# The pleural effusion microbiome in lung transplant and non-transplant patients

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29 **Key Findings:**

30 - Early- and late-stage pleural effusions in lung transplant recipients differ in
31 cellular and microbiome composition.
32 - Previous thoracocentesis influences the pleural microbiome.
33 - The pleural effusion microbiome correlates to the pulmonary microbiome in lung
34 transplant recipients, suggesting a pulmonary origin of the pleural microbiome.

35

## ABSTRACT

**BACKGROUND:** Pleural effusions are common complications of various disorders, ranging from congestive heart failure to pneumonia to a wide range of malignancies. While these have been extensively described, pleural effusions of unknown etiology occur in a substantial number of lung transplant recipients and are associated with shorter survival. Increasing evidence implicates the pulmonary microbiome in several diseases while recent studies focused on the pleural microbiome in infectious and malignant effusions. The pleural microbiome in lung transplant recipients, however, has not yet been investigated and the effects of long-term immunosuppressive and antibiotic treatment on the pleural microbiome are unclear to date.

**METHODS:** We performed a retrospective analysis of 52 pleural effusions of 47 patients with and without lung transplant. Additionally, 14 associated bronchoalveolar lavage samples of lung transplant recipients acquired within 4 weeks around the thoracocentesis procedure were included in the study. Microbiome of pleural effusion and bronchoalveolar lavage samples were analyzed by 16S rRNA sequencing. Results were correlated with clinical and microbiological data.

**RESULTS:** Early-stage pleural effusions occurring up to two months after lung transplantation differed substantially from late-stage pleural effusions regarding their cellular content and microbiome composition. Comparing late-stage pleural effusions to non-transplant patients we found a trend towards a higher α and β diversity in lung transplant pleural effusions. Long-term macrolide therapy in a subgroup of lung transplant recipients did not affect the pleural effusion microbiome. Significant differences in the pleural microbiome were found in patients with previous thoracocentesis procedures compared to pleural effusion at first thoracocentesis. With

60 corresponding bronchoalveolar lavage samples of lung transplant recipients with
61 pleural effusions available, we describe for the first time a direct correlation between
62 the pulmonary microbiome and the pleural microbiome, which was further associated
63 with an increasing exudative composition of the effusion.

64 **CONCLUSION:** Lung transplantation and time after lung transplantation seem to affect
65 the microbiome of pleural effusions. Furthermore, thoracocentesis procedures
66 influence the pleural microbiome. The composition of the pleural microbiome correlates
67 with the pulmonary microbiome, suggesting a communication of both compartments.

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69 **Key words** (9)**:** lung transplantation; lung allograft; pleura; pleural effusion; pleural
70 microbiome; pulmonary microbiome; bronchoalveolar lavage; thoracocentesis; 16S rRNA
71 sequencing;

## INTRODUCTION

Pleural effusions are fluid accumulations in the pleural cavity that can lead to dyspnea and cough. Either of transudative or exudative origin [1], etiologies range from heart, renal and liver failure to (para-)infectious and (para-)malignant pulmonary causes [2]. Pleural effusions represent a common complication of these disorders and are associated with worse prognosis [3] in virtually every of these diseases. Due to the low yield of current microbiological and cytopathological routine diagnostics, some pleural effusions remain without clear etiology, representing a major challenge in patient treatment and management [4–6]. Pleural effusions of unclear etiology also pose a frequent problem in the care of lung transplant (LTX) recipients and are associated with worse survival [7, 8].

Pleural effusion biology and in particular host-microbe interactions in the pleural space are poorly understood - in immunocompetent as well as in immunocompromised patients. While historically the pleural compartment was considered a sterile space due to the unavailability of culture-independent techniques, a few studies recently investigated the pleural microbiome in empyema and malignant pleural effusions [9–11]. Despite several factors possibly having a profound impact on the (pleural) microbiome e. g. severe immunosuppression, broad antibiotic prophylactic therapy and open chest surgery and despite being frequently affected by pleural effusions, the pleural microbiome in LTX recipients has never been studied so far.

In this study we aimed to investigate differences in the pleural microbiome in patients with and without lung transplantation, differences in patients with and without repeated thoracentesis procedures and the correlation between the pleural and pulmonary microbiome in patients with available corresponding bronchoalveolar lavage samples.

96 **MATERIALS AND METHODS**

97 *Ethics*

98 This retrospective study was approved by the local ethics committee of the Ludwig-
99 Maximilians-University (LMU) Munich. Written informed consent to participate in this
100 study was obtained from all patients, in accordance with approval by the local ethics
101 committee of LMU, Munich, Germany (Project 333-10, 454-12).

102 *Sample selection and patient cohort*

103 This retrospective study included 52 pleural effusion samples from 47 LTX recipients
104 and patients with other underlying disorders obtained between March 2021 and March
105 2022 from the CPC-M bioArchive at the Comprehensive Pneumology Center (CPC
106 Munich, Germany). Pleural effusions of LTX recipients were classified as early-stage
107 if diagnosed until 60 days after transplant. Pleural effusions of LTX recipients occurring
108 later than 60 days after transplant were classified as late-stage. 7 early-stage pleural
109 effusion samples of LTX recipients,19 late-stage pleural effusion of LTX recipients and
110 21 pleural effusions of non-transplant patients were available. 5 LTX recipients had a
111 second pleural effusion available in the biobank. Here, the first pleural effusion was
112 included into the according early- or late-stage analysis, while the second effusion was
113 only included in the previous thoracocentesis analysis. Clinical data were collected
114 from patient charts and included basic characteristics such as age and gender, as well
115 as diagnoses and medication. Laboratory data included serum and pleural laboratory
116 chemistry (including total protein, albumin, LDH, cholesterol and triglycerides), pleural
117 differential cell count as well as pleural pH, glucose, hemoglobin, and lactate levels.
118 Microbiological data included bacterial cultures as well as acid fast bacteria (AFB)
119 staining, tuberculosis polymerase chain reaction (TB PCR) and tuberculosis cultures.

120 Classification into trans- and exudates was performed according to Light's criteria [1].
121 For borderline exudates we further used the protein gradient as described by Romero
122 et al. [12].

123 *Thoracocentesis*

124 Thoracenteses were performed in the pulmonary and thoracic surgery department of
125 the university hospital of LMU Munich according to internal standards. Briefly, a
126 puncture side was identified using a portable ultrasound. The puncture site was
127 disinfected using 80% isopropyl alcohol at least thrice. Following sterile preparation, a
128 sterile drape covered the patient's torso but the puncture side. Following another
129 disinfectant application, local anesthesia of the puncture side and the chest wall was
130 achieved using 1% lidocaine solution. A soft thoracocentesis pleural catheter was
131 inserted and pleural fluid was removed by manual aspiration.

132 *Bronchoscopy*

133 Bronchoscopies were performed in LTX recipients in the pulmonary department of the
134 university hospital Munich according to internal standards. After oral bronchoscope
135 insertion, bronchoalveolar lavage was obtained with a fiberoptic bronchoscope in
136 wedge position within the selected bronchopulmonary segment of the transplanted
137 lung. The total instilled volume of normal saline (0.9 % of sodium chloride) was at least
138 100 mL, with at least 20% recovery.

139 *Nucleic acid extraction*

140 1.5 mL of pleural effusion and 5 mL of BAL was centrifuged for 10 min at 10000 x g.
141 DNA was extracted from the obtained pellet using Nucleospin Soil kit (Macherey-

142 Nagel) following manufacturer's instructions. Additionally, two blank extractions were
143 performed.

144 *Quantitative real-time PCR*

145 Quantitative real-time PCR of the 16S rRNA gene as proxy for bacterial load was
146 performed for samples, two blank extraction and 10 PCR no template controls on the
147 ABI 7300 Cycler (Applied Biosystems, Germany) using the following reaction mixture:
148 12.5 µl 2× Power SYBR Green master mix (Thermofisher Scientific, Germany), 5 pmol
149 primers (FP 16S / RP 16S), 0.5 µl 3% BSA and 2 µl DNA template in a total volume of
150 25 µl [13]. PCR conditions were 10 min at 95 ˚C; 40 cycles of 45 s at 95 ˚C, 45 s at 58
151 ˚C, 45 s at 72 ˚C; 10 min 72 ˚C; 1 cycle of 15 s at 95 ˚C, 30 s at 60 ˚C, 15 s at 95 ˚C.
152 All PCR products were checked on agarose gel. Amplification efficiency (calculated by
153 Eff=[10^(−1/slope) − 1]) was 90% with R2 of 0.993. The quantified gene copy numbers
154 were normalized to 1 mL of pleural effusion and BAL sample, respectively.

155 *16S rRNA gene sequencing*

156 Amplicon sequencing of the V3–V4 hypervariable region of the 16S rRNA gene was
157 performed on a MiSeq Illumina instrument (MiSeq Reagent Kit v3 (600 Cycle); Illumina,
158 San Diego, CA, USA) using the universal eubacterial primers 347F and 803R,
159 extended with sequencing adapters to match Illumina indexing primers [14]. To identify
160 potential contamination during DNA extraction and amplification, two blank extraction
161 and 10 PCR no template controls were performed. PCR was done using NEBNext high
162 fidelity polymerase (New England Biolabs, Ipswich, USA) in a total volume of 25 µl (10
163 ng DNA template, 12.5 µl polymerase, 5 pmol of each primer). PCR conditions were 5
164 min at 98 ˚C; 32 cycles of 10 s at 98 ˚C, 30 s at 56 ˚C, 30 s at 72 ˚C; 10 min 72 ˚C.
165 PCR products were purified using AMPure XP beads (Beckman Coulter Life Sciences,

166 Indianapolis, USA) and quantified via PicoGreen assay. Subsequently, indexing PCR
167 was performed using the Nextera XT Index Kit v2 (Illumina, Inc. San Diego, CA, US)
168 in a total volume of 25 µl (10 ng DNA template, 12.5 µl NEBNext high fidelity
169 polymerase, 2.5 µl of each indexing primer) and the following PCR conditions: 30s at
170 98 ˚C; 8 cycles of 10 s at 98 ˚C, 30s at 55 ˚C, 30s at 72 ˚C; 5min 72 ˚C. Indexing PCR
171 products were purified using AMPure XP beads, qualified and quantified via a
172 Fragment Analyzer™ instrument (Advanced Analytical Technologies, Inc., Ankeny,
173 USA) and pooled in an equimolar ratio of 4nM.

174 *Sequence data processing*

175 Sequences were analyzed on the Galaxy web platform [15]. FASTQ files were trimmed
176 with a minimum read length of 50 using Cutadapt [16]. Quality control was performed
177 via FastQC [17]. For subsequent data analysis DADA2 pipeline (Galaxy Version 1.20)
178 [18] was used with the following trimming and filtering parameters: 20 bp were removed
179 n-terminally and reads were truncated at position 280 (forward) and 200 (reverse),
180 respectively, with expected error of 6 (forward) and 8 (reverse). Subsequently,
181 amplicon sequence variants (ASV) were clustered into operational taxonomic units
182 (OTU) at 97% similarity using DECIPHER v 2.18.1 [19]. For both ASV and OTU,
183 taxonomic analysis was performed using SILVA v138.1. Reads were excluded if
184 classified as mitochondria or chloroplast or if the phylum was missing. Furthermore,
185 ASV and OTU occurring in blank extractions and PCR no template controls were
186 removed from sample data if their abundance was lower than 10% in the samples,
187 resulting in 5107 ASV and 3114 OTU remaining for final analysis. The sequence data
188 obtained in this study are deposited in the short read archive of NCBI under accession
189 number xxx.

190 *Statistical Analysis*

191 For statistical analysis the R package edgeR version 3.38.4, and the Python packages
192 pandas version 1.5.0, seaborn version 0.12.0, scikit-learn version 1.1.2, scikit-bio
193 version 0.5.6 and scikit-posthocs 0.7.0 were used. Normalization on OTU level was
194 performed using the Trimmed Mean of M-values method [20]. Differences in α diversity
195 by observed OTUs, Shannon's and Chao1 non-phylogenetic α diversity indexes were
196 analyzed using Mann-Whitney-U test. To identify bacterial genera of differential
197 abundancy OTUs were pooled on genus level. Differential abundancy was tested using
198 repeated Mann-Whitney-U-test with multiple testing adjustment using the Benjamini-
199 Hochberg procedure. Patient characteristics are presented as median (IQR) and
200 Mann-Whitney test was used for nonparametric testing. Statistical analysis was
201 performed by GraphPad Prism 8 (GraphPad Software, San Diego, CA) and SPSS
202 Statistics 25 (IBM SPSS, Armonk, NY).
203

204 **RESULTS**

205 *Comparison of early and late-stage pleural effusions in lung transplant recipients*

206 First, we compared 7 pleural effusions occurring early after transplant (median 21 days
207 post-LTX) to 19 pleural effusions occurring late after transplantation (median 188 days
208 post-LTX) (**Table 1, Figure 1A**). All the early-stage pleural effusions were exudates
209 while 4 (21%) of the late-stage pleural effusions were transudates. Microbiologic work-
210 up including culture of native pleural effusion as well as pleural effusion in blood culture
211 bottles yielded *staphylococcus epidermidis* in one late-stage pleural effusion sample,
212 all other bacterial cultures remained negative. Microscopy, PCR and culture for
213 mycobacteria yielded negative results (**Table S1**). Transbronchial biopsies at time of
214 pleural effusions were available for 7 (100%) of early-stage effusions and 17 (89%) of

215 late-stage effusions. Here, no evidence of acute rejection was seen in any of the
216 patients (all A0 according to ISHLT grading [21]). All early-stage pleural effusions were
217 considered a postoperative complication, 16 (84%) of the late-stage pleural effusions
218 were considered of unclear etiology, 2 (11%) were chylothoraces and one (5%) was
219 due to volume-overload in the setting of renal failure (**Table 1**). Early effusions
220 demonstrated significantly higher pleural LDH (473 vs. 152 U/l, $p<0.01$) and a higher
221 proportion of polymorphonuclear cells (63 vs. 4%, $p<0.01$), while late-onset effusions
222 displayed higher number of macrophages (0 vs. 9%, $p<0.05$) as well as a pronounced
223 lymphocytic predominance (21 vs. 81%, $p<0.05$) (**Table S1**).
224
225 The α diversity of the microbiome of early-stage pleural effusions was significantly
226 lower compared to late-stage pleural effusions in three different α diversity indices
227 ($p<0.001$, **Figure 1B**). The β diversity was significantly different between early-stage
228 and late-stage pleural effusions ($p=0.001$, **Figure 1C**). The most abundant bacterial
229 genera in LTX recipients were *streptococcus, veilonella, prevotella, rothia* and
230 *leptotrichia* (**Table S3**). 23 bacterial genera were more prevalent in late-stage effusions
231 than in early-stage effusions (FDR<0.05, **Figure 1D, Table S4**).
232
233 *Comparison of late-stage effusions of lung transplant recipients and non-transplant*
234 *patients*
235 Next, we compared the previous group of 19 late-stage effusions of lung transplant
236 recipients with pleural effusions of 21 non-transplant patients (**Figure 2A**). The etiology
237 of pleural effusions in non-transplant patients were heart failure in 8 (38%) patients,
238 (para-)malignant in 8 (38%) patients, renal failure in 2 (10%) patients and unclear,
239 rheumatoid and (para-)infectious in 1 patient each (5%). The non-transplant group
240 consisted of 11 exudates (52%) and 10 transudates (48%). Compared to non-

241 transplant patients, LTX recipients received extensive antimicrobial chemoprophylaxis.
242 Several LTX recipients also received long-term azithromycin therapy as an
243 immunomodulatory treatment to reverse or slow down chronic lung allograft
244 dysfunction (CLAD) [22]. A number of patients in both groups received further antibiotic
245 therapy (**Table 2**). Pleural differential cell counts revealed a predominance of
246 lymphocytes in LTX recipients compared to non-transplant effusions (81 vs 52%,
247 $p<0.05$) (**Table S2**). We found a trend towards a higher α diversity in patients after lung
248 transplantation compared to non-transplanted control, which was consistent
249 throughout different α diversity indices ($p=0.05$-$0.06$, **Figure 2B**). Pleural effusion β
250 diversity was not significantly different between lung transplant and non-transplant
251 patients, however, a trend towards a difference was seen ($p=0.078$, **Figure 2C**). Most
252 abundant bacterial genera in pleural effusions of non-transplant patients were
253 *veilonella, streptococcus, prevotella, haemophilus, neisseria* (**Table S5**). Again, there
254 was a trend towards higher pleural abundancies of a number of bacterial genera in
255 pleural effusions in lung transplant recipients (**Figure 2D, Table S6**). We further
256 compared late-stage pleural effusions of LTX recipients with and without long-term
257 azithromycin therapy. Here, no differences were seen on α and β diversity (**Figure S1**).
258

259 *Comparison of pleural microbiome in patients with and without previous thoracentesis*
260 With previous thoracentesis procedures possibly influencing the pleural microbiome,
261 we investigated the differences of the pleural microbiome in patients with (n=24, hereof
262 13 LTX) and without (n=28, hereof 18 LTX) previous thoracentesis. α diversity was
263 significantly higher in patients with previous thoracentesis ($p<0.01$, **Figure 3A**). Also,
264 significant differences in β diversity were seen ($p<0.01$, **Figure 3B**). Differential
265 abundancy analysis identified a number of bacterial genera as more prevalent in the

266 pleural microbiome of patients with previous thoracentesis, including *prevotella, rothia,*
267 *gemella, neisseria* and *streptococcus* (**Figure 3C, Table S7**).
268
269 *Correlation of the pulmonary and pleural microbiome in lung transplant recipients*
270 For 14 (74%) late-stage pleural effusions of LTX recipients, corresponding
271 bronchoalveolar lavage (BAL) samples, acquired around the time of pleural effusion
272 drainage, were available. To further delineate a possible origin for the pleural
273 microbiome, we investigated the associations of pleural and pulmonary bacterial
274 abundancies on genus level. A significant correlation of bacterial abundancies between
275 pleural and BAL microbiome was found in 11 out of 14 patients (**Figure 4A-B**).
276 Spearman's rank correlation coefficient was positively correlated with pleural protein
277 content and ratio as well as albumine ratio (**Figure 4C**). There was a trend towards a
278 negative correlation of the Spearman's R rank correlation coefficient with the albumine
279 gradient – altogether indicating an association of pleural and pulmonary microbiome
280 communication with exudative pleural effusion etiology (**Figure 4C**).
281
282 **DISCUSSION**
283 While the role of the respiratory microbiome has been increasingly recognized in
284 pulmonary diseases from COPD to cystic fibrosis and lung transplantation, the
285 microbiome of the pleural space and pleural effusions remains widely uncharacterized
286 [23]. Pleural effusions of unclear etiology pose a frequent clinical problem in lung
287 transplant recipients and are associated with reduced survival [8, 24]. For the first time,
288 we characterized the pleural microbiome in lung transplant recipients and showed
289 differential characteristics of early- and late-stage effusions. Also, pleural effusions of
290 non-transplant patients showed a distinct pleural microbiome and an influence of
291 thoracentesis procedures on the pleural microbiome was seen. We were able to

292 demonstrate a strong correlation of the pulmonary and the pleural microbiome, which
293 was more pronounced the more exudative the effusions were.

294 Early-stage and late-stage pleural effusions showed pronounced differences: early-
295 stage pleural effusions were neutrophil-rich, while late-stage pleural effusions were
296 found to be predominantly lymphocytic and had a higher share of macrophages. A high
297 percentage of lymphocytes in late-stage effusions was also noted in a study by Joean
298 et al. [24]. Acute cellular rejection as cause for lymphocytic effusions was not seen in
299 our data, which is in further agreement with Tang et al [8]. However, lymphocytic
300 effusions might also represent a localized graft vs. host interaction in the pleural
301 compartment even without recognizable pulmonary rejection. Profound changes in
302 differential cell count may reflect acute, postoperative, wound healing pleural
303 responses in the early-stage pleural effusions compared to the more chronic
304 inflammatory process in late-stage pleural effusions. Corresponding to the differences
305 in differential cell count profiles, the microbiome of the pleural effusions showed
306 reduced α and β diversity of early- compared to late-stage effusions – hinting at an
307 association between pleural immune cell characterization and the microbiome. Further,
308 extensive antibiotic coverage during the transplant procedure might have led to a
309 significant reduction in the diversity of the pleural microbiome of early-stage effusions.

310 While early-stage and late-stage pleural effusions in LTX recipients vary widely in
311 terms of α and β diversity, the differences between late-stage pleural effusions of LTX
312 recipients with those of non-transplant patients were only at the border of significance.
313 However, a trend was noted throughout different measures of α diversity as well as for
314 a range of bacterial genera. It is possible that the small number of patients together
315 with the diversity of diagnosis in the non-transplant group mitigated specific differences
316 e. g. of heart failure transudates or (para-)malign exudates compared to LTX effusions.

317 Long-term immunomodulatory treatment with azithromycin has been demonstrated to
318 be effective in the prevention and treatment of chronic lung allograft dysfunction [25–
319 27]. Azithromycin achieved high pleural concentrations in an animal model of
320 empyema [28]. Interestingly, a subgroup-analysis of late-stage effusions of LTX
321 recipients with long-term azithromycin therapy compared to effusions of LTX recipients
322 without long-term azithromycin showed no differences in the composition of the pleural
323 microbiome. This is in line with data by Spence et al. demonstrating that there were no
324 differences in the pulmonary microbiome in LTX recipients with and without
325 azithromycin therapy and that thus the beneficial effects of azithromycin might rather
326 be of an immunomodulatory nature [29].

327 Interestingly, we found a significant influence of previous thoracentesis in transplant
328 and non-transplant patients on the pleural microbiome. The microbiome in patients with
329 previous thoracentesis showed amongst others a higher abundancy of some genera
330 of the cutaneous microbiome. This implies that thoracentesis procedures might effect
331 changes in the microbiome, potentially through the introduction of skin commensals
332 and consecutive changes of the pleural microbiome.

333 Several studies elucidated the microbiology of empyema and established a
334 multimicrobial composition with a prominent role for bacteria originating from the
335 oropharynx, e.g. from the *streptococcus anginosus group*, and anaerobes, e.g.
336 *fusobacteria* [10, 30, 31]. In the recently published TORPIDS study, around 80% of
337 243 empyema samples had a polymicrobial and only around 20% of the empyema
338 samples had a monomicrobial infection [10]. Around half of the samples with
339 monomicrobial infection revealed *streptococcus pneumoniae* as the causative
340 pathogen [10]. Animal models with *streptococcus pneumoniae* demonstrated that a
341 transpleural spread of infection is possible by an intracellular translocation through

342 visceral mesothelial cells into the pleural space [32]. However, in empyema originating
343 from a parapneumonic etiology bacterial composition of pneumonia and the
344 subsequent empyema does not always overlap [31]. Further, around 30% of empyema
345 in the MIST2 study developed without pneumonia, which raises the question for other
346 forms of transmission or another local origin of the pathogen [31]. While empyema is
347 reported to be frequent in lung transplant recipients [8], we did not find any empyema
348 in our cohort neither in the lung transplant nor in the non-transplant group.
349 Nevertheless, in our study even non-transplant patients with transudates in the setting
350 of heart or renal failure displayed a diverse pleural microbiome, incorporating bacteria
351 that have been implicated in polymicrobial pleural infection, e. g. of the *streptococcus*
352 *anginosus group* etc. Thus, it is conceivable that some pleural empyema may originate
353 from the resident pleural microbiome itself, especially in the context of older age or
354 immunosuppression. These data further raise the question if a communication
355 between the pulmonary and the pleural compartment results in the formation of a
356 pleural microbiome. With corresponding bronchoalveolar lavage samples available in
357 the majority of the patients with lung transplantation, we were able to demonstrate a
358 robust correlation of the pleural and pulmonary microbiome in these patients.
359 Exudative markers were further correlated with a positive association between pleural
360 and pulmonary microbiome, suggesting an increased communication between these
361 two compartments especially in the setting of inflammation. Mechanisms of the pleuro-
362 pulmonary microbiome communication might play a pivotal role in the understanding
363 of empyema development. While these findings might not be simply extrapolated from
364 lung transplant recipients under antibiotic long-term therapy to non-transplant
365 individuals, this is regardless the first evidence for a quantitative correlation between
366 pleural and lower respiratory tract microbiome.

367 There are several limitations to this study: Due to the retrospective nature of the study,
368 the number of available samples was limited and no negative controls at sampling
369 timepoint were available. Negative controls were carried throughout the DNA
370 extraction and analysis course. Although contamination of the BAL by the
371 oropharyngeal bronchoscope passage is thought to be relatively small [33], we cannot
372 fully exclude contamination as bronchoscopies were not protected and performed in
373 the setting of clinical routine. The indication for pleural drainage was set solely for
374 clinical reasons by the responsible physician, thus introducing a selection bias towards
375 bigger, more symptomatic effusions.

376 Nevertheless, we believe that this study provides novel insights into the pleural
377 microbiome pointing out changes in pleural effusion microbiome due to thoracentesis,
378 correlation of the lower respiratory tract microbiome and the microbiome in pleural
379 effusions as well as differences between neutrophilic early-stage and lymphocytic late-
380 stage pleural effusions in LTX recipients.

381 Further prospective studies are needed to provide information about longitudinal
382 changes of the pleural microbiome in immunocompetent and immunocompromised
383 patients, as well as diagnostic and prognostic dues in the pleural microbiome. Further,
384 it will be of great interest to characterize the pleural microbiome in patients without
385 pleural effusions.

386 **CONCLUSIONS**

387 This study provides insights into the pleural effusion microbiome in patients with and
388 without lung transplantation. The microbiome composition in pleural effusion varies
389 between early-stage and late-stage pleural effusions. Differences in the pleural
390 microbiome between patients with and without previous thoracentesis might imply the

391 influence of pleural drainage on the pleural microbiome. Similarities between the
392 pleural effusion and pulmonary microbiome, particularly in patients with exudative
393 pleural effusion characteristics imply a communication between the pleural space and
394 the lower respiratory tract.

395 **Author contributions:**

396 Conceptualization of the study: CM, MG, SG, NK
397 Data collection: CM, MG, GS, IB, LE, PP, DKG, KM, CS, SM, MI, JB, NK
398 Data analysis: CM, MG, SG, GS, NK
399 Writing of the original draft: CM, MG, SG, NK
400 Editing of the original and final draft: CM, MG, SG, GS, IB, LE, PP, DKG, GB, AÖY, MS, KM,
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407 **Declarations of interest:**

408 **CM, MG have no conflict of interest to disclose.**
409 **SG:**
410 **GS:**
411 **IB:**
412 **LE:**
413 **PP:**
414 **DKG:**
415 **GB:**
416 **AÖY:**

417 **MS:**

418 **KM:**

419 **CS:**

420 **SM:**

421 **MI:**

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