to esophageal atresia, but both signs have high false-positive rates.[29] Additional findings may be generated by other abnormalities like VACTERL

or CHARGE association. There should be a high suspicion of esophageal atresia in case of a small stomach bubble associated with a direct visualization of the fluid-filled blind-ending esophagus during fetal swallowing, which is called the “upper neck pouch sign”.[57]

The prenatal detection rates in sonography are approximately 45%.[29] Fetal Magnetic Resonance Imaging (MRI) may be a useful diagnostic addition in suspicious ultrasound findings, but reported results are varying and can also have a high number of false-positive diagnosis.[29]

1.7. Postnatal Diagnosis and Management

Most newborns become symptomatic in the first hours of life by distinct salivation and coughing or choking following the first feeding attempts. The clinical diagnosis can be made by the inability to pass a nasogastric tube into the stomach. In the consecutive radiograph a curled tube in the proximal esophageal pouch and air-free abdominal cavity in patients with pure EA can be seen. In case of a proximal TEF respiratory symptoms can be severe, including life-threatening episodes of cyanosis or apnea. A nasogastric tube can eventually be passed through the fistula, but will never be seen below the diaphragm radiographically and will result in severe respiratory compromise.[29] Usually minor symptoms will occur in infants with distal TEF and abdominal gas can be seen in the radiograph **(Figure 1)**. However, severe respiratory compromise can occur by reflux of gastric fluid through the fistula into the airways and the distended abdominal cavity can even worsen the pulmonary function.[29]

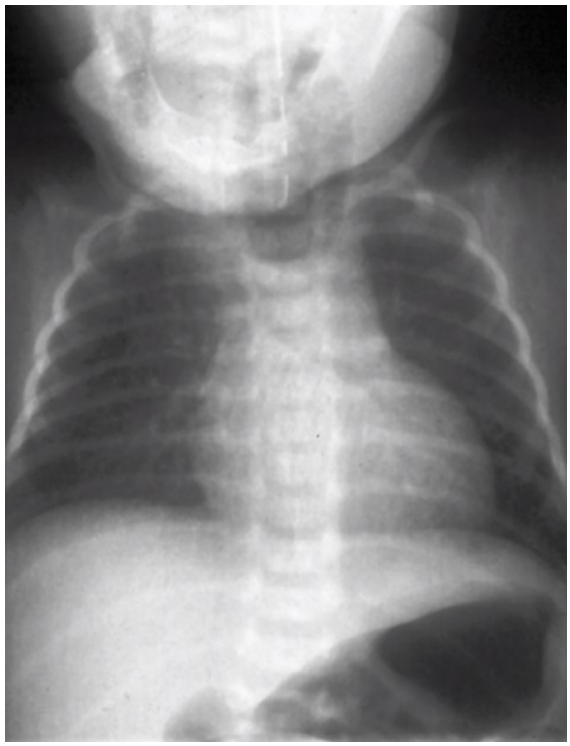

Figure 1: Prediction of gap length by plain chest radiograph with nasogastric tube in the upper esophagus in relation to the carina. Distal TEF is confirmed by presence of abdominal gas. Left sided aortic arch is another important preoperative information for the pediatric surgeon.

In case of an H - type fistula the severity of respiratory symptoms depends on the size of the fistula.[29] Small fistulas can have a prolonged history of recurrent respiratory infection prior to the diagnosis.

Presence of esophageal atresia requires a meticulous diagnostic evaluation to exclude additional anomalies, which includes an echocardiography, sonography of the kidneys and urinary tract, radiographs of the spine and limbs and genetic tests; in addition, echocardiography can exclude a right-sided aortic arch, which would change the surgical approach.[29]

To limit the risk of further aspirations, a special probe with permanent suction, so called Replogle tube, is inserted in the upper pouch and left there until EA repair.

1.8. Classification

The history of esophageal atresia classification started 1929, when E.C. Vogt, a radiologist, classified 4 different types of esophageal atresia **(Figure 2)**.[58] Type I describes an absent esophagus, Type II an esophageal atresia without a TEF, Type IIIa an esophageal atresia with a proximal TEF, Type IIIb an esophageal atresia with a distal TEF, Type IIIc an esophageal atresia with a proximal and distal TEF. Type IV is considered as a tracheoesophageal fistula between an intact esophagus and the trachea.

The Vogt classification is still frequently used in Central-Europe, while the Gross classification seems to be more internationally used and refers to Gross, who altered a numeric form of classification from Ladd to an alphabetic system in 1953 (Figure 2).[28,59]

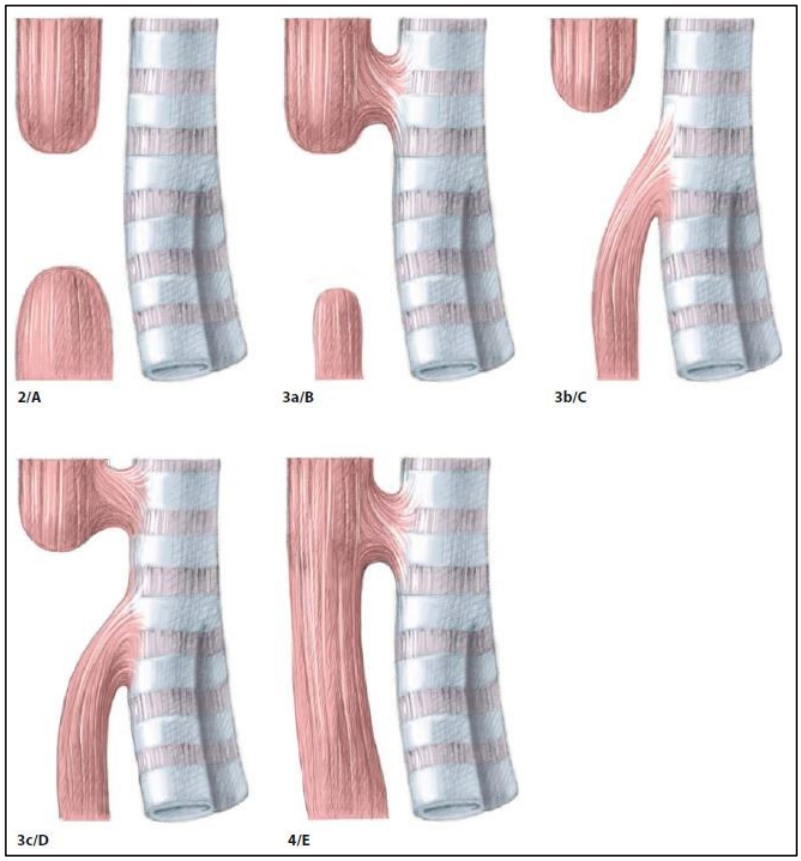

Figure 2: Classification of Esophageal Atresia (left…Vogt classification / right…Gross classification; reproduced from Puri P, Höllwarth ME. Pediatric Surgery. Second Edition. Berlin Heidelberg: Springer Verlag; 2019 with permission of publisher, Springer Nature).[2]

In 1962, Waterston et al. developed a risk stratification in infants with EA.[60] This classification system allows identification of risk factors influencing the survival rate and the surgical treatment. The Waterston Risk Groups are based on birth weight and associated noncardiac and cardiac anomalies and offer the possibility to compare outcomes and survival rates **(Table 3).**

Group	Survival (%)	Waterston risk classification
A	99	Birth weight > 2,500g and otherwise healthy
B	93	Birth weight 2,500-2,000g and well or higher weight with moderate associated anomalies
C	71	Birth weight < 2,000g or severe associated cardiac defects

Table 3: The Waterston risk classification as a predictor of survival in patients with EA.[25,60,61]

An alternative risk categorization was proposed by Spitz et al, who defined two criteria as the most important predictors of outcome: birth weight below 1,500g and presence of a complex congenital cardiac anomaly **(Table 4).**[61] Major cardiac anomalies were defined as cyanotic congenital heart disease requiring palliative or corrective surgery or non-cyanotic congenital heart disease requiring medical or surgical treatment for cardiac failure.[61]

Group	Spitz classification	Survival (%)
I.	Birth weight > 1,500g without major congenital heart disease	98
II.	Birth weight <1,500g or major congenital heart disease	59
III.	Birth weight > 1,500g and major congenital heart disease	50

Table 4: The Spitz classification of esophageal atresia with recent survival rates (2006).[22,25,61]

1.9. Surgical Approach

The surgical repair of EA requires general anesthesia with endotracheal intubation and a broad-spectrum intravenous antibiotic prophylaxis is given preoperatively. The endotracheal tube should be placed close to the tracheal bifurcation to avoid an over-inflation of the fistula. The Replogle probe is left in the upper pouch to facilitate its intraoperative identification.

Preoperative tracheo-bronchoscopy is often required to confirm the diagnosis and to exclude a proximal TEF and tracheomalacia **(Figure 3)**.[29] During bronchoscopy the distal TEF can be splinted with a 3 French ureteric catheter passed through the bronchoscope to facilitate the consecutive surgical repair. Routine tracheoscopy to exclude a proximal TEF is usually done in infants with a weight of more than 1,500 grams, because the size of the endoscope is limited. The distal TEF is usually located slightly above the carina or at the carina, indicating a short gap EA; the dorsal membranous part of the trachea must be inspected carefully up to the cricoid cartilage, since small proximal TEF can be easily missed.[2]

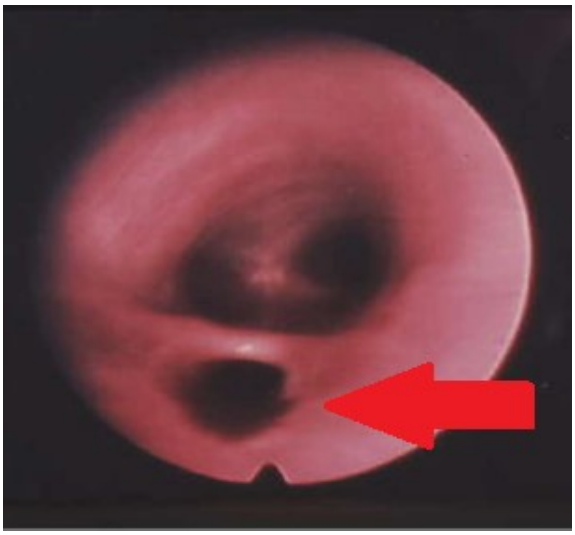

Figure 3: Tracheo-bronchoscopic inspection, localization of the fistula and exclusion of proximal TEF.

1.9.1. Short Gap Esophageal Atresia

A right dorso-lateral thoracotomy is the standard approach for EA repair, except in case of a right-sided aortic arch. This approach requires a left-sided position of the infant with an over-head suspension of the right arm **(Figure 4)**.

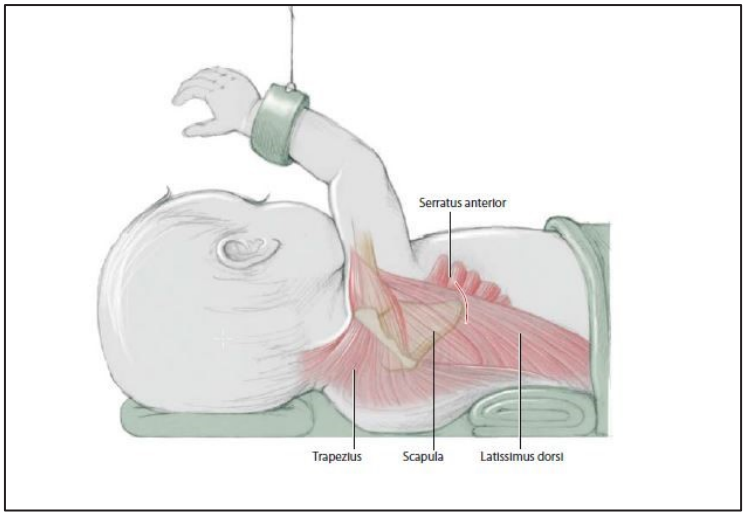

Figure 4: Patient positioning and standard surgical approach for EA repair (reproduced from Puri P, Höllwarth ME. Pediatric Surgery. Second Edition. Berlin Heidelberg: Springer Verlag; 2019 with permission of publisher, Springer Nature).[2]

Care must be taken to avoid tension or traction on the arm which can result in injuries of the brachial plexus.[2] A skin incision is placed 1cm below the tip of the scapula to the midaxillary line, a vertical incision in the midaxillary line can be used

alternatively for cosmetic reasons.[2] Following the incision of the anterior thoracic fascia and retraction of the latissimus dorsi muscle, the serratus anterior muscle is mobilized; the thoracodorsal nerve which runs on the deep surface of the latissimus muscle should be spared.[2] The intercostal incision is placed at the upper border of the 5th rib. For an extrapleural approach the exposed pleura is pushed off the thoracic wall towards the dorsal mediastinum. While preparing to the dorsal mediastinum, the azygos vein appears as a lead structure **(Figure 5)**. The azygos vein can be mobilized and divided; however, some surgeons prefer its preservation. The vagus nerve must be identified, running from the lateral border of the upper pouch to the trachea-esophageal fistula towards the lower esophagus and all vagal fibres supplying the distal esophagus must be preserved.[2] Meticulous handling of the thin and hypoplastic distal esophagus is obligatory.

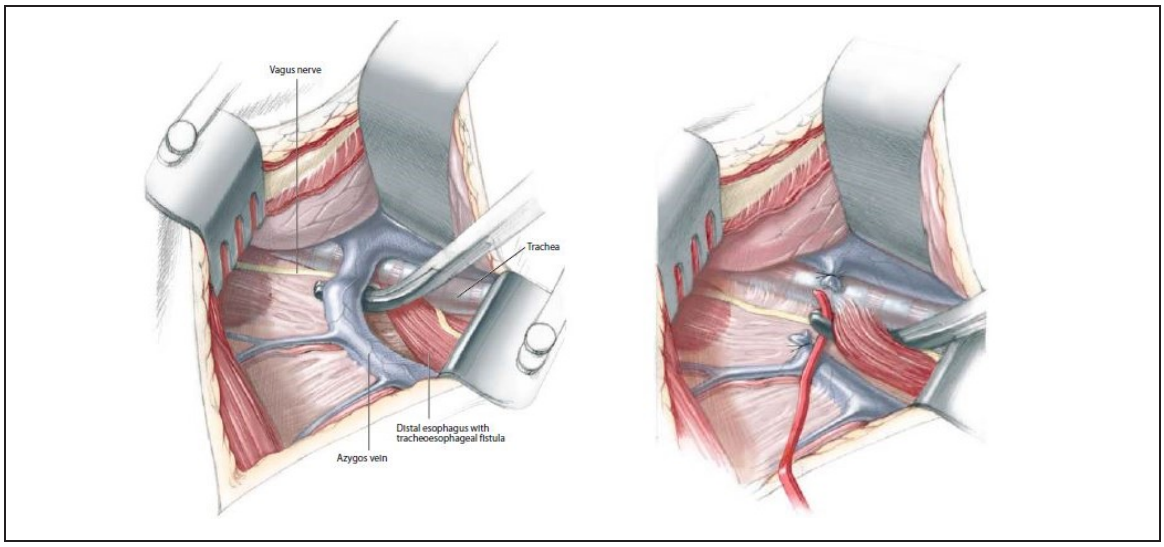

Figure 5: The azygos vein can be dissected or preserved during EA repair (reproduced from Puri P, Höllwarth ME. Pediatric Surgery. Second Edition. Berlin Heidelberg: Springer Verlag; 2019 with permission of publisher, Springer Nature).[2]

After placing two traction sutures, the distal fistula is carefully divided and closed with a continuous, absorbable, monofilament, 6.0 suture close to the trachea but without tension or traction which could narrow the airway **(Figure 6)**.[2] Preparation of the proximal pouch can be challenging due to its high retraction into the neck and the firm connective tissue to the trachea.

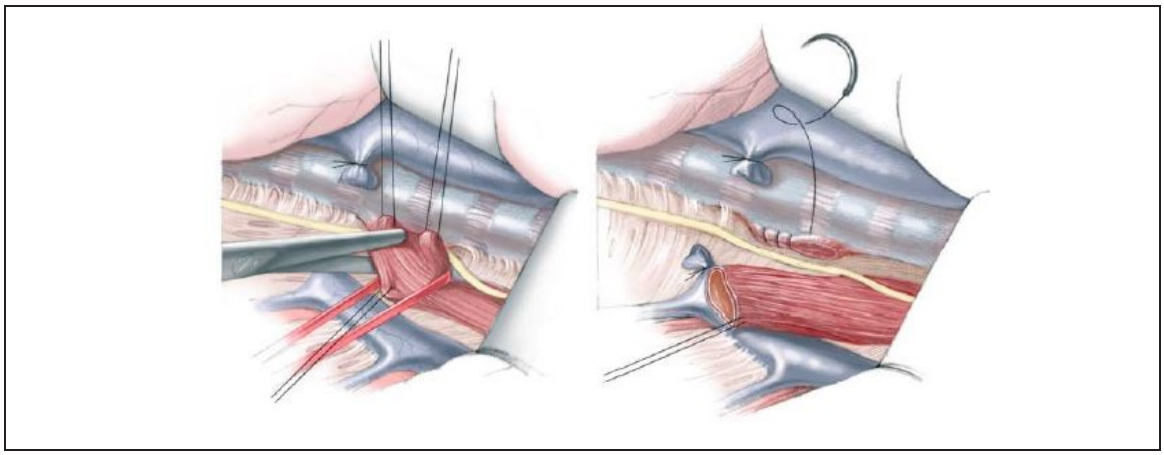

Figure 6: Division of the distal TEF and closure the tracheal opening (reproduced from Puri P, Höllwarth ME. Pediatric Surgery. Second Edition. Berlin Heidelberg: Springer Verlag; 2019 with permission of publisher, Springer Nature).[2]

The upper pouch is opened and an end-to-end anastomosis is applied with interrupted absorbable 6.0 sutures. Extreme care must be taken to get sufficient tissue of the inner mucosal layer with every stitch **(Figure 7)**. Before completing the anterior aspect of the anastomosis, a 5 French silastic feeding tube is passed into the stomach, serving as a trans-anastomotic splint, feeding tube and gastrointestinal decompression probe.[2] In the end, a pleural drain is inserted through a separate intercostal incision and placed near the anastomosis; the ribs are then approximated with some pericostal stiches.[2] The thoracic muscles are sutured to their fascial insertion and the subcutis and skin are closed with absorbable sutures.

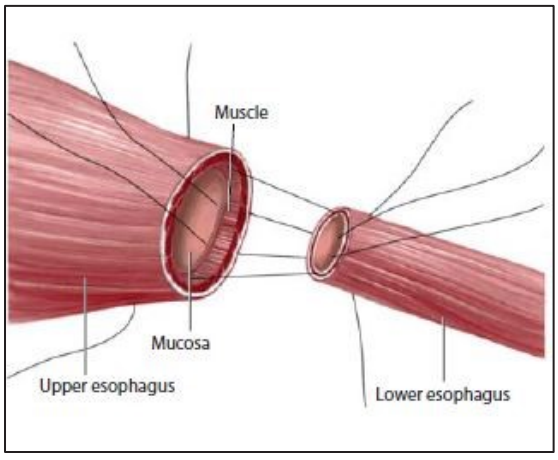

Figure 7: End-to-end anastomosis of the two esophageal openings; sufficient tissue of the mucosa must be taken with every stitch (reproduced from Puri P, Höllwarth ME. Pediatric Surgery. Second Edition. Berlin Heidelberg: Springer Verlag; 2019 with permission of publisher, Springer Nature).[2]

Disadvantages of thoracotomy are frequently recognized following open EA repair, such as chest wall asymmetry, rib fusion, maldevelopment of thoracic muscles, scoliosis or winged scapula; furthermore, chronic thoracic pain is reported in up to 50% of patients.[62] Thoracoscopic transpleural EA repair is therefore seen as an alternative to the conventional approach and has the great advantages of sparing the wall of the thoracic cavity and a more detailed view of the anatomy.

Thoracoscopic EA surgical repair is feasible without any absolute contraindications, but requires experienced anesthetic care with decreased ventilatory pressure and increased frequency ventilation.[63] A pneumothorax is insufflated with a standard pressure of 5 mmHg and a flow of 0.1 l/min. In term infants a 5 mm 30° telescope and 3 mm instruments are used **(Figure 8)**. It is essential that the procedure is only started, if the infant achieves a stable condition. The surgical steps are the same as described above.

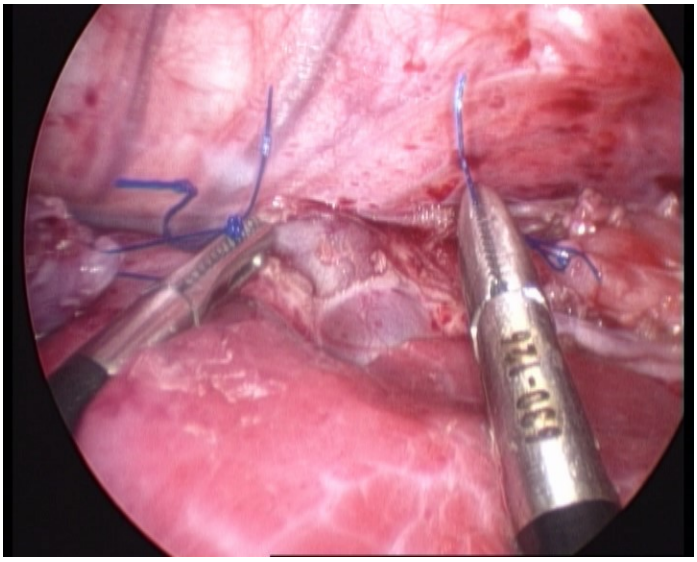

Figure 8: Thoracoscopic repair of EA-TEF.

1.9.2. Long Gap Esophageal Atresia

The definition of long gap esophageal atresia (LGEA) varies in the literature and ranges from the inability to achieve a primary anastomosis to a measured gap-length of not less than two centimeters or covering not less than two thoracic vertebras.[39] An initial gastrostomy is essential in LGEA and for surgical repair there are two different strategies: preserving the native esophagus with elongation methods or esophageal replacement.[2] Delayed repair in LGEA can be possible over

time, especially the upper pouch shows spontaneous growth in 8 to 12 weeks.[2] Furthermore, the elongation can be promoted by regular longitudinal bougienage under fluoroscopic control **(Figure 9)**. This longitudinal stretching is performed twice a day and requires a mild sedation. Surgical repair can be planned if the two segments are slightly overlapping, so that a tension-free anastomosis can be achieved.

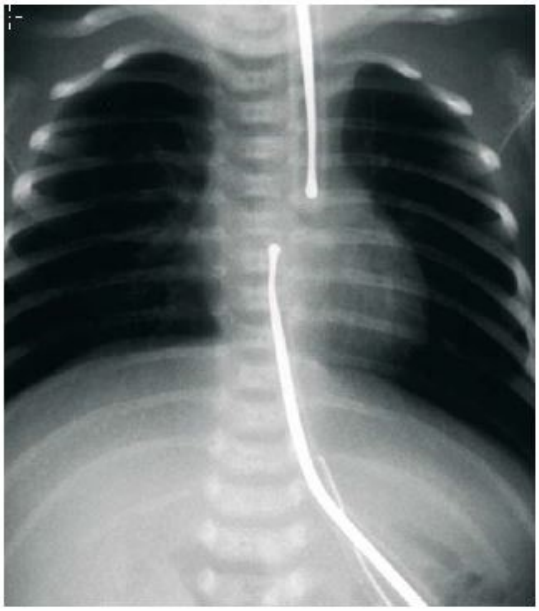

Figure 9: Longitudinal bougienage under fluoroscopic control in case of long gap EA (reproduced from Puri P, Höllwarth ME. Pediatric Surgery. Second Edition. Berlin Heidelberg: Springer Verlag; 2019 with permission of publisher, Springer Nature).[2]

1.10. Postoperative Complications

Stadil et al. reported major complications in newborns with long-gap EA within the first year of life in a systematic literature review and found anastomotic stricture (53.7%), GER (32.2%) and anastomotic leak (22.7%) as most common complications.[39] Another recent retrospective nationwide database analysis with 3,157 EA patients revealed that 10% of all infants operated on EA had to be readmitted within 30 days and 26% within the first year of life, due to GER (54%), infections (42%), failure to thrive (17%), tracheomalacia (14%) or esophageal stricture (10%).[32]

1.10.1. Anastomotic Insufficiency

The rate of anastomotic leakage varies between 14% to 32.4%, but most of the cases can be managed conservatively with adequate drainage and nutritional support **(Figure 10).**[25,39] A recently published literature review reported a frequency of reoperations due to leakage of 2.5%.[39] Even in case of leakage following transthoracic repair sufficient drainage can usually be achieved and allows spontaneous closure.[25] However, anastomotic leaks often result in stricture formation and can also be followed by recurrent TEF.[25]

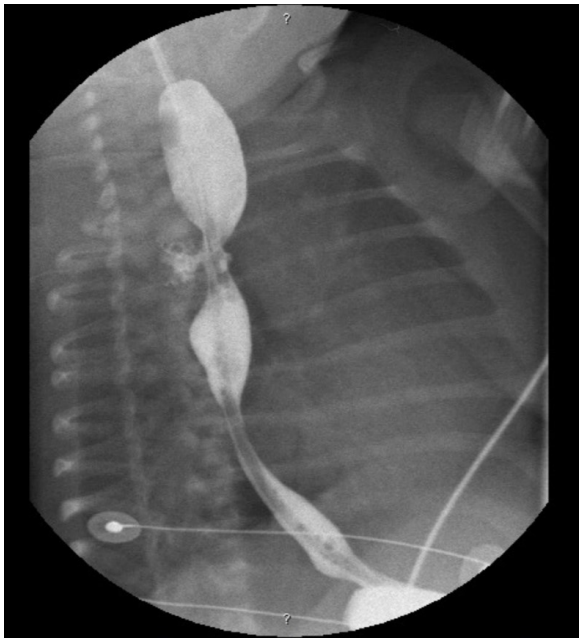

Figure 10: Postoperative fluoroscopy with anastomotic leakage.

Complete breakdown of the anastomosis is rare, often presents with a tension pneumothorax and requires adequate drainage and in some cases a cervical esophagostomy and delayed esophageal replacement.[22,25]

1.10.2. Anastomotic Strictures

Anastomotic strictures occur in 30% to 80% of patients with long-gap EA with delayed primary repair **(Figure 11)**.[25,37,39] A significant association between recurrent stricture formation and anastomotic leak as well as GER is reported in the recent literature.[64] Furthermore, poor surgical technique, anastomotic tension and ischemia at the ends of the esophagus are mentioned as additional factors.[22,25] Spitz

mentions a meticulous surgical technique with including the mucosa in every suture of the anastomosis as crucial step in stricture prevention.[22]

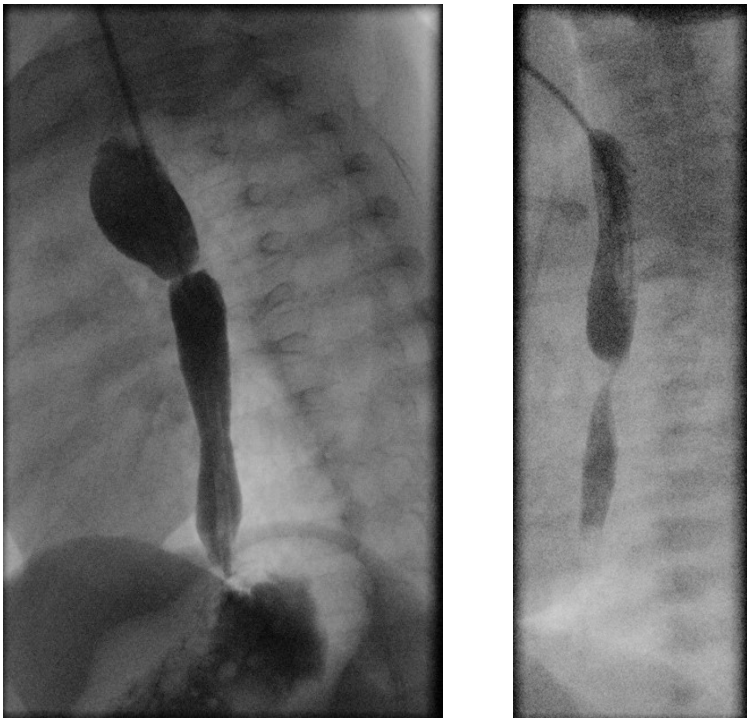

Figure 11: Radiographic diagnosis of esophageal stenosis following EA repair

Most strictures respond well to anastomotic dilatations but the timing and the number of dilatations remain unclear. Esophageal strictures can be dilated either endoscopically with balloon or with longitudinal bougienage. If the stricture does not respond to repeated esophageal dilations, intralesional steroid injection, mitomycin C, esophageal stent placement, and endoscopic incisional therapy are common alternatives.[65] In this case it is crucial to exclude gastroesophageal reflux.

1.10.3. Recurrent Tracheoesophageal Fistula

Recurrent TEFs occur in 3% to 14% following TEF closure and are related to anastomotic insufficiency or local erosion and inflammation.[25] There are some techniques described to reduce the risk of recurrent TEF, like pleural or pericardial flap, with vague medical evidence. The majority of patients with recurrent TEF presents with respiratory symptoms and recurrent pulmonary infections at a mean period of 20 weeks following surgical repair.[22]

Gold-standard diagnostic approaches for recurrent fistula are tube cine-esophagogram and bronchoscopic cannulation of the fistula, since up to 50% of recurrent fistulas can be missed in routine contrast swallow studies.[22,25]

The classic open surgical repair is associated with a significant mortality, morbidity and high rates of recurrence.[66,67] Postoperative complications following repair of recurrent TEF occur in 40% and result in a mortality rate of 8.6%.[67] Thus, active observation as well as repeated endoscopic procedures can be safe alternatives to the open approach.[68]

1.11. Microbiome

The term microbiome was first proposed by Joshua Lederberg in 2001, describing the ecological community of commensal, symbiotic, and pathogenic microorganisms that literally share our body space.[69] In 2007, the Human Microbiome Project (HMP) was launched by the National Institutes of Health with sequencing of 11,000 primary specimens from 18 body sites in 300 healthy individuals, but the lung was initially not included.[70] In a second part, the HMP was expanded to analyze host and microbiome relationship in three longitudinal cohort studies of representative microbiome-associated conditions: pregnancy and preterm birth, inflammatory bowel diseases and prediabetes.[71]

However, there is a huge variability in the determination of gut microbiome composition, which can lead to different results depending on study design and sample collection. Thus, the *International Human Microbiome Standards Project*, was introduced to standardize general practices in sampling, processing and bioinformatical methods.[72]

The composition of microorganisms changes over time, the diversity of the microbiome can be linked to important health parameters and refers to specific bacterial colonization as predisposing disease factor.[13] Early colonization of the intestinal and pulmonary microbiome seems to have a substantial role for the future respiratory health and disease.[13,15] Recent scientific work has identified associations between changes of the microbiome and several medical conditions ranging from autism to cancer.[71] Furthermore, the microbiome can affect the

efficacy of medical therapy or survival during graft-versus-host disease.[71] However, microbiome analysis shows a broad spectrum of interpersonal diversity even in the absence of disease, which complicates the identification of a disease related microbial dysbiosis.[71]

1.12. General Terminology

Any microscopic life form, including bacteria, fungi, protozoa or viruses, is called microbe. The microbiota is defined as the microbial community membership in a given population, such as the human body and the microbiome describes the genetic information of the gene products of a microbiota.[15,72]

Phylogeny is the study of evolutionary relationships among individuals or groups of organisms evaluated by deoxyribonucleic acid (DNA) sequences. Phylogenetic analyses are of central importance for understanding the biodiversity and genomes. Taxonomy describes the identification and classification of different organisms and 16S rRNA is still the standard for taxonomic identification. Organisms can be classified by different taxonomic levels, from genus (low taxonomic level) to phylum (high taxonomic level).[72] The three domains of Bacteria, the Archaea and the Eucarya can be divided into kingdoms, phyla, classes, orders, families, genera, species and subspecies **(Figure 12)**.

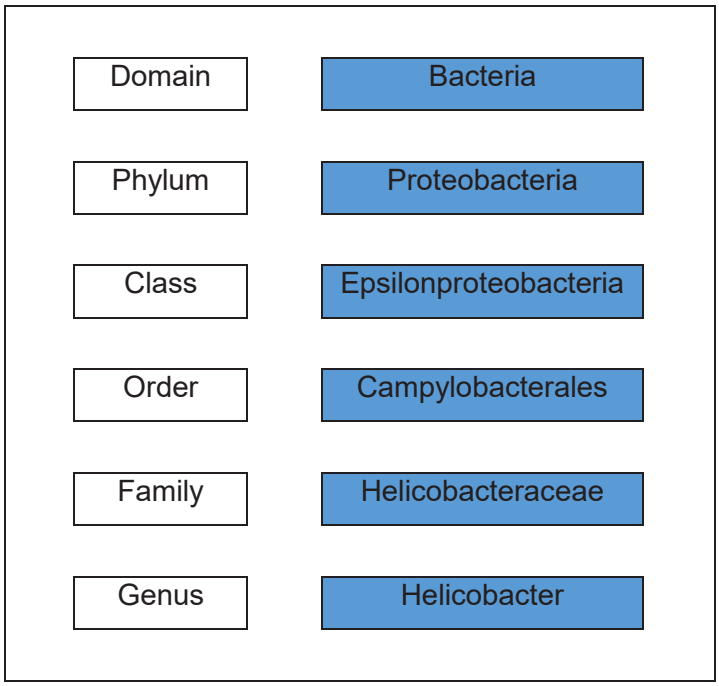

Figure 12: Taxonomic classification of Helicobacter.[73]

Dysbiosis is defined as a change in the microbiota composition that is associated with a disturbance in local ecological conditions and is generally associated with impaired interactions between the host and the microbe.[72]

The DNA amplification method of polymerase chain reaction (PCR) in molecular biology describes a laboratory technique leading to multiple copies of a chromosomal region within a chromosome arm. Metagenomics or shotgun random sequencing is a specific method for DNA sequencing in which the DNA is fragmented into segments and identified by using sequence databases.[72]

1.13. 16S rRNA Gene Sequencing

The 16S rRNA is part of the small 30S subunit of prokaryotic ribosomes and is used in the reconstruction of phylogenies because the extremely slow rate of evolution of this gene and the presence of both variable and constant regions make amplification possible.[72] Several variable segments of the 16S rRNA gene can be used for identification of organisms and its sequencing is inexpensive and fast.[72] This gene has 9 constant regions and 9 hypervariable regions, which enable a sequence-specific characterization of different bacteria.[74] Out of the nine hypervariable regions in the 16S rRNA gene the V1-V3 and the V3-V5 regions are

commonly used for discrimination of bacterial diversity, but these two regions are known to give also different and inconsistent results.[15] Diversity between different bacterial species can be shown in DNA sequences of the hypervariable regions of the 16S rRNA gene; the 16S rRNA gene analysis is an established amplicon sequencing method commonly used to identify and compare bacteria present in a particular sample.[72] Recent public 16S rRNA gene databases, including *Greengenes, Silva* and *EzBioCloud*, provide taxonomy for more than 1,700,000 bacteria and archaea.[75]

Nevertheless, the 16S rRNA gene sequencing has also some limitations. Inappropriate inclusion of too many analyses can lead to false positive significance results. Furthermore, variability in sampling methods, patient diversity, amplification techniques or DNA extraction procedures can be an important study bias.[72] Processing variability can occur as species bias due to different wall composition and limit the thresholds for abundance.[72] The batch effect describes the bias, that in a single batch some samples can be incomplete and the adapter addition can influence the sequencing results.[72] Finally, species-level identification may not be feasible due to unresolved information for some gene sequences and the differentiation of homogeneous species can result in different database records.[72]

1.14. Operational Taxonomic Units (OTUs)

Different clusters of microorganisms, assigned by DNA sequence similarity in 16S rRNA are summarized as operational taxonomic units (OTU). With implementation of OTUs, microbial cohorts can be assigned in richness, evenness, dominance and the diversity index.[15] OTUs define clusters of microorganisms grouped by DNA sequence similarity of at least 97% at the species level or 94% at the genus level with the reference database.[72]

1.15. Diversity Indices

The quantitative identification of microorganisms can be done by using diversity indices. The diversity describes the distribution of specific OTUs in one sample.

Alpha diversity is high in areas like the gut with lots of different microbes. The alpha diversity is analyzed to assess differences between organism according to its richness and evenness, or both; the richness refers to the number of different taxonomic groups and the evenness measures the distribution of abundances of different OTUs in the group.[72] The dominance describes the emergence of a single OTU.[15] Alpha diversity can only be compared between different groups with nearly identical samples sizes, since diversity increases with an increase of sample size number. There are several different diversity indices, the most widely used are the Shannon-Wiener index or the inverse-Simpson index. Species richness can be estimated with richness estimators like Chao-1.

The beta diversity defines the diversity between the habitat or inter community.[72] Beta diversity is low if there is high conformity between two samples; thus, the highest amount of beta diversity is reached when two communities have almost no identical species. Beta diversity can be estimated with the Bray Curtis dissimilarity score.

The abundance or ubiquity describes the frequency of an OTU compared to other OTUs in the originating population and is measured as a percentage of the total number of OTUs.[72]

1.16. The Airway Microbiome

Standard microbiological culture techniques of healthy lungs revealed negative results. Thus, the lungs were considered as sterile in the last decades. With the introduction of culture-independent molecular techniques several microbiological organisms could be identified, including bacteria, fungi and viruses.[72] The optimal microbiome composition is considered to be most likely found in term-born, vaginally delivered, breast fed infants. Nevertheless, comparable, longitudinal data of these patients are lacking.[13] The microbial colonization of the airways seems to begin very early, eventually even before delivery, but seems to be highly dynamic with a time period to establish a stable pulmonary microbiome throughout the first three years of life.[11,13] Recent scientific work suggests that initially a dominant organism is present as pioneer colonizer, depending on the initial exposure of the infant.[13] Overlapping core pulmonary microbiota in health and early

disease suggests that the pediatric respiratory microbiome can change over time with certain diseases.[14]

The airway microbiome in a healthy population shows marked variety between individuals and is dominated by Firmicutes, Bacteriodetes (around 80%) and Proteobacteria (around 10%) at the phylum level and *Prevotella, Veillonella* and *Streptococcus* at the genus level.[13,72,74] *Pseudomonas, Haemophilus* and *Neisseria* are the most common species within Proteobacteria.[13] In healthy lungs, the pulmonary microbiome is determined by the balance of three factors: **microbial immigration** through micro-aspiration, inhalation of bacteria or direct mucosal dispersion, **microbial elimination** through cough, muco-ciliary clearance or host defense mechanisms and the **relative reproduction rates** of the members.[11,72] Gastroesophageal reflux and esophageal dysmotility can accelerate the microbial immigration and on the other hand chronic pulmonary diseases like cystic fibrosis (CF) or bronchiectasis can impair the muco-ciliary clearance capacity and decrease the rate of microbial elimination.[11]

In chronic pulmonal disease regional growth conditions are the most important determinants of microbiome composition, including pH, temperature, oxygen tension, local microbial competition, nutrient availability, host epithelial cell interactions and activation or concentration of inflammatory cells.[72] A significantly increased bacterial burden was observed in patients with severe destructive pulmonary disease such as pulmonary fibrosis or emphysema.[11] The pulmonary microbiota in patients with asthma shows a high bacterial burden and diversity, which is in contrast to findings of the intestinal microbiome, where chronic inflammatory diseases are related with depletion of bacterial diversity.[18] On the other hand, decreased microbial diversity and richness was found to be associated with a poor pulmonary function in chronic airway diseases.[17]

The relationship between the lung microbiome and developing pulmonary disorders remains unclear and the identification of microbiological organisms as prognostic markers in different chronic inflammatory disorders remains a future perspective.[72] Early pulmonary colonization in infancy with Streptococcus was found to be a risk factor for later allergic sensitization and asthma.[18] Airway colonization with *Streptococcus pneumoniae, Haemophilus influenzae* or *Moraxella catarrhalis*

leads to a significantly higher risk for respiratory infections in 3-years old children.[13] In contrast to these *risk microbiota* some authors also proposed *resilience microbiota*, which may decrease the incidence and severity of acute respiratory infections in children, such as a high abundance of *Lactobacillaceae*.[10] The genus *Lactobacillus* was also found to be less abundant in the early microbiome of infants with extreme low birthweight (ELBW) who later developed a bronchopulmonary dysplasia.[18]

Recent literature provides microbiome composition data for chronic pulmonary diseases, including chronic obstructive pulmonary disease (COPD), cystic fibrosis, bronchiectasis and interstitial lung disease, but the distribution of these changes remains unclear.[72,76] Dickson et al. have described a *dysbiosis-inflammation cycle* as bidirectional model of the relationship between an altered pulmonary microbiome and the host response: pulmonary inflammation leads to an alteration of the microbiome growth condition as well as increased mucus production and pulmonal wall permeability.[11] An altered pulmonary microbiome compared to healthy controls was found in every lung disease.[11] A significantly different pulmonary microbiome, with a severity-related loss of diversity in patients with COPD was demonstrated in several studies, with Proteobacteria, Bacteroidetes, Actinobacteria and Firmicutes as common phyla and *Pseudomonas, Streptococcus, Prevotella* and *Haemophilus* as common genera.[11,72,76]

There are concerns that lower airway samples may be contaminated by the oropharyngeal microorganisms. However, the microbiome of the bronchial tree and the alveolar surface seem to be similar but distinct to the upper respiratory tract.[11,72] Different microbial diversities can result from different sampling methods; thus, adequate sample procedures should be considered to answer a specific question. Sampling from the distal airways can be achieved by bronchoalveolar lavage or induced sputum; however, in both methods contamination of the upper airways occurs and a pure lower airway microbiome is difficult to achieve.[14] In addition, potential contamination of laboratory reagents can significantly influence the determination of microbial composition, particularly in pulmonary microbiome studies, where the biomass of lung samples is low compared to other body regions.[11,15]

The implementation of the pulmonary microbiome for identification of pathomechanisms of acute and chronic lung diseases and potential therapeutic approaches must be future issues.

1.17. Gene Sequencing in Rare Pediatric Diseases

Current microbiome research in special pediatric diseases is limited but next generation sequencing techniques seem to be highly relevant for neonatal diseases, because the development of the gut microbiome already starts in the perinatal period.

The pulmonary microbiome seems to be associated with the development of bronchopulmonary dysplasia (BPD) in infants with ELBW. The airway microbiome at birth of ELBW and term infants is similar at birth, but different and less diverse in infants who later develop BPD; these infants show a consistent temporal dysbiosis.[18] Especially the presence of *Ureaplasma spp*. in the pulmonary microbiome may increase the risk for BPD.[17]

Van der Gast et al. compared three cohorts of symptomatic children with clinically distinct airway diseases, such as cystic fibrosis, bronchiectasis and protracted bacterial bronchitis and found a common early core microbiota; however, the core microbiota was different in adults with the same disease.[77] Airway microbiome studies in patients with asthma showed an increased bacterial burden and diversity, suggesting an association between pulmonary microbiome and airway inflammation, disease control or even susceptibility to exacerbations.[14]

With next generation sequencing techniques, rare pediatric diseases can be researched and change current surgical concepts. For example, the indication and timing of early resection of a congenital pulmonary airway malformation (CPAM) remains questionable. A recent study showed pathogenic microbes, such as pseudomonas and fungi, in asymptomatic infants with CPAM, emphasizing the necessity of early thoracoscopic resections.[78]

Fecal microbiome studies in infants with GERD and proton pump inhibitor (PPI) treatments showed a significantly decreased relative abundance of *Lactobacillus* and *Stenotrophomonas* and an increase of *Haemophilus*.[79] However,

within-individual comparison (“before PPI” vs. “on PPI”) of oral PPI administration did not influence alpha- or beta - diversity and was not associated with increased risk of Clostridium difficile infections.[79]

In patients with necrotizing enterocolitis (NEC) an intestinal dysbiosis with a significant reduction of the bacterial diversity could be detected prior to any clinical symptoms.[80] The use of probiotics seemed to influence the gut microbiota and promoted a healthy microbiome; significant effects could be shown in the prevention strategies of necrotizing enterocolitis in preterm infants treated with *Lactobacillus* and *Bifidobacterium*.[81] Thus, neonatal probiotic strategies should be an important point for future research.

In short bowel syndrome (SBS) the microbial diversity seems to be reduced compared to controls.[80] Another study proposed a personalized treatment with probiotics based on gene sequencing fecal microbiome analysis in children with Hirschsprung’s associated enterocolitis (HAEC), knowing that the fecal microbiome differed significantly between healthy episodes and HAEC.[82] One common finding in NEC, SBS and HAEC seemed to be an overabundance of Proteobacteria.[80]

Furthermore, malignant tumors can have significant impact on the intestinal microbiota. Human neuroblastoma induced tumor cachexia and changes of the gut microbiome in a murine model; the authors suggested that modulation of the gut microbiome in cancer patients could eventually prevent tumor-related cachexia.[83] Future investigations in this field may have an impact on the understanding and non-operative treatment of such diseases.

1.18. Lessons from the Gut Microbiome

The human gastrointestinal tract harbors more than 100 trillion microorganisms and its composition is determined by numerous environmental and genetic factors.[72] The initial composition of the gut microbiome in infants correlates with the mode of delivery, methods of feeding and the perinatal administration of antibiotics.[13] Early colonization patterns are essential for immune development and can influence the risk of allergies or asthma.[13] Early infant exposures are known to alter the intestinal microbiome and are related to an increased risk of atopic

diseases such as asthma or eczema.[14] Another example can be found from CF research, showing that the early nutritional status in infants with CF is associated with disease morbidity and mortality.[14]

The gut microbiota may also influence several body functions, including host defense mechanisms and immune system responses as well as respiratory or neurological disorders and is considered as an important modulator of the systemic immune system.[14,72,84] Furthermore, the gut microbiome also has a significant impact on neuroanatomical pathways and CNS function, summarized as *gut-brain axis*.

The intestinal microbiome controls local defense mechanisms against enteral pathogens and influences systemic immunity or the severity of allergic inflammation.[14,84] A restoration of the gut microbiome in CF patients to similar levels as healthy controls can be associated with a reduced frequency of pulmonary exacerbations.[12] The role of the gut microbiota in the host response to bacterial pneumonia is essential.[84] Schuijt et al. showed that mice with a depleted gut microbiome had an increased bacterial dissemination, inflammation, organ failure and accelerated mortality after pneumococcal infection; consecutive fecal microbiome transplantation 6 h after pneumococcal infection led to a decrease of pulmonary bacterial counts, TNF-α and Interleukin-10 levels.[84] The gut microbiota seems to have a significant influence on metabolic pathways within alveolar macrophages as well as Th2-dominant immune response.[10,84] This phenomenon of *gut-lung-axis* is well known in the literature and several models of underlying mechanisms are described: cells activated in the gut-associated lymphoid tissue (GALT) or mesenteric lymph nodes are transported to the respiratory mucosa by T-cell related immune homing molecules and lead to an anti-inflammatory response.[13]

However, there is a huge variability in the determination of gut microbiome composition, which can lead to different results depending on study design and sample collection. Thus, the *International Human Microbiome Standards Project*, was introduced to standardize general practices in sampling, processing and bioinformatical methods.[72]

1.19. Aims and Hypothesis

The major aims of this study were:

(1) to investigate the cardiopulmonary performance capacity and presence of ventilation disorders following EA repair

(2) to identify differences of the airway microbiome composition in EA patients compared to a healthy control group

We hypothesized that:

(1) Cardiopulmonary performance capacity is significantly reduced following EA repair

(2) Ventilation disorders occur significantly more often in EA patients

(3) Airway microbiome is significantly different between EA patients and controls

(4) Impaired pulmonary function is associated with differences of the airway microbiome composition

2. Patients and Methods

All consecutive children treated at the Department of Pediatric and Adolescent Surgery of the Medical University of Graz for EA between 1980 and 2010 were invited to participate in a prospective study examination consisting of spirometry, cardiopulmonary exercise performance testing and analysis of the airway microbiome. The results were compared to a healthy age- and sex matched control group which was recruited from our day clinic or from the personal environment of the Department´s employees or patients.

The medical records of our EA patients were reviewed retrospectively for associated congenital anomalies, postoperative complications and consecutive interventions or disease-related co-morbidities. Gastro-esophageal reflux disease was assessed by pH-metry or 24h-impedance pH-metry. All patients were classified according to Spitz risk group.[61] The patients were asked to assess their quality of life regarding the presence of abdominal discomfort, swallowing difficulties, regurgitation or acid belching, recurrent cough, allergies or food intolerances.

At the study visit a 12-lead resting electrocardiography (ECG) was obtained to exclude cardiac arrhythmias and blood pressure (NIBP) at rest was measured non-invasively. The clinical examination included investigation of the following anthropometric data: height, body weight (BW) and body mass index (BMI). The body fat in % was determined by the caliper method using a 4-site skin fold procedure: on the right-side triceps muscle (vertical fold on the posterior midline halfway between the acromion and the olecranon), the biceps muscle (vertical fold on the anterior midline above the belly of the biceps muscle), subscapular (the diagonal fold 1–2 cm caudal to the inferior angle of the scapula) and supra-iliacal (diagonal fold 1 cm cranial of the anterior superior iliac crest).[48] The sum of all skin folds was used to estimate the body fat according to a standardized table as previously published.[85]

Appendicular muscle mass was measured by a multi-frequency impedance spectroscopy (Combyn^{TM} ECG, Academic Technologies at the Institute of

Cardiovascular Medicine GmbH, Graz, Austria) as previously described in the literature.[86]

The state of training was determined as hours of training per week and the assessment of the individual physical activity level as previously published and expressed as PAL values between 1.1 - 1.2 to 2.0 - 2.5, according to inactive - sedentary, moderately or vigorously active lifestyle.[87]

2.1. Spirometry

The lung function was measured by a small spirometry (Oxycon Pro® Carl Reiner GmbH, Vienna, Austria) at rest and after exercise. Spirometry is a simple and commonly used lung function examination for the diagnosis of obstructive ventilation disorders and for the determination of lung volumes.[88] It includes the measurements of static and dynamic lung function parameters as well as respiratory flows in the mouth. The measurements are carried out with flow sensors, where the volume is calculated numerically from the integration of the flow over time. The patient must breathe into a mouthpiece according to a standardized test cycle so that inspiratory and expiratory volumes and their flow rates can be measured. In the spirometric measurement parameters a distinction is made between static and dynamic lung function parameters: static lung volumes are lung volumes whose measured values do not depend on the time course of the spirogram, while dynamic pulmonary function parameters depend on the temporal course; the most important spirometric parameters are summarized below:[88]

- **Maximum vital capacity (VC_{max})**: The vital capacity (VC) belongs to the static lung volumes and includes the expiratory reserve volume, the tidal volume and the inspiratory reserve volume. The VC_{max} corresponds to the highest value of all vital capacity maneuvers and is expressed as observed over age and gender corrected expected maximum vital capacity.

- **Forced expiratory volume in 1 second (FEV 1):** Volume that can be exhaled within one second after maximum inspiration with maximum forced expiration. It belongs to the dynamic lung volumes and is the most important diagnostic

parameter for obstructive ventilation disorders in the central areas of the lower airways.

- **Tiffeneau index:** The Tiffeneau-index or relative one-second capacity was calculated as FEV1 / VC_{max}. It is a measure of the airway flow resistance and describes how much volume of the VC_{max} can be exhaled within a second.

Based on the measured volumes and the created diagrams, obstructive ventilation disorders can be diagnosed and indications of restrictive ventilation disorders can be obtained: an obstructive ventilation disorder was documented if the Tiffeneau index was less than 75%, a restrictive disorder if VC_{max} was found below 80%.[88] Examples of normal, obstructive and restrictive spirometric flow-volume curves are shown in **Figures 13 - 15.**

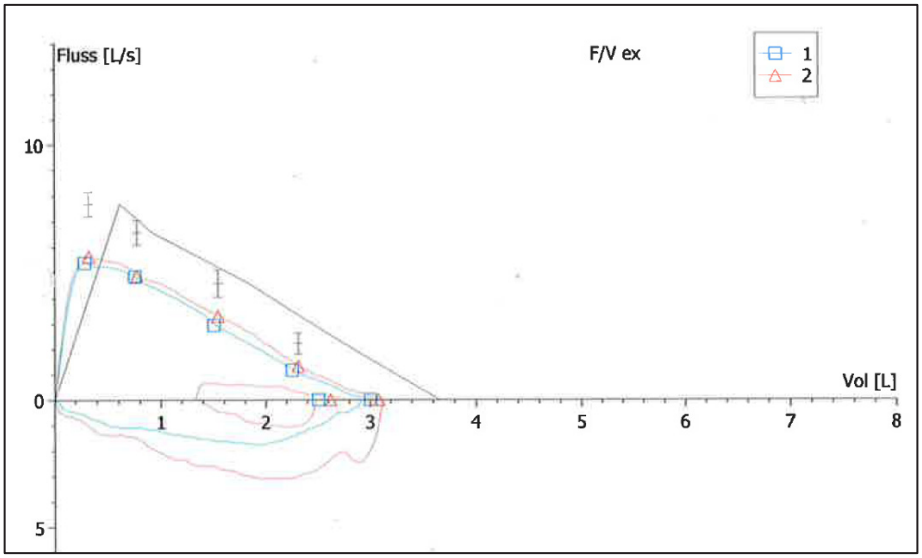

Figure 13: Normal spirometric flow-volume curve.

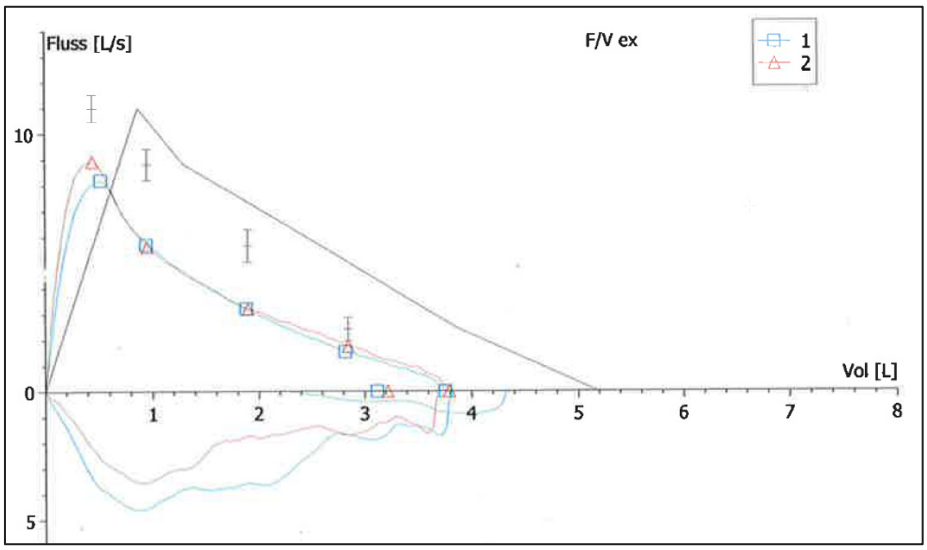

Figure 14: Obstructive ventilation disorder. Obstructive pulmonary disease generates a concave curve that represents the slowing of expiratory flow through the respiratory system. The degree of deformation reflects the severity of the obstruction.

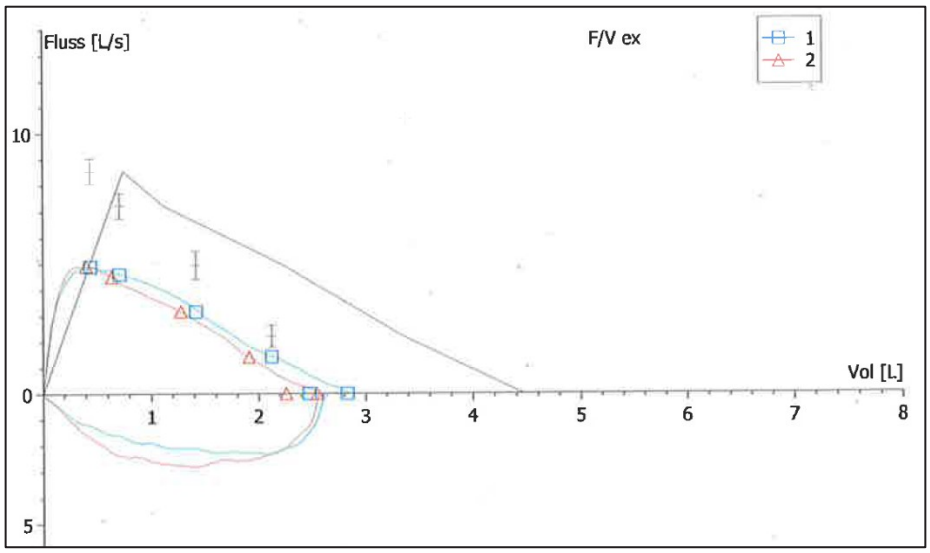

Figure 15: Restrictive ventilation disorder. The curves of patients with a restrictive respiratory disease have an almost normal shape, while the lung volumes and flows are considerably reduced.

Spirometry was repeated after the cardiopulmonary exercise performance testing (CPET) to rule out an exercise-induced asthma (EIA). EIA leads to a transient increase in the airway resistance after an intensive exercise and can be measured as a decrease of FEV 1 in a consecutive spirometry.[89]

2.2. Cardiopulmonary Exercise Performance Testing

All equipments were calibrated according to the instructions of the manufacturer before testing. Each test was supervised by a physician of sports medicine and a medical technical analyst.

The cardiopulmonary exercise performance was determined by CPET with a bicycle ergometer (Excalibur Sport®, Lode B.V., Groningen, The Netherlands) and a spirometer (Oxycon Pro® Carl Reiner GmbH, Vienna, Austria) in an upright position **(Figure 16)**. Spiroergometry was performed with a gender and age adapted protocol using a stepwise load increase as previously published **(Table 5).**[48] During the test, participants breathed through a facemask (Hans Rudolph, Kansas City, MO, USA) connected to a calibrated respiratory gas analysis system (Oxycon Pro® Carl Reiner GmbH, Vienna, Austria). Expired gas was passed through a flow meter (Triple V volume transducer), an oxygen analyzer and a carbon dioxide analyzer.

Figure 16: Standard setting in CPET (with friendly permission from M. Kanizaj / LKH-Univ. Klinikum Graz)

The spiroergometry was carried out to subjective exhaustion or until the participants were unable to maintain the required pedaling speed (cadence) of greater than 60 revolutions per minute (rpm). The exercise phase was followed by a 3-min recovery period of slow pedaling (60 rpm) with the same workload as at the beginning of the test.

Heartrate (HR) was measured by continuous twelve-lead ECG (Combyn ECG TM®) and oxygen saturation was continuously monitored (finger pulse oximeter Habel Medizintechnik®). Lactate levels were obtained by earlobe sampling (20 µl of blood per measurement were sampled to heparinized capillaries per test) before the test, at the end of each step and after the recovery phase (enzymatically amperometric measurement with a Biosen C_line®, EKF Diagnostics for life, Cardiff, UK).

Body Weight [kg]	Min. Performance (Start) [Watt]	Step (Increase) [Watt]	Duration of Step [min]	Performance at 3 min Recovery [Watt]
25 – 30	15	10	1	15
31 – 35	20	10	1	20
36 – 40	25	10	1	25
41 – 45	30	15	1	30
46 – 50	35	15	1	35
51 – 55 ♀	40	15	1	40
56 – 60 ♀	45	15	1	45
> 60 ♀	50	20	1	50
51 – 55 ♂	40	20	1	40
56 – 60 ♂	45	20	1	45
61–70 ♂	50	25	1	50
>70 ♂	50	30	1	50

Table 5: Protocol for bicycle spiroergometry testing.

The flow meter and gas analyzers were connected to a computer, which calculated the following parameters:

- **Minute ventilation (V_E):** The tidal volume (V_T) represents the lung volume of the normal volume of air displaced between normal inhalation and exhalation when no extra effort is applied. The minute ventilation (V_E) is that volume of air breathed in and out of the lungs and is given in l / min; it is calculated as V_T multiplied by the respiratory rate according to the values before maximum load is reached.

- **Oxygen uptake (VO_2):** The inspiratory (FiO_2) and expiratory (FeO_2) fraction of oxygen are measured; the accuracy for FiO_2 and FeO_2 is given with ± 0.01 vol % by the manufacturer. Using these values and the minute ventilation, the oxygen uptake is calculated as $VO_2 = (FiO_2 – FeO_2) * V_E$.[48]

- **Peak oxygen uptake (peak VO_2):** The peak VO_2 is defined as the average VO_2 over the last 30 seconds prior to termination of the test and is expressed in ml/kg/min. The peak VO_2 defines the upper limit of the cardiopulmonary system and is considered a standard measure of aerobic performance.[90]

- **Relative performance capacity:** The relative performance capacity is calculated from the achieved maximal wattage in relation to the age and gender-specific standard values.[91]

- **Respiratory exchange ratio (RER):** The RER depends on the metabolic substrate of the energy production and can be used to estimate the percentage of fat or carbohydrate utilization.[90] With pure carbohydrate metabolism the RER is 1, with pure fat burning 0.7; an average nutrition leads to an RER of approximately 0.82-0.85.[90] In high performance intensities, the CO_2 production exceeds the O_2 uptake, so that the RER can rise to values above 1.[90] A calculated RER is also used as an indicator that the participants are nearing exhaustion and their cardio-pulmonary limits; thus a RER > 1.10 is used as criterion to determine that the peak VO_2 reflects the peak physiological workload.[92]

- **Breathing reserve (BR):** The BR is calculated from the difference between the maximum voluntary ventilation (MVV) and the measured V_E at maximum physical exertion.[90] To measure the MVV, the patient is asked to breathe quickly, deeply, and vigorously for 12 or 15 seconds, then multiply by 5 or 4 to get the maximum value for one minute; when performing a resting spirometry, the forced expiratory one-second capacity (FEV1) can alternatively be multiplied by 35–40 in order to obtain the MVV.[90]

Healthy untrained participants are normally not limited in their performance capacity by pulmonary factors. They can reach a respiratory minute volume of 20–50% of the MVV, which gives a relative breathing reserve of approximately 50-80%.[90] Patients with chronic pulmonary disease, on the other hand, approach their MVV much earlier under exercise, which indicates that these patients are limited in their exercise capacity by a respiratory factor; the respiratory reserve in these patients is typically below 45–50%.[90]

- **Oxygen pulse (O_2/HR):** The O_2/HR is regarded as the correlate of the heart stroke volume, it is determined from the quotient of VO_2 and heart rate.[90] The oxygen pulse is an important parameter for estimating myocardial function under stress; values of 4–6 ml are normal at rest with increases to values of approximately 10–20 ml at maximum load.[90]

- **Aerobic capacity (ΔVO_2 / ΔWR):** The ΔVO_2 / ΔWR expresses the change in oxygen uptake (ΔVO_2) in relation to the change in load or work rate in watts (ΔWR) with a continuous increase in load; the normal value for healthy volunteers is approximately 10–12 ml/min/W.[90] The parameter of the aerobic capacity allows a statement about the ratio of aerobic and anaerobic energy production.[90]

- **Respiratory equivalent for oxygen (EQO_2):** The EQO_2 is an index of ventilation efficiency and formed by dividing the ventilation (V_E) by the oxygen uptake (VO_2); it reflects the amount of air, to inhale 1 Liter (L) of oxygen.[90] At rest the EQO_2 is relatively high, so breathing economy is poor. This is explained by the sections of the lung that are not supplied with blood at rest, which thus increase the dead space. With altered load, the circulation of the lungs improves and the breathing economy increases.[90] Training can improve breathing economics and leads to a lower curve; therefore it is used as an index for ventilation efficiency and breathing economy. The normal values of the EQO_2 should be lower than 25L at rest, but can be increased in case of cardiopulmonary diseases. [90]

In healthy subjects, the increase in V_E at low exercise levels primarily takes place via an increase in the tidal volume; in higher exercise levels, the increase in ventilation is ensured by an increase of the respiratory rate.[90] Patients with obstructive or restrictive respiratory diseases can show typical pathological breathing patterns.[90]

2.3. Total DNA Isolation, 16S Amplicon-Based Library Preparation and Sequencing

Deep induced sputum was collected as described previously.[1,93] Briefly, patients were asked to inhale hypertonic saline with the help of a nebulizer. Thereafter, breathing deepening exercises were performed with the help of a thoracic physical therapist. With the combination of these exercises and inhalation, sputum from the lower airways was sampled in a sterile vial and stored at -80°C upon total DNA isolation. Before nucleic acid extraction samples were treated with an equal volume of 1M (100 µg/ml) DL-Dithiothreitol solution in water and incubated at 37° Celsius (C) for 20 minutes in a water bath. After centrifugation at 4,000 g and 30 minutes at room temperature (RT), the supernatant was decanted and the cell pellet was resuspended in PBS (Carl Roth, Karlsruhe, Germany) up to a volume of 500 µl. 250 µl of the suspension was used for total DNA extraction with the MagNA Pure LC DNA Isolation Kit III (Bacteria and Fungi) (Roche, Mannheim, Germany) according to manufacturer's instructions and as published in Klymiuk et al. 2017.[94] The cell suspension of each sample was mixed with 250 µl bacterial lysis buffer and bead beated in a Magna Lyser tube in a Magna Lyser instrument (Roche, Mannheim, Germany) at 6,000 rpm for 30 seconds twice.

After incubation with lysozyme (25 µl, 100mg/ml) at 37°C for 30 minutes and proteinase K (43.4 µl, 20mg/ml) digestion at 65°C for one hour, samples were heat inactivated at 95°C for 10 minutes. After centrifugation for 5 minutes at 14,000 rpm 200 µl of the sample was loaded to the MagNA Pure LC 2.0 instrument (Roche, Mannheim, Germany) and isolated according to manufacturer's instructions with the MagNA Pure LC DNA Isolation Kit III (Bacteria, Fungi) (Roche, Mannheim, Germany). Total DNA was eluted in 100 µl elution buffer. For PCR reaction, five µl

of total DNA were used in a 25 µl PCR reaction in triplicates using a Fast Start High Fidelity PCR system (Roche, Mannheim, Germany) according to Klymiuk et al. with the target specific primers F27- AGAGTTTGATCCTGGCTCAG and R357-CTGCTGCCTYCCGTA.[94,95] Finally, the triplicates of each sample were pooled and the PCR for indexing was performed as described in Klymiuk et al. with 8 amplification cycles.[94] Five µl of the indexing PCR was pooled of each sample and the final library was purified by conventional gel electrophoresis and quantified using the QuantiFluor ONE dsDNA Dye (Promega, Hilden, Germany) on a Quantus™ Fluorometer (Promega, Hilden, Germany). Quality control was performed on a BioAnalyzer 2100 instrument using a DNA 7500 lab Chip. For sequencing the pool was diluted according to standard procedures and the 6 pM library was sequenced on an Illumina MiSeq desktop sequencer (Eindhoven, Netherlands) with 20% PhiX control DNA (Illumina, Eindhoven, Netherlands) and v3 chemistry for 600 cycles in paired end mode according to manufacturer's instructions and FastQ raw reads were used for data analysis. For data analysis a total of 2,363,371 (per sample minimum 39,514, maximum 81,288, median 63,061) raw sequence reads were used in the Galaxy based workflow (Medical University of Graz, funded by the Austrian Federal Ministry of Education, Science and Research, Hochschulraum-Strukturmittel 2016 grant as part of BioTechMed Graz). Briefly, raw reads were quality-filtered, de-noised, de-replicated, merged and checked for chimeras using DADA2 pipeline with standard settings in QIIME2.0.[96,97] For taxonomic assignment SILVA rRNA database 1.3 with 97% identity was used.

2.4. Statistical Analysis

All data were entered into an Excel 2019® (Microsoft Corporation. Microsoft Excel [Internet]. 2018, USA) spreadsheet and SPSS Statistics 21© (IBM Corp. Released 2012. IBM SPSS Statistics for Windows, Version 21.0. Armonk, NY: IBM Corp) was used for data analysis. A Kolmogorov Smirnov Test was used to assess normal distribution. In case of normal distribution, data was displayed as mean and standard deviation and statistical group comparison was performed using a 2-sided, unpaired t-test. In case of absent normal distribution, data was displayed as median and interquartile range (IQR) and a Mann-Whitney-U-Test was used for group comparison. The Fisher exact test or the Chi-square test was used to compare

categorical data. Correlation analysis was performed with Pearson correlation tests. Statistical significance was defined as $p<0.05$.

To interpret and compare taxonomic information from the 16S rRNA datasets Calypso online software Calypso® (Version 8.84) was used.[98] Species richness was calculated using the Chao-1-estimator, the Shannon's Index and inversed-Simpson Index. As a measure for beta-diversity redundancy analysis (RDA), color-coded principal component analysis plot (PCoA) and the Anosim Bray Curtis dissimilarity score were used. Differences in taxa abundance were calculated with Calypso® after log10- transformation of data. P-values were adjusted for multiple testing by false discovery rate (FDR). The top 300 most abundant taxa were included in the analysis. Linear discriminant effect size (LEfSe) analysis was performed to detect statistically relevant bacteria in different groups.

2.5. Ethical Standards

This study was performed according to the declaration of Helsinki. Informed written consent was obtained from all patients and controls or their legal guardians. The study was approved by the institutional review board (EK 29-276 ex 16/17).

3. Results

Out of 47 eligible EA/TEF patients treated in the regarded time period, 21 agreed to participate. Three patients refused to take part and 23 patients were unavailable. At the follow-up examinations, one patient had to be excluded because of mental impairment and another one was unable to donate a sufficient sputum sample, leaving 19 patients for further analysis.

3.1. EA Patient Group

The mean age of the EA group was 24.7 ± 7 years (range: 14 - 40 years) and consisted of 10 male and 9 female patients. 16 patients (84.2%) had a Gross type C atresia, 1 had a type A, 1 a type B atresia and 1 had a type D esophageal atresia. All patients underwent right thoracotomy for EA repair.

Five patients (26.3%) suffered from at least 1 congenital anomaly: 1 patient was born with an imperforate anus and a unilateral renal agenesis. Four patients had a congenital cardiac anomaly; 3 of these patients underwent cardiac surgery: 1 patient with an atrial septal defect (ASD) type II, one patient with a muscular ventricular septal defect (VSD) and another patient with a congenital aortic coarctation. The fourth patient with a congenital cardiac anomaly had an ASD type II, which was not hemodynamically relevant. No patient had a birth weight below 1,500g, only the three patients with hemodynamically relevant cardiac anomalies were classified as risk group II, according to the Spitz risk classification **(Table 6).**[61]

ID	Gest. Age	Birth Weight	Vogt	Gross	Waterston	Spitz	TM	Congenital Anomalies
P_1	36	2,300	IIIb	C	B	I		
P_2	NK	NK	IIIb	C	NK	NK		
P_3	40	3,000	IIIb	C	A	I		Imperforate anus, unilateral renal agenesis
P_4	37	2,800	IIIb	C	A	I	✓	
P_5	37	2,735	IIIb	C	B	II		ASD II, butterfly vertebrae Th10-12
P_6	41	3,070	IIIb	C	A	I	✓	
P_7	42	2,800	IIIb	C	A	I		
P_8	35	2,100	IIIb	C	B	I		ASD II
P_9	38	2,150	IIIb	C	B	I	✓	
P_10	28	2,600	IIIa	B	A	I		
P_11	34	1,680	IIIb	C	C	I		
P_12	36	2,200	IIIb	C	B	I		
P_13	36	1,980	IIIb	C	C	I		
P_14	36	2,350	IIIc	D	B	II	✓	muscular VSD
P_15	NK	NK	IIIb	C	NK	NK		
P_16	40	3,150	IIIb	C	A	I		
P_17	37	2,670	IIIb	C	A	I	✓	
P_18	40	3,070	IIIb	C	A	I	✓	
P_19	37	2,264	II	A	B	II		Congenital aortic coarctation

Table 6 : Patient cohort. (ID…identification, TM…tracheomalacia, NK…not known, ASD…atrial septal defect, VSD…ventricular septal defect)

3.2. Control Group

The mean age of the control group was 24.6 years ± 8 years (range: 12 - 43 years) and consisted of 10 male and 9 female participants. There was no statistically significant difference regarding patient age between EA and control groups (*unpaired t-test*; *p=0.949).*

3.3. Complications and Subsequent Interventions

The following post-operative complications were recorded: 1 patient (5%) had an anastomotic insufficiency, 7 patients (37%) required dilatations because of esophageal stenosis. Clinically relevant tracheomalacia was diagnosed in 6 patients (32%). Fifteen patients (79%) had a documented GERD, 9 of them (47%) required a fundoplication in the further course **(Table 7).** In summary, 15 patients (79%) required a follow-up intervention.

At the study visit 9 EA patients (47%) complained about abdominal discomfort. Five patients (26%) reported swallowing difficulties. Regurgitation and acid belching were recorded in 6 patients (32%). The prevalence of recurrent cough was 21% and occurred only in the EA patient group *(Chi-square test, p=0.034)*. Six patients (32%) mentioned allergies, another 3 reported (16%) food intolerances.

ID	Leakage	Esophageal Stenosis	GERD	Follow-up Interventions
P_1		✓		bougienage
P_2			✓	fundoplication
P_3			✓	fundoplication
P_4		✓	✓	bougienage
P_5			✓	jejunal probe, fundoplication, cardiac surgery
P_6			✓	pleural drainage, bougienage
P_7				
P_8		✓	✓	bougienage, fundoplication
P_9				aortopexy
P_10	✓	✓	✓	gastrostomy, bougienage, fundoplication, jejunal probe
P_11			✓	
P_12				fundoplication, distal esophageal myotomy
P_13			✓	fundoplication
P_14			✓	cardiac surgery, prox. TEF closure
P_15			✓	
P_16		✓	✓	bougienage
P_17			✓	fundoplication, pyloromyotomy
P_18		✓	✓	
P_19		✓	✓	gastrostomy, bougienage, fundoplication, cardiac surgery

Table 7: Postoperative complications, GERD and follow-up interventions.

3.4. Anthropometric Data

There was no statistically significant difference in the amount of physical activity and training per week, the height, weight, body mass index or body fat contents between the study and the control group. EA patients showed a significantly lower muscle mass compared to the control group (*unpaired t-test; $p<0.001$*) (**Table 8**).

	EA Patients *n=19*	Controls *n=19*	p-value
Age	24.7 ± 7.0	24.6 ± 8.0	0.949
Anthropometry			
Height [m]	1.69 ± 0.09	1.70 ± 0.10	0.812
BW [kg]	62.6 ± 13.9	68.5 ± 12.4	0.181
BMI	22.0 ± 4.9	23.8 ± 4.3	0.311
Body fat [%]	21.2 ± 9.0	18.6 ± 6.3	0.331
Muscle mass [kg/Ht²]	6.6 ± 1.5	8.6 ± 1.5	**< 0.001**
PAL	1.6 ± 0.2	1.7 ± 0.2	0.216
Training (h/week)	0.0 ± 1.9	4.0 ± 3.1	0.057
Spirometry			
VC_{max} [%]	73.8 ± 15.6	103.7 ± 10.3	**< 0.001**
Tiffeneau index [%]	82.1 ± 7.2	82.8 ± 5.4	0.705
Spiroergometry			
Performance capacity [%]	114.4 ± 22.4	144.2 ± 28.2	**0.001**
peak VO_2 [ml/(min*kg)]	37.5 ± 8.6	45.0 ± 9.0	**0.013**
O_2/HR [ml]	12.5 ± 3.4	16.2 ± 4.4	**0.012**
EQO_2	21.4 ± 1.8	19.7 ± 2.8	**0.014**
BR	5.0 ± 11.9	15.0 ± 9.4	0.246
ΔVO_2 / ΔWR	10.5 ± 0.9	10.5 ± 1.1	0.751

Table 8: Anthropometric data, results of spirometry and spiroergometry of EA patients and controls (n=19 each). Data are displayed as mean ± standard deviation (SD). (PAL…physical activity level; VC_{max}…maximum vital capacity; peak VO_2…peak oxygen uptake, O_2/HR…oxygen pulse; EQO_2…respiratory equivalent for oxygen, BR… breathing reserve, ΔVO_2 / ΔWR… aerobic capacity) (Reproduced from Arneitz C et al. Pediatr Res. 2020,[1] with permission of publisher, Springer Nature)

3.5. Spirometry

Spirometry revealed a significantly lower VC_{max} for EA patients compared to controls (*unpaired t-test, p<0.001*). Twelve patients showed a reduced VC_{max}; 9 of these had a restrictive and 3 a combined ventilation disorder. Restrictive ventilation disorders occurred only in the EA patient group *(EA n=9 vs. controls n=0; Chi-square test, p=0.001);* two EA patients were obstructive and one patient had an

exercise-induced asthma, indicated by a reduced Tiffeneau index following CPET (FEV 1 / VC_{max} at 1st spirometry: 84% vs. 2nd spirometry: 70%) **(Table 9 and 10).**

	EA				p-value
Ventilation Disorders	**Patients**	**%**	**Controls**	**%**	
Restrictive	9	47.4%	0	0%	**0.001**
Obstructive	2	10.5%	4	21.1%	0.374
Combined	3	15.7%	0	0%	0.071
Exercise-induced asthma	1	5.3%	0	0%	0.311
Normal pulmonary function	4	21.1%	15	78.9%	**0.001**
Total	**19**	**100%**	**19**	**100%**	

Table 9: Pulmonary function in spirometry.

ID	1st spirometry		2nd spirometry	
	VC_{max} [%]	FEV 1 / VC_{max} [%]	VC_{max} [%]	FEV 1 / VC_{max} [%]
P_1	61.2	85.4	65.7	85.4
P_2	77.0	70.3	85.7	70.2
P_3	85.5	73.9	86.1	72.8
P_4	85.1	71.1	82.3	75.1
P_5	52.4	82.1	49.3	87.4
P_6	94.3	84.5	92.4	89.4
P_7	72.6	81.7	77.5	81.5
P_8	66.4	93.8	62.0	98.3
P_9	72.2	97.7	76.3	95.9
P_10	80.4	93.8	79.6	94.1
P_11	82.1	83.4	84.7	84.4
P_12	73.0	82.4	73.2	84.8
P_13	49.2	85.2	50.0	89.9
P_14	39.6	71.6	41.7	71.0
P_15	63.4	87.4	57.3	88.4
P_16	82.0	81.7	81.8	85.3
P_17	99.7	84.0	104.1	70.0
P_18	72.3	92.3	67.9	94.1
P_19	76.0	78.4	78.3	77.8

Table 10: Results of the spirometry of EA patients before and following CPET.

3.6. Cardiopulmonary Exercise Performance

All subjects included in the study had a RER value greater than 1.10. Spiroergometry revealed a significantly lower relative performance capacity (*unpaired t-test; p=0.001*) and a significantly lower peak VO_2 (*unpaired t-test; p=0.013*) in the EA group compared to the control group (Table 8).

While seven patients (36.8%) had a reduced V_E, none of the controls presented with a reduced V_E (Chi-square test; *p=0.008*).

The values for O_2/HR, and EQO_2 were within the normal ranges in both groups. However, the O_2/HR was significantly lower (*Mann-Whitney-U test*; *p=0.012)* and the EQO_2 was significantly higher in the EA group *(Mann-Whitney-U test; p=0.014)* (Table 10). Exclusion of the four EA patients with congenital cardiac anomalies and their respective controls still revealed a significantly decreased performance capacity (mean 116.7 ± 24.6 vs. 146.1 ± 31.5; *unpaired t-test, p=0.008)* and peak VO_2 *(*mean 37.6 ± 8.8 vs. 44.7 ± 8.9; *unpaired t-test; p=0.036)* of EA patients in the remaining participants.

Patients with restrictive ventilation disorders showed a significantly lower relative performance capacity compared to controls (*Mann-Whitney-U test*; *p=0.029*) **(Figure 17).** A reduced respiratory minute volume lead to a significantly reduced relative performance capacity (*Mann-Whitney-U test; p=0.002*).

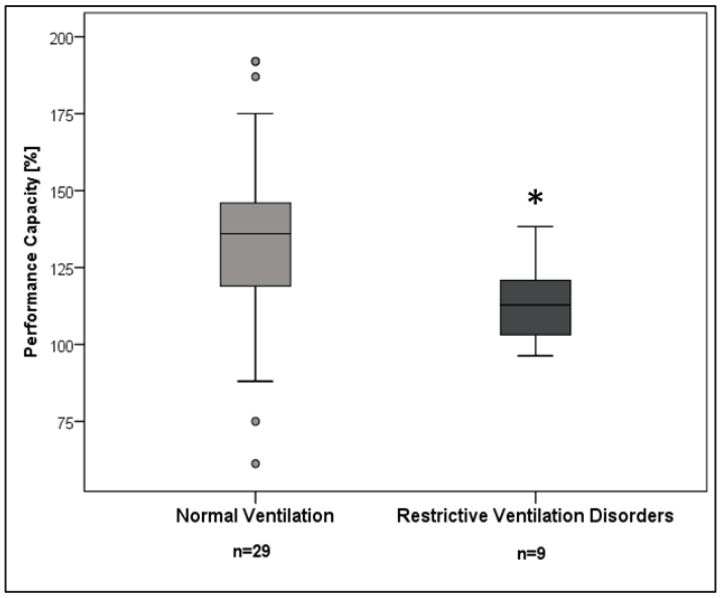

Figure 17: Relative performance capacity [%] in participants (n=38) with and without restrictive ventilation disorders (…p=0.029); restrictive ventilations disorders occurred only in the EA patient group. (Reproduced from Arneitz C et al. Pediatr Res. 2020,[1] with permission of publisher, Springer Nature)*

Moreover, neither tracheomalacia nor prematurity had a significant impact on the relative performance capacity or spirometry results **(Table 11 and 12).**

	Normal n=13	**Tracheomalacia n=6**	**p-value**
VC_{max}	70.9 ± 11.6	77.3 ± 21.5	0.416
Tiffeneau-index	83.0 ± 6.7	80.0 ± 8.4	0.521
Performance capacity	116.1 ± 20.5	110.7 ± 27.7	0.898
peak VO_2	35.6 ± 7.5	41.6 ± 9.9	0.179
O_2/HR	11.7 ± 2.7	14.2 ± 4.5	0.323
EQO_2	21.5 ± 2.1	21.2 ± 1.4	0.966

Table 11: Spirometry and spiroergometry results in EA patients with and without tracheomalacia. Mann-Whitney-U tests were used to calculate statistical significances. Data are displayed as mean ± standard deviation. (VC_{max}…maximum vital capacity; peak VO_2…peak oxygen uptake, O_2/HR…oxygen pulse; EQO_2…respiratory equivalent for oxygen) (Reproduced from Arneitz C et al. Pediatr Res. 2020,[1] with permission of publisher, Springer Nature)

	EA full-term n=8	EA preterm n=11	p-value
VC_{max}	79.6 ± 10.2	69.6 ± 17.8	0.238
Tiffeneau-index	81.0 ± 7.3	82.8 ± 7.3	0.492
Performance capacity	120.2 ± 24.6	110.2 ± 20.7	0.310
peak VO_2	38.5 ± 9.9	36.7 ± 7.9	0.968
O_2/HR	13.7 ± 4.1	11.7 ± 2.8	0.351
EQO_2	21.6 ± 2.3	21.3 ± 1.5	1.000

Table 12: Spirometry and spiroergometry results in full-term and preterm EA patients. Mann-Whitney-U tests were used to calculate statistical significances. Data are displayed as mean ± standard deviation. (VC_{max}…maximum vital capacity; peak VO_2…peak oxygen uptake, O_2/HR…oxygen pulse; EQO_2…respiratory equivalent for oxygen, BR… breathing reserve, $\Delta VO_2 / \Delta WR$… aerobic capacity) (Reproduced from Arneitz C et al. Pediatr Res. 2020,[1] with permission of publisher, Springer Nature)

No significant difference could be shown in the breathing reserve (BR): 12 of the patient cohort and 12 of the controls had a depletion of the BR (*Chi-square test*; *p=0.631*); a limited BR had no significant impact on the cardiopulmonary exercise performance (*Mann-Whitney-U test; p=0.120*).

All spiroergometry results of the EA patients are listed in **Table 13**.

ID	Performance capacity	peak VO_2	O_2/HR	EQO_2	RER	BR	ΔVO_2 / ΔWR
P_1	103.1	28.1	12.5	23	1.04	0	11.3
P_2	75.0	26.9	14.5	19.7	1.34	4	11.4
P_3	144.0	40.6	13.7	20.9	1.13	4	11.4
P_4	61.3	27.1	10.4	22.8	1.21	34	10.5
P_5	98.9	31.6	6.3	21.9	1.06	14	10.1
P_6	131.0	55.8	17.6	20.2	1.12	0	12.4
P_7	112.8	37.3	16.5	21.2	1.1	9	10.3
P_8	112.1	48.1	11.8	22.2	1.1	22	11.1
P_9	138.3	47.1	19.5	19.1	1.16	0	10.5
P_10	125.0	46.3	14	20.1	1.26	27	10.1
P_11	138.5	33.5	11	22.2	1.27	3	10.1
P_12	114.0	43.4	12.3	17.7	1.29	22	9.3
P_13	134.3	33.2	8.9	20	1.05	0	10.3
P_14	100.0	40.8	12.2	22	1.19	0	8.9
P_15	96.3	26.0	9.2	24.1	1.1	17	10.7
P_16	143.6	39.3	10.2	25.7	1.18	5	10.8
P_17	113.0	43.9	17.2	21.1	1.14	18	11.6
P_18	120.9	34.9	8.4	22.2	1.08	35	9.2
P_19	111.6	28.0	11.8	20.8	1.11	3	10

Table 13: Results of the EA patient group spiroergometry data. (VC_{max}…maximum vital capacity; peak VO_2…peak oxygen uptake, O_2/HR…oxygen pulse; EQO_2…respiratory equivalent for oxygen, BR… breathing reserve, ΔVO_2 / ΔWR… aerobic capacity)

3.7. Airway Microbiome

Alpha-diversity of the deep induced sputum samples as measured by Shannon Index, Inversed Simpson's and Chao 1 did not differ significantly between EA patients and controls **(Figure 18)**. Likewise, beta-diversity was not significantly different between the two groups in Anosim Bray Curtis, PCoA and RDA analysis (Figure 18).

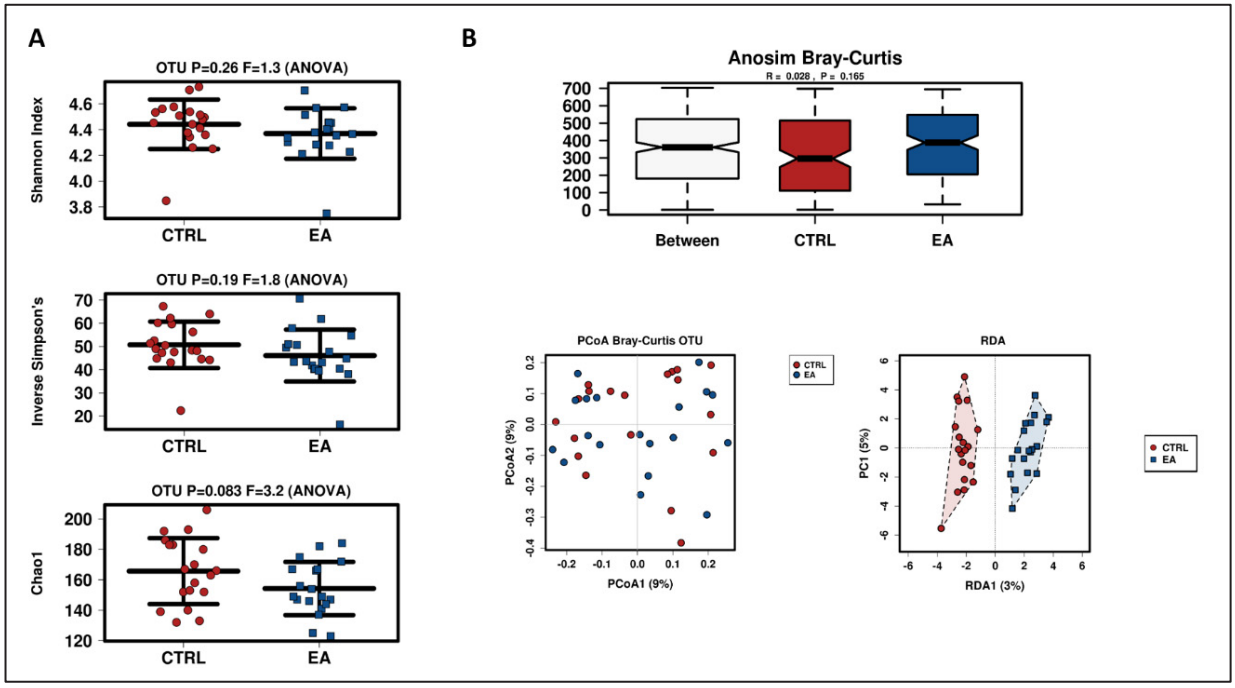

Figure 18: Parameters of alpha (A) and beta (B) diversity. RDA (p=0.134). CTRL...control group; EA...esophageal atresia group (Reproduced from Arneitz C et al. Pediatr Res. 2020,[1] with permission of publisher, Springer Nature)

Figure 19 depicts the relative abundances at the phylum and order levels comparing EA patients and controls.

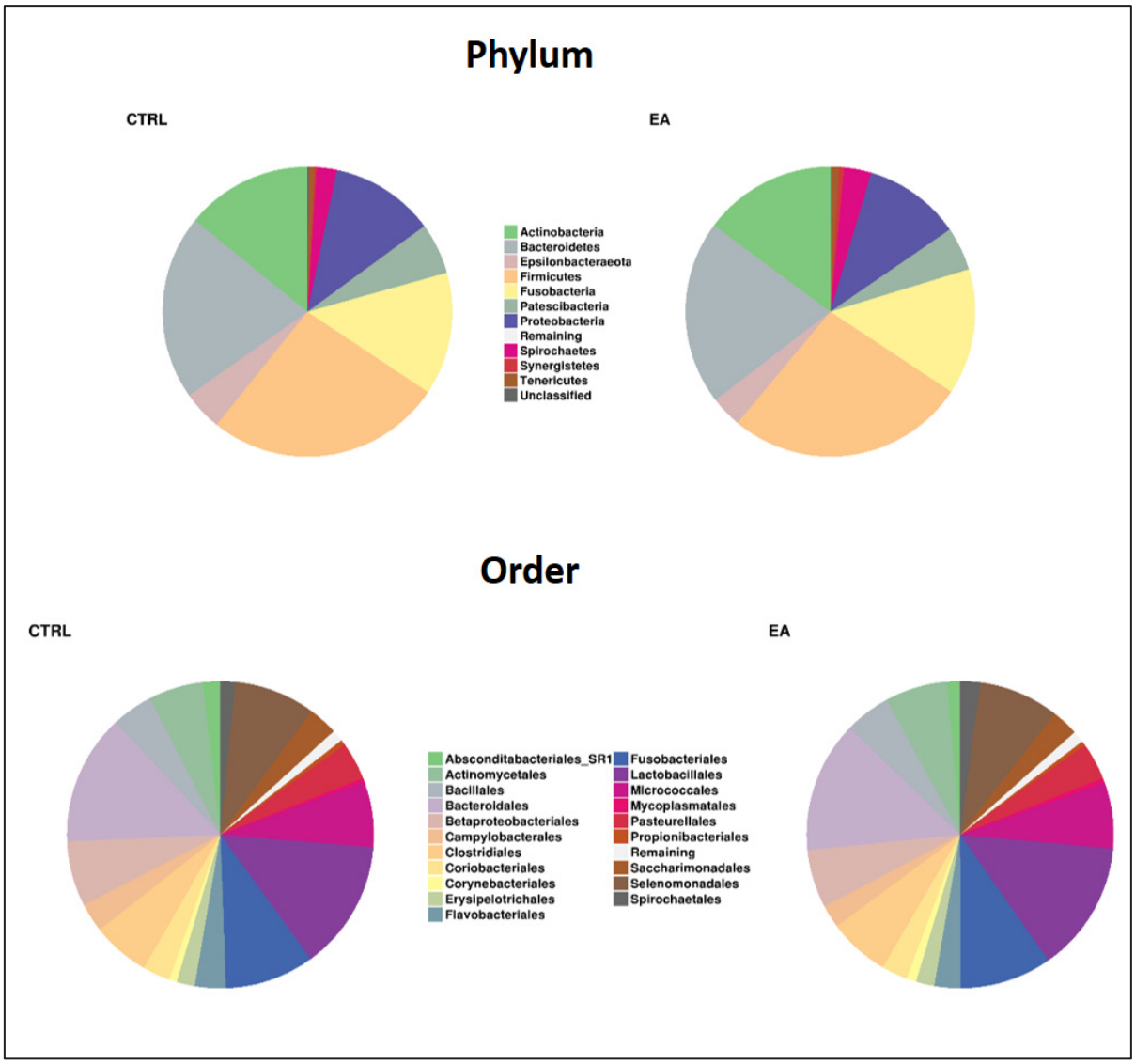

Figure 19: Relative abundances at the phylum (A) and order (B) levels. CTRL...control group; EA...esophageal atresia group (Reproduced from Arneitz C et al. Pediatr Res. 2020,[1] with permission of publisher, Springer Nature)

LEfSe analysis at the species level is shown in **Figure 20**. *Prevotella uncultured* (p=0.0081), *Streptococcus anginosus* (p=0.0078), *Prevotella 7 prevotella enocea* (p=0.026) and Mogibacterium timidum (p=0.047) were significantly enriched in the EA patient group. Species from *Alloprevotella uncultured* (p=0.0079) and *Campylobacter uncultured* (p=0.0072) were significantly higher in the control group than in the EA group (Figure 20).

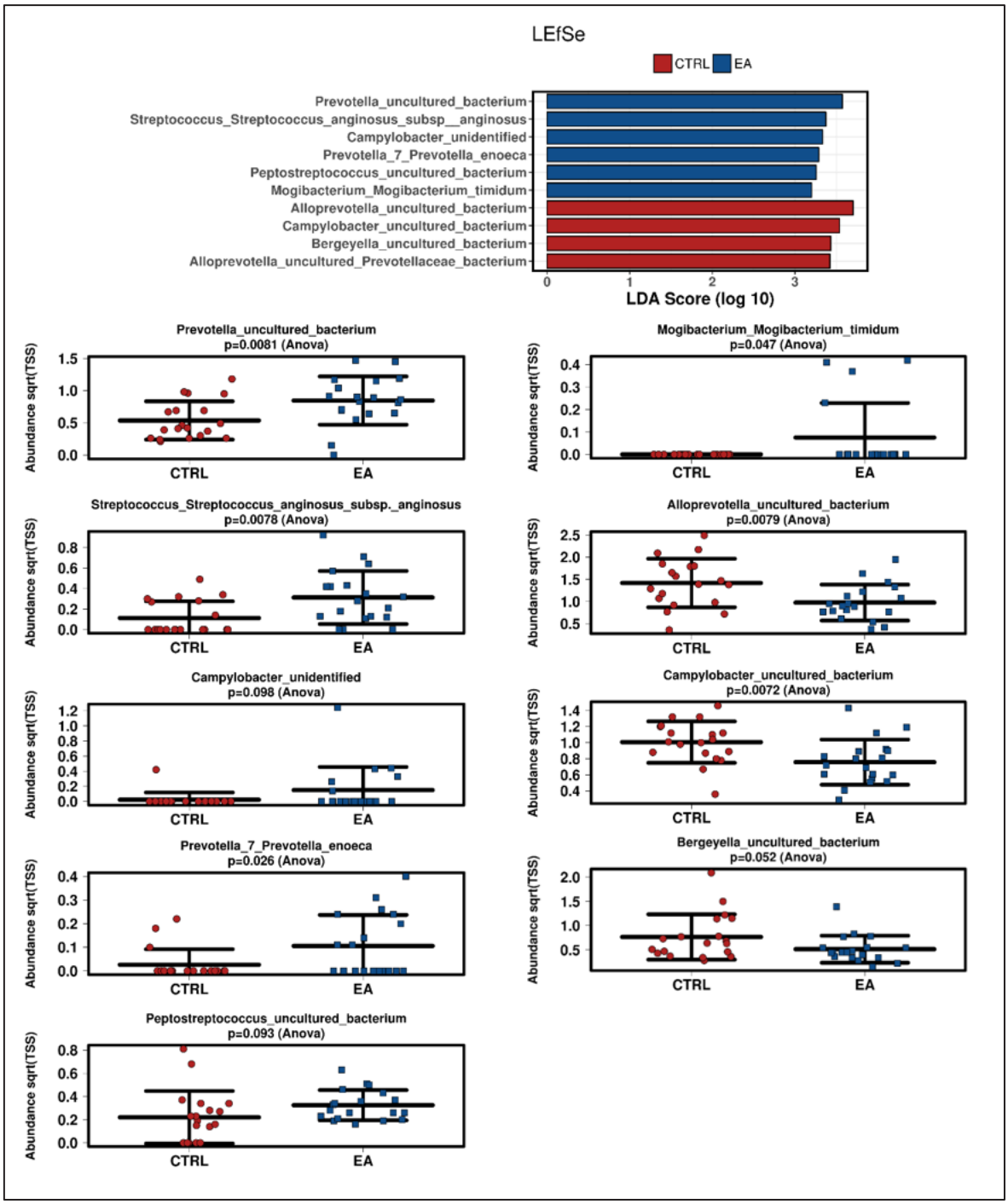

Figure 20: Histograms of linear discriminant analysis (LDA) effect size (LEfSe) comparison of deep induced sputum samples at the species level comparing EA patients (n=19) and controls (n=19). Log-level changes in LDA score are displayed on the x axis. Only results with p<0.1 are displayed as pairwise comparisons. (Reproduced from Arneitz C et al. Pediatr Res. 2020,[1] with permission of publisher, Springer Nature)

3.7.1. Subgroup Analysis of EA Patients with Reduced VC_{max}

Analysis of the airway microbiome composition in EA patients with a reduced VC_{max} and their respective controls revealed no significant differences of alpha- and beta diversity **(Figure 21).**

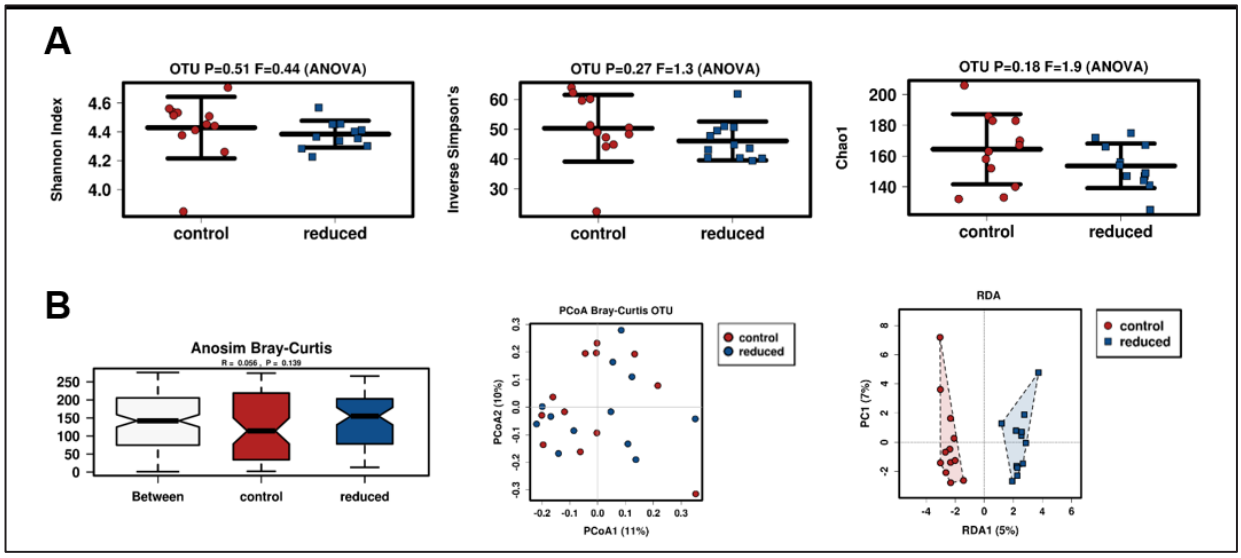

Figure 21: Microbiome analysis of EA/TEF patients with reduced VC_{max} (n=12) and their respective controls (n=12). Parameters of alpha diversity (A) and beta diversity (B; p=0.164 for the RDA analysis). (Reproduced from Arneitz C et al. Pediatr Res. 2020,[1] with permission of publisher, Springer Nature)

The relative abundances at the phylum and order levels comparing EA patients with and without a reduced VC_{max} and their respective controls are shown in **Figure 22**.

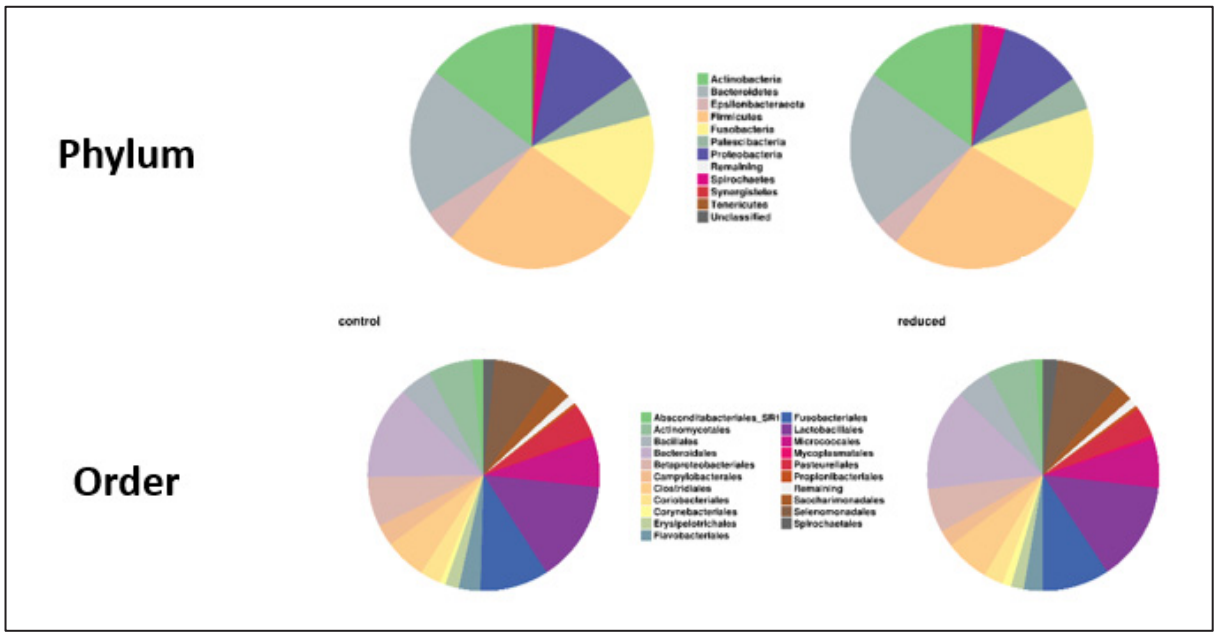

Figure 22: Relative abundance at the phylum (left graph) and order (right graph) level. (Reproduced from Arneitz C et al. Pediatr Res. 2020,[1] with permission of publisher, Springer Nature)

LEfSe revealed significantly enriched *Prevotella uncultured bacterium* (p=0.004) *and Streptococcus anginosus* (p=0.021) and decreased *Alloprevotella uncultured Prevotellacae* (p=0.035) of patients with a reduced VC_{max} **(Figure 23).**

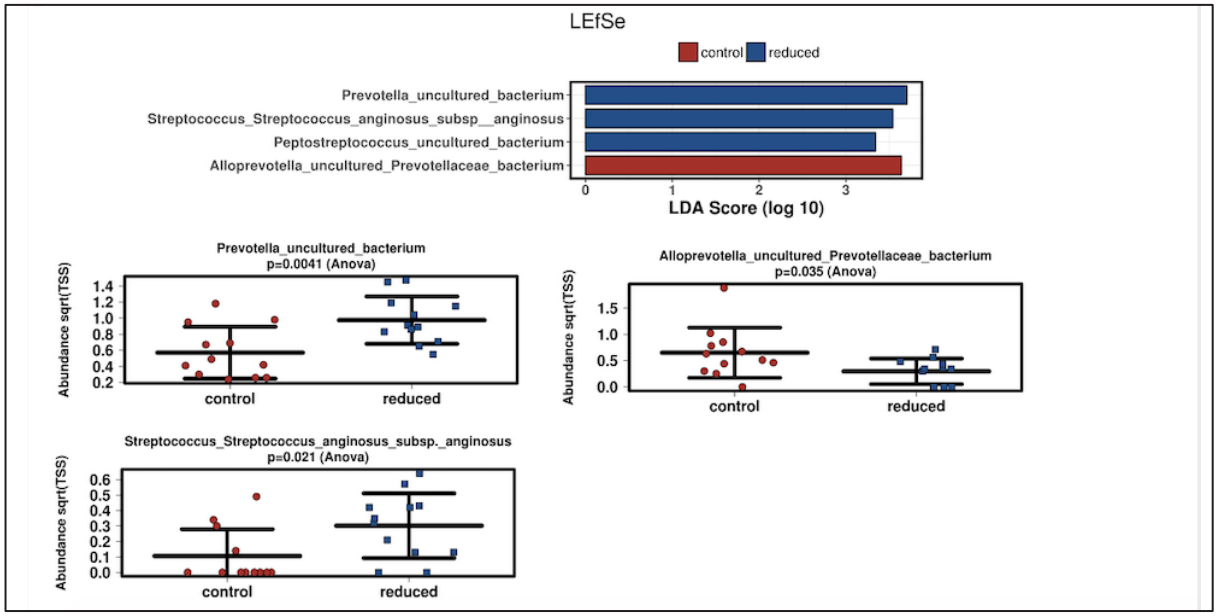

Figure 23: Results of LEfSe subgroup analysis of patients with reduced VC_{max}. Only parameters with p<0.1 in the LEfSe analysis are displayed as strip charts. (Reproduced from Arneitz C et al. Pediatr Res. 2020,[1] with permission of publisher, Springer Nature)

3.7.2. Subgroup Analysis of EA Patients with Reduced V_E

Matched pair analysis of the airway microbiome composition in EA patients with a reduced minute ventilation (V_E) revealed a significant difference of the Shannon diversity Index (F=9.1, *p=0.011*) and a statistical trend in Chao1 (F=4.4, *p=0.058*) **(Figure 24).** As a measure for beta-diversity RDA analysis showed significance (variance 64.34, F=1.13, p=0.044) (Figure 24).

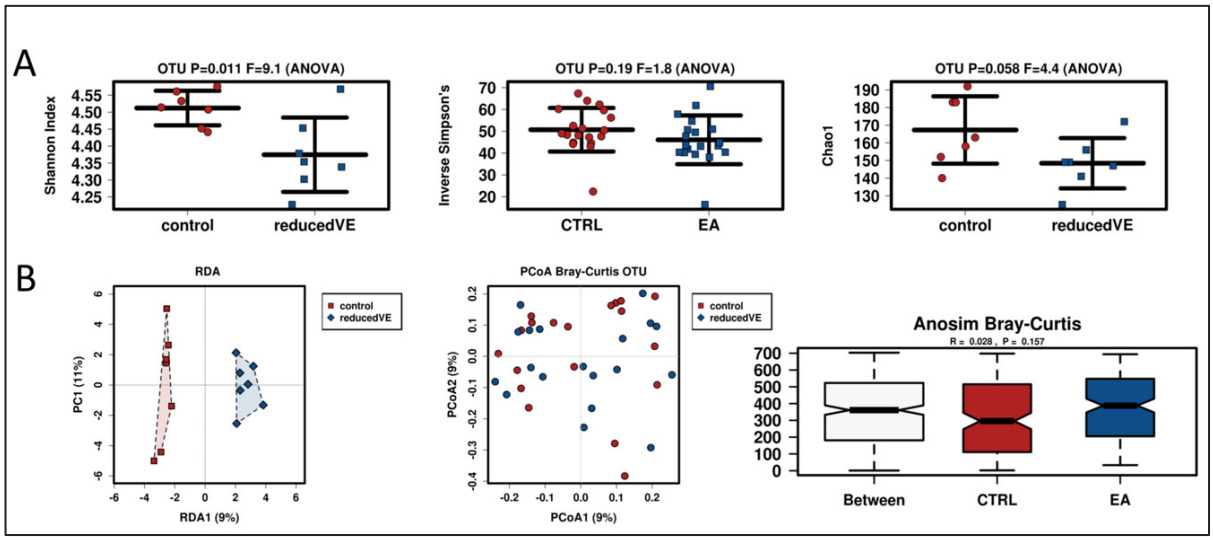

Figure 24: Parameters of alpha diversity (A), beta diversity (B; p=0.042 for the RDA analysis) in EA patients with a reduced V_E and their respective controls.

Significant differences in the taxa of *Epsilonbacteraeota* (p=0.046) were identified at the phylum level. At the order level significantly different abundances of *Saccharimonadales* (p=0.027) and *Campylobacterales* (p=0.046) were observed.

Marker bacteria of LEfSe analysis in species level revealed significant higher OTUs of *Actinomyces graevenitzii* (p=0.013), *Dialister* (p=0.041) and *Prevotella* (p=0.028) **(Figure 25).**

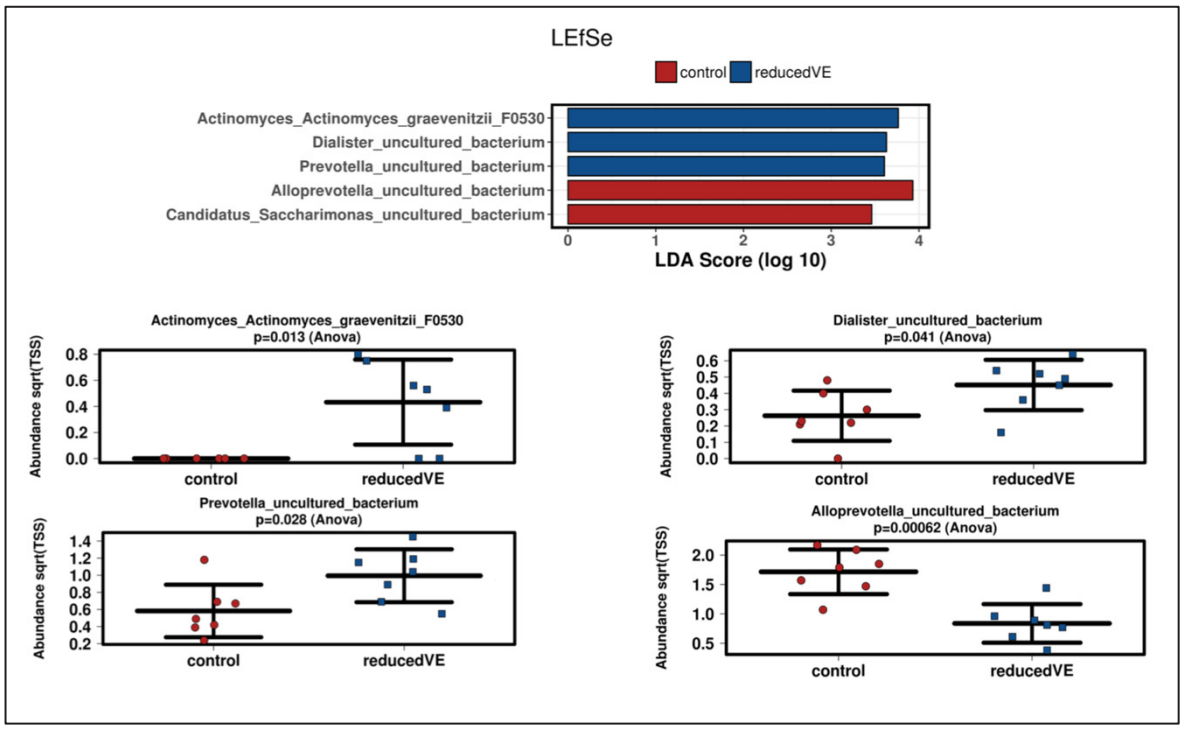

Figure 25: Results of LEfSe subgroup analysis of patients with reduced V_E. Only parameters with p<0.1 in the LEfSe analysis are displayed as strip charts.

3.7.3. Subgroup Analysis of Preterm EA Patients

Comparison of the airway microbiome in formerly preterm EA/TEF patients (n=11) to formerly full-term patients (n=8) revealed no differences of alpha- and beta diversity **(Figure 26).**

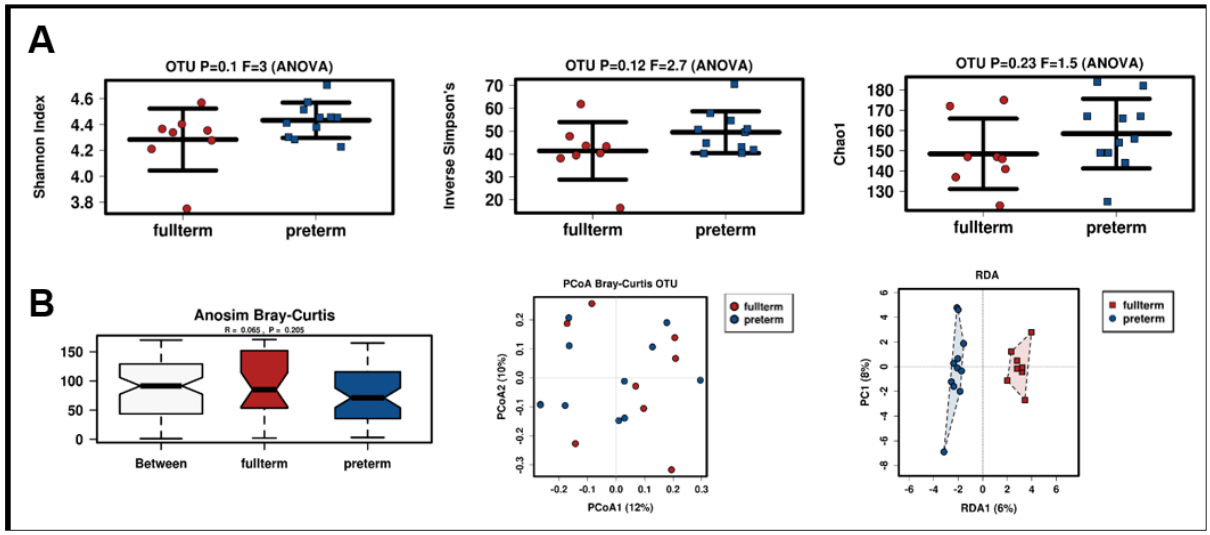

Figure 26: Microbiome analysis of EA/TEF patients born full-term (n=8) and preterm (n=11). Parameters of alpha diversity (A), beta diversity (B; p=0.274 for RDA analysis).

(Reproduced from Arneitz C et al. Pediatr Res. 2020,[1] with permission of publisher, Springer Nature)

The relative abundances at the phylum and order levels comparing EA/TEF patients born full-term and preterm are shown in **Figure 27**.

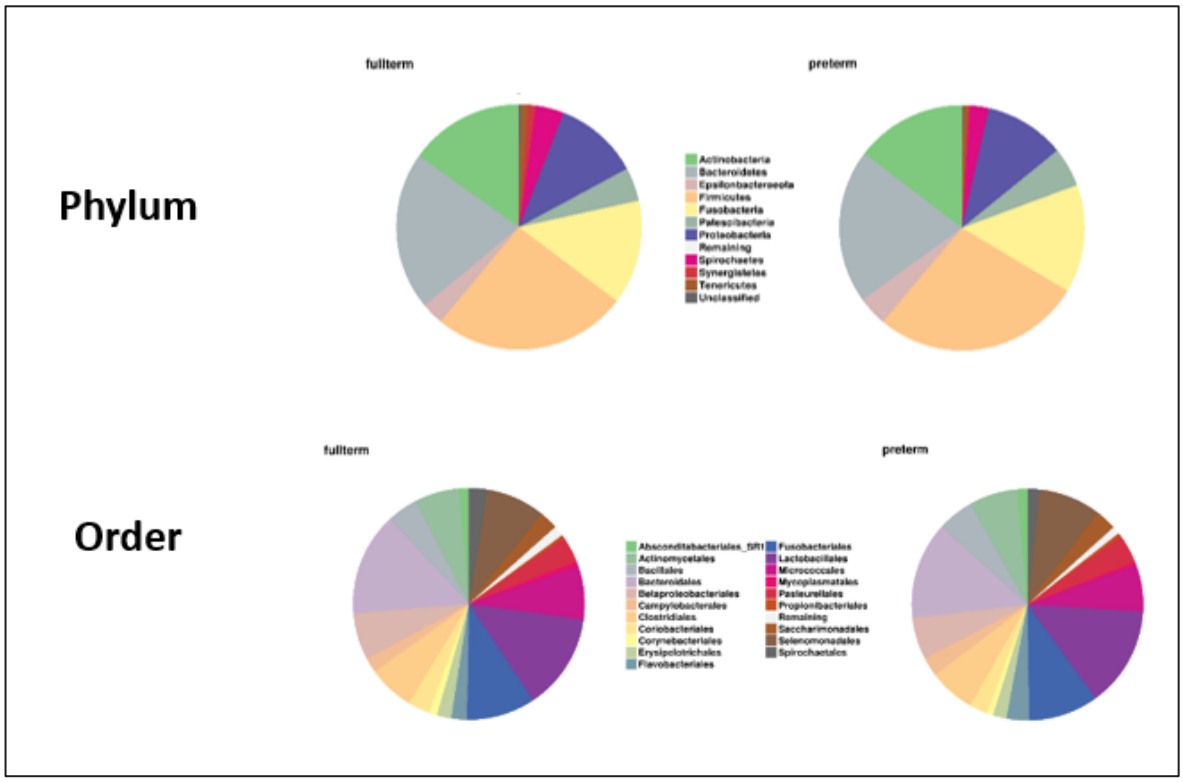

Figure 27: Microbiome analysis of EA/TEF patients born full-term (n=8) and preterm (n=11). Relative abundance at the phylum and order level. (Reproduced from Arneitz C et al. Pediatr Res. 2020,[1] with permission of publisher, Springer Nature)

LEfSe analysis revealed significantly enriched *Campylobacter uncultured bacterium* (p=0.0075), *Bergeyella uncultured bacterium* (p=0.041) and *Neisseria meningitides* (p=0.03) and significantly decreased *Filifactor uncultured bacterium* (p=0.03) in formerly preterm patients **(Figure 28).**

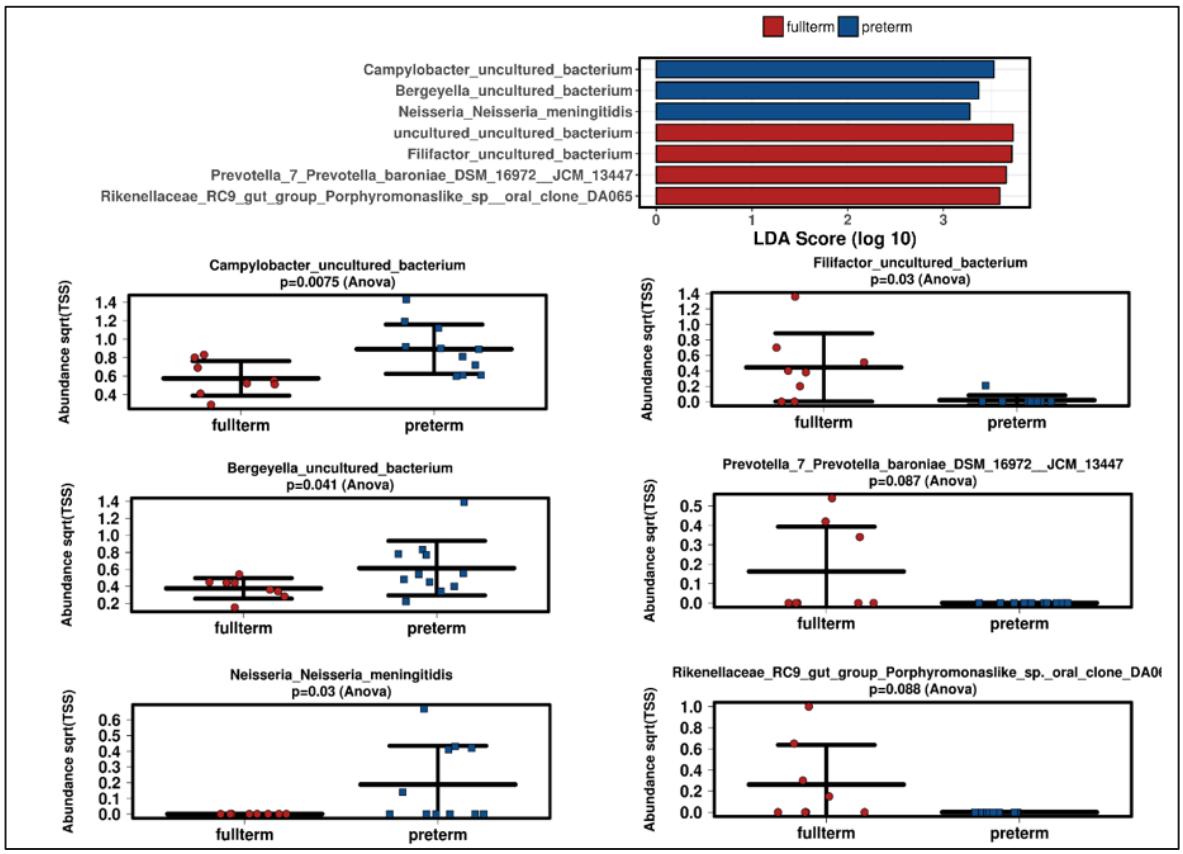

Figure 28: Microbiome analysis of EA/TEF patients born full-term (n=8) and preterm (n=11). Results of LEfSe subgroup analysis, only parameters with p<0.1 in the LEfSe analysis are displayed as strip charts. (Reproduced from Arneitz C et al. Pediatr Res. 2020,[1] with permission of publisher, Springer Nature)

4. Discussion

4.1. Answer to Research Questions and Summary of Results

The main findings of the present study were a significantly decreased VC_{max} and exercise performance as well as discreet airway microbiome differences in EA patients compared to healthy age and sex matched controls at a mean follow-up of 24 years.

We could verify our hypothesis 1 and 2:

(1) Cardiopulmonary performance capacity was significantly reduced following EA repair.

(2) Ventilations disorders occurred significantly more frequent in EA patients.

We had to reject our hypothesis 3 and 4:

(3) Airway microbiome was not significantly different, in terms of alpha- and beta diversity.

(4) Impaired pulmonary function was not associated with differences of the airway microbiome composition.

However, significant different marker bacteria between EA patients and controls were shown in the LEfSe analysis and subgroup analysis of patients with reduced minute ventilation revealed significant differences.

4.2. Anthropometric Data

Our EA patients had a significantly lower muscle mass compared to the control group. This could be associated with a decreased physical fitness and impaired locomotor function as described in previous studies.[8,9,99] However, there was no statistically significant difference in the amount of physical activity and training per week. Therefore, a routine cardiopulmonary function evaluation in

school-aged patients and a referral to physiotherapy if required seems to be necessary in EA patients.

4.3. Spirometry

Spirometry revealed that the majority of EA patients in our study (78.9%) had some sort of ventilatory impairment confirming other reports of long-term pulmonary function in patients following EA repair.[3–8,49,52] EA patients showed a significantly lower VC_{max} than their age and sex matched peers in our series.[1] Reduced lung volumes following EA repair were found frequently in long-term pulmonary function tests.[3,6]

Our spirometry results correlate with previously published data on long-term pulmonary function in patients following EA repair.[3–8,49,52] Sistonen et al. found significantly increased respiratory morbidity in adults following EA surgery with a mean age of 36 years.[5] While patients with an obstructive ventilation disorder did not show any significant differences in the performance capacity, the presence of a restrictive ventilation disorder was significantly linked with a reduced relative performance capacity. The proportion of restrictive pulmonary dysfunction in patients after esophageal atresia compared to healthy controls is strikingly high; it was documented in almost half of our EA patients. Nevertheless, it remains unclear whether an altered lung parenchyma or a restricted thoracic mobility as a sequelae of the operation acts as the underlying cause. A comparison with patients who had thoracoscopic surgery would be very interesting in this regard. Furthermore, restrictive ventilatory defects were significantly correlated to the inter-pouch distance, the duration of post-operative ventilation, recurrent aspiration pneumonia during infancy and GERD in recently published reports.[7] Peetsold et al. summarized possible explanations for restrictive pulmonary impairment, including recurrent infections, micro-aspirations due to gastro-esophageal reflux, scaring and scoliosis.[6] Pedersen et al. suggest that restrictive ventilation disorders occurring in EA is probably due to poor lung growth after thoracotomy.[100] Furthermore, the right dorso-lateral thoracotomy as a standard surgical approach for tracheoesophageal fistula seems to lead to significant late musculoskeletal consequences.[101]

4.4. Cardiopulmonary Exercise Performance

Studies on exercise capacity in patients following esophageal atresia repair are rare, contain small patient cohort, show divergent results and especially long-term evaluations are missing. Sistonen et al. found significantly increased respiratory morbidity in adults following EA surgery with a mean age of 36 years, but did not perform cardiopulmonary exercise testing.[5] Our prospective study can provide a long-term follow-up with spirometry and bicycle ergometry: patients following esophageal atresia repair show a significant lower relative performance capacity, significant lower maximal oxygen uptake and lower vital capacity compared to controls at a mean follow-up of 24 years.[1]

Many previous studies used standardized treadmill testing, using the Bruce protocol.[3,6–8,49,50] Treadmill testing was preferred over bicycle testing, because underdeveloped knee extensors can be a limiting factor in children.[8] Zaccara et al. found significantly lower exercise duration (*p=0.01*) in 8 patients with a mean age of 11.6 years. Gischler et al. and van der Cammen-van Zijp et al. tested EA patients 5 years following surgery and showed that the maximal exercise tolerance was significantly below the norm.[3,50] The reduced exercise tolerance may contribute to persistent respiratory morbidity and impaired growth.[3] We found no significant difference in size, weight or body mass index in our cohort. Further, we doubt that cardiopulmonary exercise testing is reliable in children younger than 7 years of age, because voluntary exhaustion in small children is difficult to achieve.

Only three groups have studied the maximum exercise capacity of EA patients using bicycle ergometry and presented divergent results; none of these studies, however, has prospectively compared the cardiopulmonary exercise results to a healthy age- and sex-matched control group.[4,51,52] Olbers and co-workers performed a cardiopulmonary exercise test in 24 EA patients at the 7-year follow-up.[52] They found a significant correlation between desaturation and antibiotic prophylaxis users (*p=0.024*).[52] Beucher et al. found in their retrospective study a normal maximal oxygen uptake ($VO_{2\,max}$) in all cases, except one, who did not reach maximal effort.[4] The cardiopulmonary function tests showed a poor ventilatory response in almost half of their patients. Patients with an limited ventilatory reserve

had also an abnormal cardiopulmonary testing, which could not be shown in our study.[4] However, a reduced respiratory minute volume lead to a significantly reduced relative performance capacity. Montgomery et al. compared EA patients with and without severe respiratory complications and found no difference in maximal working capacity; overall they reported a slightly decreased exercise capacity in EA patients at a mean age of 14 years.[51]

Peetsold et al. reported normal maximal oxygen uptake in 38 tested EA patients with a mean age of 13 years, except in one patient, probably because of airway obstruction.[6] Toussaint-Duyster et al. showed a significantly lower mean endurance time ($p<0.001$) in a relatively large patient cohort, including 55 8-years old EA patients.[8] 17 of these patients have been previously tested at the age of 5 years; their exercise capacity was not significantly different, suggesting that a reduced exercise performance may persist.[8]

Neither congenital anomalies of the heart nor the trachea had a significant impact on the relative performance capacity or spirometry results. Thus, early repair of a congenital heart malformation or tracheomalacia seems to achieve normal values in the relative performance capacity. Further, a poor risk classification (Spitz II) does not lead to a reduced relative performance capacity. Montgomery et al. compared EA patients with and without severe respiratory complications and found no difference in maximal working capacity; overall they reported a slightly decreased exercise capacity in EA patients at a mean age of 14 years.[51]

A strong correlation between ventilation disorders and exercise performance capacity has also been reported in previous studies.[3,6–8] Furthermore, earlier exhaustion in CPET significantly correlated with the inter-pouch distance, duration of postoperative ventilation, gastro-esophageal reflux disease and recurrent aspiration pneumonia during infancy.[7]

4.5. Airway Microbiome

In our study, we have examined the airway microbiome following esophageal atresia repair and compared the results to an age- and sex-matched healthy control group. The determination of the pulmonary microbiome composition in healthy lungs is still very limited, only a few papers addressed the “normal human lung microbiome”.[72]

Alpha- and beta-diversity of the deep induced sputum samples did not differ significantly between EA patients and controls. Even chronic pulmonary impairment like in tracheomalacia or restrictive ventilation disorders revealed no significant dysbiosis of the airway microbiome.[1]

LEfSe analysis between the two groups showed marker bacteria such as *Prevotella, Streptococcus anginosus* or *Mogibacterium timidum* on the species level. These bacteria are considered normal bacterial inhabitants of the oral cavity.[102] However, *Prevotella* and *Streptococcus anginosus* were also found among the respiratory microbiota of patients with chronic lung diseases.[103,104] Furthermore, higher relative abundances of *Prevotella* in airway microbiota have been shown to be associated with an increased host inflammatory response, protracted bacterial bronchitis, asthma development and higher risk of progressing chronic obstructive pulmonary disease (COPD).[103,105,106] However, obstructive airway disease was uncommon in our study cohort, but recurrent cough occurred in 20% of our patients and may be related to prolonged respiratory tract infection. Recurrent respiratory tract infections are common in infants with EA, but are described to become less frequent with increasing age.[107,108] Investigations of the aerodigestive microbial composition in children with chronic cough revealed that the lower airway microbiota was enriched with *Prevotella* in the bacterial bronchitis group.[109]

Airway microbiome studies have also found a high relative abundance of oral microbes, suggesting that recurrent microaspirations might influence the lower airway microbiota.[102,109] Especially in EA patients with a high number of possible aspirations associated with esophageal dysmotility and gastroesophageal reflux,

the oral microbiota could have a major impact on the composition of the airway microbiome, but still, only little is known about the microbial functionality and the kinetics of microbiome changes.[72]

The microbiota composition can be influenced by exogenous forces including diets, environmental biodiversity, infection and antibiotics.[110] For instance, antibiotic-induced changes of the microbiome composition may play an important role in the formation of allergies, autoimmunity and infectious diseases.[110] The exclusion criteria of our study were designed to avoid as many of these confounding factors as possible. However, the possibility to positively shape the airway microbiome in EA/TEF patients with for instance pro- or prebiotics is subject for further examinations.

Airway microbiome composition of EA patients with a reduced VC_{max} or preterm EA patients revealed no significant dysbiosis and similar taxa in the LEfSe analysis.

The composition of the airway microbiome in EA patients with a reduced V_E showed significant differences of alpha and beta diversity in a matched pair analysis. The V_E is an important parameter for oxygen intake under physical exertion. A reduced pulmonal capacity can be caused by restrictive and obstructive ventilation disorders or lack of utilization of lung capacity due to other performance-limiting factors. In our patient cohort, the reduced V_E seems to be caused by the presence of ventilation disorders. Differences in the taxa abundances were similar as described above. LEfSe analysis in these patient group revealed significant higher OTUs of *Actinomyces graevenitzii*, *Dialister* and *Prevotella. Actinomyces graevenitzii* is an increasingly recognized pathogen of respiratory infections, causing pulmonary actinomycosis or abscess formation, and should be included in the differential diagnosis for organizing pneumonia.[111] *Dialister* (phylum Firmicutes) belongs to the normal nasal microbiota and was found to be significantly more abundant in subjects with non-exacerbated asthma or ventilator-associated pneumonia.[112,113] Recent literature shows, that different pulmonary diseases can change the bacterial load and patients with COPD show a significantly decreased

bacterial burden compared to controls.[72] Whether or not a reduced V_E can progress to a pulmonary disease is a hypothesis that needs future research.

Loss of diversity and dysbiosis play an important role in several chronic inflammatory diseases, but still, only little is known about microbial functionality and the kinetics of microbiome changes.[72] Prospective, long term studies are needed to understand the interactions between changes of the pulmonary microbiome and disease progression.

4.6. Limitations and Strengths

Possible limitations of this study include that the deep induced sputum samples were used to examine the airway microbiome. There are concerns that these samples may be contaminated by the oropharyngeal microorganisms; however, deep induced sputum appears to be the only method that is ethically justifiable, since assessment of the pulmonary microbiome is only possible with bronchoalveolary lavage or bronchoscopy, which can only be obtained under anesthesia. Thus, deep induced sputum is frequently used to describe the airway microbiome.[12,72]

Other possible limitations are the relatively small sample size and the mode of testing by bicycle spiroergometry instead of treadmill testing. Treadmill testing was preferred over bicycle testing in previous EA studies, because underdeveloped knee extensors could be a limiting factor in children.[8] However, in our long-term follow-up, the youngest patient was already 14 years old and median age was 24.7 years. Large sample sizes in orphan pediatric diseases are difficult to achieve and other follow-up studies of EA patients have included between 8 and 63 patients .[3,4,6–8,49,51,52]

The strengths of our study are the long-term follow up of 24.7 years and the age- and sex-matched control group.

4.7. Conclusion

EA patients frequently showed restrictive ventilation disorders and impaired cardiopulmonary function and discreet changes of the airway microbiome. Long-term examinations of patients with congenital esophageal atresia consisting of routine locomotor function evaluation, spirometry and spiroergometry are necessary in order to detect impaired cardiopulmonary function and to prevent the progression of associated complications. Prospective, long-term studies are needed to unravel possible interactions between alterations of the airway microbiome and impaired pulmonary function.

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