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Journal:	*BMJ Open*
Manuscript ID:	bmjopen-2014-006677
Article Type:	Research
Date Submitted by the Author:	22-Sep-2014
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Primary Subject Heading:	Respiratory medicine
Secondary Subject Heading:	Pathology
Keywords:	Chronic airways disease < THORACIC MEDICINE, Emphysema < THORACIC MEDICINE, Microbiology < PATHOLOGY

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A Possible Role for *Lactobacillus* in the Pathogenesis of COPD

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Key Words: Lactobacillus, COPD, Inflammation, small airways

Abstract Word Count: 270
Total Word Count: 2979

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Abstract:

Objectives: The present study assesses the relationship between the status of GD1 positivity, presence of *Lactobacillus,* and the inflammatory response measured in host lung tissue in mild to moderate COPD. We hypothesize that there will be a loss of GD1 producing *Lactobacillus* with increasing severity of COPD and that GD1 has anti-inflammatory properties

Setting: Secondary care, 1 participating centre in Vancouver, BC, Canada.

Participants: 74 individuals who donated non cancerous portions of their lungs or lobes removed as treatment for lung cancer (normal lung function controls (n=28), persons with mild (GOLD 1) (n=21), and moderate (GOLD 2) COPD (n=25)).

Outcome Measures: Primary outcome measure was GD1 positivity within each group and whether or not this impacted quantitative histological measures of lung inflammation. Secondary outcome measures included *Lactobacillus* presence and quantification and quantitative histological measurements of inflammation and remodeling in early COPD.

Results: Total bacterial count (P>0.05) and prevalence of *Lactobacillus* (P>0.05) did not differ between groups. However, the GD1 gene was detected more frequently in the controls (14%) than in either mild (5%) or moderate (0%) COPD (P<0.05) samples. Macrophage and neutrophil volume fractions (0.012 ± 0.005 (mean ± SD) versus 0.026 ± 0.017 and 0.005 ± 0.002 versus 0.015 ± 0.014 respectively) in peripheral lung tissue were reduced in samples positive for the GD1 gene (P<0.0035).

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Conclusion: A reduction in GD1 positivity is associated with an increased tissue immune inflammatory response in early stage COPD. There is potential for *Lactobacillus* to be used as a possible therapeutic, however, a larger sample set needs to be tested and analyzed before an anti-inflammatory role of *Lactobacillus* in COPD can be confirmed.

Strengths and limitations of this study:

Strengths

- Study performed directly on human lung tissue
- Data shows a novel potential mechanism for *Lactobacillus* in COPD pathogenesis
- Patient population is well controlled and stratified between disease groups

Limitations

- Small sample size (however, it is relatively large for a lung tissue study)
- Small number of total positive GD1 samples
- Sample population derived from lung resection patients mostly for cancer therapy

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Introduction:

Chronic obstructive pulmonary disease (COPD) is a worldwide public health problem that affects approximately 10% of persons over 40 years of age [1] and predicted to become the 4th leading cause of death in the United States by 2020 [2]. Previous studies have shown an association between the decline in FEV_1 and the infiltration of peripheral lung tissue by neutrophils, macrophages, CD4+ and CD8+ T-cells, and B cell lymphocytes that have an increasing tendency to form tertiary lymphoid follicles in lungs from persons with severe (GOLD 3) and very severe (GOLD 4) COPD [3]. This data provide evidence that both an innate and adaptive inflammatory immune response may be present and initially begin in early grade COPD. However, what the target is for this adaptive immune response is not yet known. Evidence has been shown to support a potential auto-immune mechanism against elastin [4].

However, others believe that it could be environmental causes like bacteria that can drive this immune response [5]. Recently several groups have suggested that the lung itself is not sterile and that there is a detectable bacterial microbiome present [6-10]. They have also shown that this bacterial microbiome changes in COPD [7, 9]. It may be possible that some of these bacteria could be potential targets of the adaptive immune response observed in COPD while others, in contrast, may have beneficial roles.

One of these previous reports on the bacterial microbiome in lung tissue identified *Lactobacillus* as a potential bacteria that could discriminate between control lung tissue and COPD GOLD 4 [9]. In order to investigate how this might contribute to the pathogenesis of COPD, this study examines the host response to differences between *Lactobacillus* positive and negative samples as well as on *Lactobacillus* species either positive or negative for the bacterial Glycerol Dehydratase (GD1) gene. This gene is most commonly found on plasmids within the *Lactobacillus reuteri* species and along with potassium and 1,2 propanediol converts glycerol to reuterin via a dehydration reaction mechanism [11]. Reuterin is a broad spectrum antibiotic whose mechanism of action is postulated to act via an oxidative stress mechanism [12]. Expansion of *Lactobacillus* is associated with a reduction in reuterin production [13], potentially due to quorum sensing. This means that less *Lactobacillus* will be able to convert glycerol to the broad spectrum antibiotic reuterin when these bacteria are found in high abundance [14, 15]. Based on this information along with the previous findings within the lung tissue bacterial microbiome we hypothesize that there will be a loss of GD1 producing *Lactobacillus* with increasing severity of COPD and that GD1 has anti-inflammatory properties.

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Methods:

Preliminary Data Sample Group:

Samples from these individuals were from a previously published study [9] and the processing, tissue sampling methods, and DNA extraction methods have already been published. Demographic information was also previously published but in brief it consisted of tissue from 8 Non-smokers, 8 smokers, and 8 COPD GOLD 4. The non-smokers and smokers had normal lung function without obstruction. Their FEV1 percent predicted was 88.8 ± 13.4 and 94.3 ± 15.3 respectively, while their FEV1/FVC was 80.80 ± 4.82 and 76.65 ± 5.07. The COPD GOLD 4 group had an FEV 1 of 15.4 ± 2.4 and an FEV1/FVC of 26.83 ± 7.85. There was no significant difference between pack years smoked between the smokers (46.00 ± 12.24) and COPD GOLD 4 group (38.83 ± 14.97).

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Early COPD Group:

Tissue Collection:

The methods used to collect and preserve lung tissue for both PCR and qPCR are previously published [3, 9, 16, 17]. Briefly, lung tissue donated by 74 individuals, treated for lung cancer by either lobectomy or pneumonectomy were entered into this study. All 74 subjects provided informed consent for the use of their lung tissue in this study under conditions approved by the appropriate committees of all the institutions involved. The post bronchodilator FEV_1 and FEV_1/FVC measurements made during the pre-operative assessment of lung function were used to assign these donated tissues to "At Risk" controls (n=28), mild COPD (GOLD 1) (n=21) and moderate COPD (GOLD 2) (n=25). 3 out of the 74 individuals (2 in the control group and 1 in the GOLD 1 group) had used inhaled corticosteroids and all 74 were free from clinically apparent respiratory infection at the time of surgery. No recent antibiotic treatment information was recorded in these individuals.

Tissue Processing:

Following completion of the pathological examination each lung specimen was inflated, frozen, and stored at -80^0 C as previously described [3, 9, 17]. The specimen was kept frozen on dry ice while cut from apex to base into 2cm thick slices, and a drill press with a sharpened hollow cylinder removed cores of tissue from each lung slice. Two tissue cores were examined for 27/28 cases in the control group, 19/21 cases in GOLD 1 and 25/25 cases in GOLD 2. In the remaining three individuals only one tissue core was examined. Five series of 20 consecutive frozen sections were cut from each tissue core and sections 1-5, 8-12, and 14-18 were allocated

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for DNA extraction and sections 6-7, 13, 19-20 allocated to coded glass slides for histological staining.

qPCR for Total Bacteria and Lactobacillus:

For all qPCR assays a correction factor to account for plate variation was applied as described in the online supplement. A previously described assay was used to determine the total bacterial load and spanned the 16S hypervariabe V2 region. One modification was made to the analysis in that total bacteria were expressed as 16S/ng of DNA instead of bacteria/Rpp40 [9]. The assay for total *Lactobacillus* has been previously reported and no modifications were made [9, 18]. The forward primer sequence was 5-ACG AGT AGG GAA ATC TTC CA-3 and the reverse primer sequence was 5-CAC CGC TAC ACA TGG AG-3 and was designed to target all species in the genera *Lactobacillus* and *Lactococcus*. The values for total *Lactobacillus* were expressed as a percent of total 16S bacteria and was obtained from normalization to total 16S (e.g., (*Lactobacillus* value/16S value) x 100). The standard curve formulas for both assays can be found in the online supplement [Table S1].

PCR for glycerol dehydratase (GD1):

A 40 cycle PCR was performed on all DNA samples and yielded an approximately 560bp sized band resolved on a 1% agarose gel. The forward primer was 5-GTTCAGTCCGCCGCATATC-3 and for the reverse primer 5-GCCGCTCTTCGTGGATTTC-3. The cycling conditions have been published previously [19]. If at least one of the two samples from an individual tested positive then the individual was considered positive for bacteria containing the glycerol dehydratase (GD1) gene.

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Quantitative Histology:

Sections on coded glass slides were stained for macrophages, neutrophils, eosinophils, NK cells, dendritic cells and follicular dendritic cells, B-lymphocytes, CD8+ T-lymphocytes, and CD4+ T-lymphocytes. A more detailed breakdown of the staining can be found in [Table S2]. ImagePro Plus software v4.0 (MediaCybernetics Inc., Bethesda, MD) was used to compute the volume fraction (Vv) of the tissue taken up by specifically stained inflammatory cells in the small airway wall tissue using established point counting methods [3]. Intraobserver and interobserver error for the quantitative histology was assessed (Figure S1 & S2) and there was a good correlation between both (R^2 of 0.9046 and 0.9485 respectively).

Data Analysis:

Data from a previously published data set that consisted of non-smokers (n=8), smokers (n=8), and COPD GOLD 4 (n=8) [9] was first analyzed for the prevalence for GD1. Subsequent analysis was performed on the current data set for the role of *Lactobacillus* in early COPD. A multivariate analysis was performed to asses associations between the bacterial load, relative abundance of lactobacillus, inflammatory cell Vv, and airway wall thickness. Where applicable the mean ± standard deviation for the different groups was listed. Categorical data comparing the distribution of *Lactobacillus* detection through increasing disease severity and GD1 detection throughout GOLD stage utilized a chi-square test. Analysis of GD1 positive versus GD1 negative samples utilized multiple comparisons and a Bonferroni correction was applied. All other data utilized the Student's t-test for significance testing. A P-value of <0.05 was

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considered statistically significant unless otherwise stated due to Bonferroni correction. Multivariate analysis was performed and a correlation was considered significant if it was below a p-value of 0.05.

Results:

Early COPD Group Demographics:

Table 1 shows there was no significant difference in age, gender, or smoking history between the three groups of subjects (P>0.05) and the difference in FEV_1/FVC and FEV_1 percent predicted shown in table 1 is consistent with their status as either control, or mild, or moderate COPD.

qPCR for Total Bacteria and Lactobacillus:

Table S4 displays the location breakdown within the lung of each respective tissue sample. There was no difference between the top, middle, and lower thirds of the lung with respect to total bacteria (P values ranging from 0.30-0.90) [Table S5 and Figure S3]. Since no significant difference was found between the two samples from each individual they were averaged together

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for all subsequent analysis. There was no significant difference (P>0.05) between the sample groups based on total 16S/100ng of DNA [Figure S4]. The control group had an average value of 22.0 ± 21 (mean ± SD), the GOLD 1 group had 20.9 ± 13.2, and the GOLD 2 group had 15.5 ± 16.8 16S/100 ng of DNA respectively. There was also no difference in total bacteria with respect to any drug use (P>0.05) [Figure S5] or steroid use only (data not shown).

There was no significant difference between the three sample groups in the percent total *Lactobacillus* after Bonferroni correction (P>0.05) [Figure S6]. The percent total *Lactobacillus* for the control group was 8.7% ± 15.0% (mean ± SD), 2.5% ± 3.8% for the GOLD 1 group, and 8.1% ± 11.6% in the GOLD 2 group. There was no difference in the relative abundance of *Lactobacillus* based on any drug use or steroid use only (data not shown). Further, there was no significant difference in clinical characteristics (lung function, smoking history, age, and gender) between *Lactobacillus* positive and negative individuals [Table S6].

PCR for Glycerol Dehydratase (GD1):

Preliminary data showed that there was a clear decrease in the prevalence of GD1 producing *Lactobacillus* in the COPD GOLD 4 lung tissue when compared to both the non-smoking and smoking controls (P < 0.05) [Figure 1A]. Following up on this data in the early COPD data set a total of 5/74 (7%) of samples tested positive for the GD1 gene and 4/5 (80%) of these individuals also tested positive for *Lactobacillus*. There was a significant decrease in the individuals that tested positive for the GD1 gene as the disease severity increased (P < 0.05) [Figure 1B]. A total of 14% of individuals were positive for GD1 in the control group versus 5% and 0% in the GOLD 1 and GOLD 2 groups respectively [Figure 1B]. There was no significant difference in

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steroid use, bronchodilator use, or use of both types of drugs between those that were GD1 positive and those that were GD1 negative (P>0.05).

Quantitative Histology:

The two samples from each individual were averaged for the final Vv measurement data used for all inflammatory cells. The multivariate analysis on all the Vv small airway measurements as well as the total bacterial 16S and percent total *Lactobacillus* showed that the Vv of CD1a+ cells correlated with the percent total *Lactobacillus* (r = 0.27, P = 0.038) [Table 2]. These CD1a+ dendritic cells were also positively correlated with CD35+ Follicular dendritic cells (r=0.39, P= 0.009) and CD8+ T-cells (r=0.27, P=0.04). Total 16S bacterial load positively correlated with CD21+ Follicular dendritic cells (r=0.30, P=0.013) [Table 2]. These CD21+ Follicular dendritic cells were also positively correlated with NK-1 cells (r=0.32, P=0.013). The strongest correlation from the multivariate analysis was between CD4+ T-cells and CD68+ macrophages (r=0.44, P=0.001).

The inflammatory cell Vv was then investigated between GD1 positive versus GD1 negative individuals [Figure 2]. There was a significant reduction in the Vv of CD68+ macrophages and neutrophils in the GD1 positive group versus the negative group (P<0.0035) [Figure 2B & C]. There was no difference in inflammatory cell Vv in the small airways with steroid (data not shown) or with any drug use [Figure S3].

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Discussion:

The present results confirm earlier reports showing no difference in total bacteria within the microbiome between control and COPD lungs and extend them by showing that this applies to tissue in mild to moderate COPD [7, 9]. It also shows that although no detectable difference in total *Lactobacillus* could be made between controls, GOLD 1, and GOLD 2 differences in the genotypic make up of the *Lactobacillus* could be found in the loss of GD1 positivity in mild and moderate COPD. Furthermore, *Lactobacillus* was found to have a positive correlation with CD1a+ dendritic cells suggesting that *Lactobacillus* may influence the host response through these cells. Finally, this study also shows that GD1 positive individuals have lower macrophage and neutrophil Vv than GD1 negative individuals. Overall this data supports the hypothesis that GD1 positive *Lactobacillus* may have an anti-inflammatory role in the pathogenesis of COPD.

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These observations are of particular interest because of a previous report by Forsythe, et al. [20] who showed that in mice sensitized to albumin, oral treatment with GD1 positive *Lactobacillus reuteri* prior to challenging their airways with albumin resulted in a reduction in macrophage and neutrophil infiltration into their lung tissue and reduced the cellular and inflammatory mediator content of their BAL fluid compared to controls treated with saline alone [20]. In addition, these same investigators subsequently reported that the non-specific CD4 (+) CD25 (+) Foxp3 (+) regulatory T-cells exerted a potent immunoregulatory action on the response to challenge by a specific antigen [21]. These observations clearly suggest the possibility that GD1 positive *Lactobacilli* induce immunoregulatory mechanisms capable of controlling the host immune response in mice. Our study builds upon these previous observations by investigating *Lactobacillus* in human lung tissue and is consistent with Forsythe,et al 's macrophage and neutrophil data.

The preliminary data that was investigated suggests that, like in GOLD 1 and GOLD 2 grade disease, GOLD 4 also has a significant reduction in GD1 positive individuals versus controls. This would imply that reduction in GD1 positivity occurs early on in disease and could be a contributing factor to the increased inflammation seen as disease progresses [3]. More studies designed at investigating *Lactobacillus* across all grades of disease will need to be completed to elucidate the true scope to which this genus can influence the progression of COPD.

Previous studies have shown that CD8+ T-cells are important in the progression of COPD [22]. The correlation between CD1a+ dendritic cells and CD8+ T-cells along with the CD1a+ dendritic cell correlation with *Lactobacillus* provides a possible mechanism by which these

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bacteria may exert their action in COPD. Although, in the early COPD data set, there was a significant reduction in GD1 positive macrophage and neutrophil Vv when compared to the GD1 negative group, no correlations with macrophages or neutrophils could be found with respect to *Lactobacillus*. This suggests that the effect between these specific inflammatory cells and GD1 positive *Lactobacillus* may not be a simple linear correlation and may act through other inflammatory cells. Additionally, CD1a+ dendritic cells did not correlate with the other major cells that have been found to be important in COPD (macrophages, CD4 T-cells, and B cells) [3, 22, 23]. This also implies that *Lactobacillus* may not directly impact these cells in disease. However, since this study focused mostly on early COPD it is possible that *Lactobacillus* do have an impact in later stages of the disease but this study was not designed to examine this possible relationship.

There are some important limitations that need to be mentioned in this study. First, although there was a significant reduction in the Vv of macrophages and neutrophils between GD1 positive and GD1 negative groups the total number of positive samples was relatively small and larger studies will need to be carried out in order to confirm these initial findings. Interestingly, the preliminary data set that was used for this study consisted of tissue samples from different individuals than those used in the early COPD data set and showed consistent data with respect to GD1 positivity: That there was a significant reduction in GD1 positivity as COPD increased in severity. Second, the tissue was obtained from patients with lung cancer and even though tissue was obtained from areas well away from the tumor the global bacterial load as well as the composition could have been affected. However, none of the total percent *Lactobacillus* values for the control, GOLD 1, and GOLD 2 groups are out of the range of what would be expected

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based on previous research on the bacterial microbiome in lung tissue [9]. Third, although previous research has shown that there may be a difference between the bacterial microbiome when steroids are used [24] there were very few individuals on steroids (n=3) in this patient population and, for this study, steroids probably had no effect on the reported findings. Fourth, although GD1 was analyzed reuterin was not directly measured in this study and the mechanism of action in which GD1 positivity exerts its anti-inflammatory effect may not be through this particular pathway. Further studies, aimed at investigating reuterin directly, will need to be performed to confirm that GD1 acts through this antibiotic to have anti-inflammatory effects within COPD.

As more information about how bacteria can influence and change our innate and adaptive immune system becomes known, it is becoming increasingly evident that many bacteria have beneficial effects that can positively influence our immune system [25]. Although the effect is small these results provide preliminary evidence of the potential benefit of GD1 positive *Lactobacillus* in small airway inflammation in COPD. Future studies may show that it could be possible to utilize *Lactobacillus* with the GD1 gene as a clinical intervention to help reduce inflammation or as a prophylaxis in those with mild and moderate COPD.

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Contributorship Statement: MAS (performed, & designed experiments, data analysis, wrote first draft), SU (performed experiments), WME (tissue procurement), JCH (conceived the study, intellectual contributions), RGH (conceived the study, tissue procurement, intellectual contributions)

Competing Interests: The authors have no significant competing interests to declare.

Funding: Supported by Merck external studies agreement IIS 38970 (UBC #F1038978), NIH #HL084948, and CIHR #CIF-97687. None of the agencies had any input into the study design; in collection, analysis and interpretation of data; in writing of the report and in the decision to submit the report for publication.

Data Sharing: Upon acceptance data will be uploaded to Dryad

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Table 1: Clinical Characteristics of the Sample Groups (average ± SD)

	Controls (n=28)	GOLD 1 (n=21)	GOLD 2 (n=25)
Age	65.7 ± 9.6	66.0 ± 8.9	63 ± 9.2
Gender (M:F:Unknown)	16:11:1	14:7:0	17:8:0
Smoking History (cigarette-years)	895.4 ± 622.8	1061.8 ± 410.5	945.0 ± 555.7
FEV_1/FVC	77.4 ± 4.9	64.3 ± 4.3*	62.0 ± 7.0**
FEV_1 (percent predicted)	100.0 ± 12.5	89.9 ± 9.0†	69.0 ± 6.6**

* P<0.0001 between controls versus GOLD 1
**P<0.0001 between controls versus GOLD 2
†P<0.0001 between GOLD 1 versus GOLD 2

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Table 2: Significant comparisons from multivariate analysis of all measurements made on lung tissue.

ALL SAMPLES

Variable	by variable	n=	Signif Prob	Correlation
CD1a+ Dendritic Cell	lacto/Total (16S)	60	0.0383727	0.26807019
CD21+ Follicular Dendritic Cell	16S Total	66	0.0131415	0.30380793
CD35+ Follicular Dendritic Cell	CD1a+ Dendritic Cell	45	0.0088667	0.38576746

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NK-1	CD21+ Follicular Dendritic Cell	59	0.0131419	0.3211255
CD4 T-Cell	CD68+ Macrophage	51	0.001058	0.44532966
CD8 T-Cell	CD1a+ Dendritic Cell	56	0.0457076	0.26814134
CD79a+ B-Cell	CD4 T-Cell	51	0.0389584	0.29004891

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Figure Legend:

Figure 1: A) GD1 distribution in the preliminary data set; Non-smokers (n=8), Smokers (n=8), and COPD GOLD 4 (n=8), $P > 0.05$ between groups **B)** GD1 distribution displayed as a percentage. The graph shows the percentage of GD1 positive individuals in controls, GOLD 1, and GOLD 2. The table shows the absolute number of individuals in each group positive for GD1 as well as the percentage in brackets, $P < 0.05$ between groups.

Figure 2: A) Inflammatory cell volume fraction in GD1 positive and GD1 negative samples. No significant difference was seen between the adaptive immune cell Vv and GD1 ($P>0.05$). A significant difference was seen in the Vv of macrophages and neutrophils based on GD1 positivity ($P<0.0035$). **B)** Small airway Vv of macrophages by GD1 positivity ($P<0.0035$) **C)** Small airway Vv neutrophils by GD1 positivity ($P<0.0035$).

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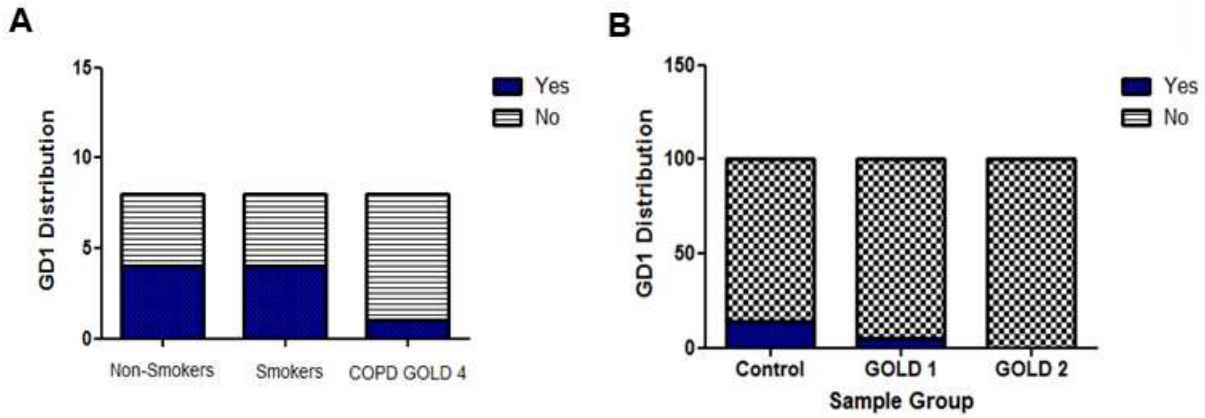

Figure 1

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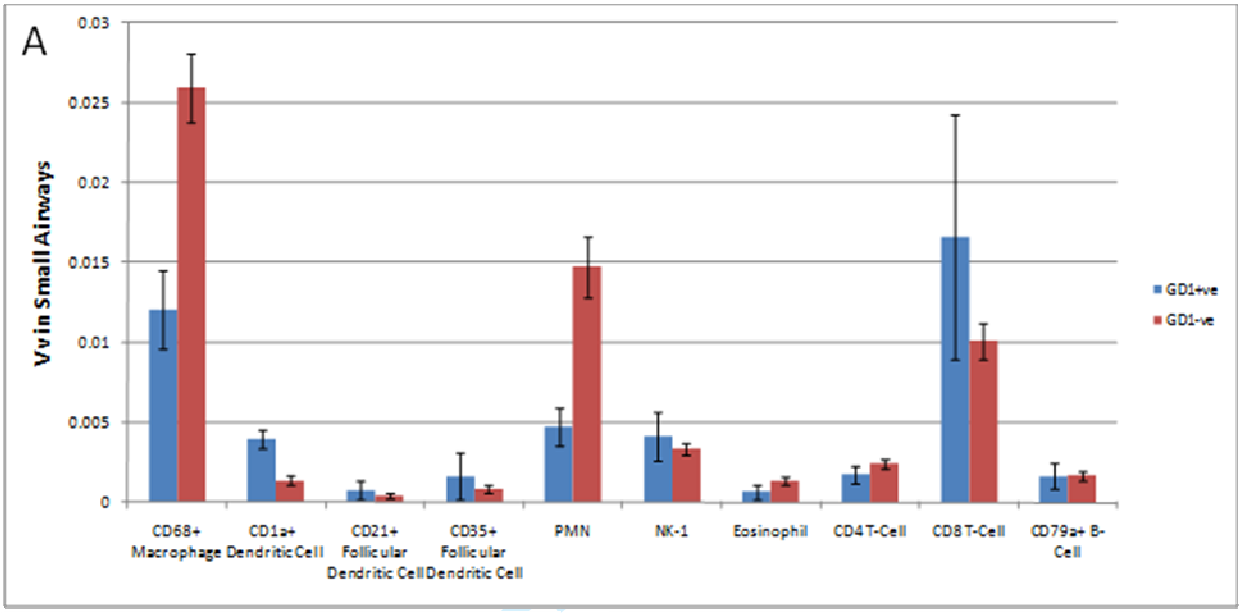

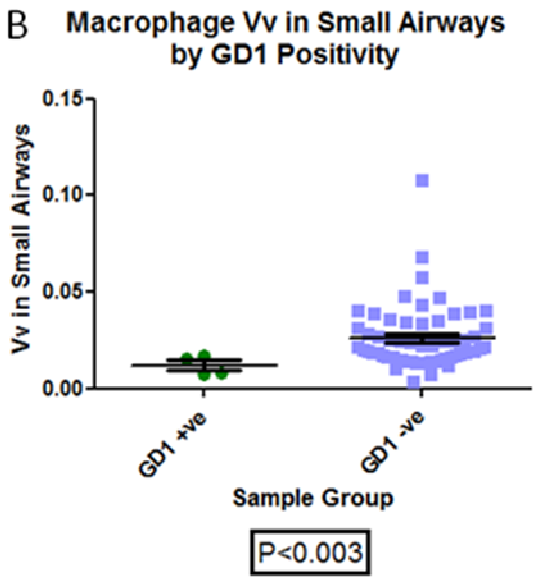

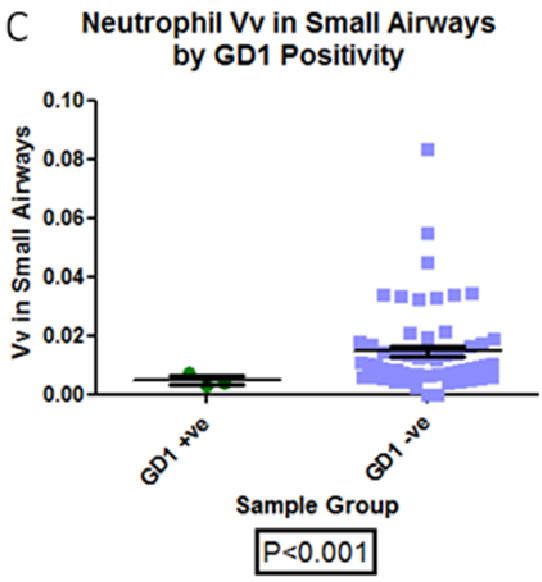

Figure 2

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Online Data Supplement:

Methods:

qPCR Analysis:

Table S1: qPCR Standard Curve Formulas

	16S Total Bacteria Assay	***Lactobacillus* Assay**
Plate 1	y = -3.25x + 29.03	y = -3.15x + 30.65
Plate 2	y = -3.29x + 29.53	y = -3.22x + 30.75

qPCR Plate Correction:

In order to accurately compare plates against each other four samples were run on both plates and the plate that gave larger values was divided by the plate with smaller values to obtain a multiplicative factor. This was averaged for all four samples and an average multiplicative factor was obtained and used for all samples in the plate with smaller values. This was done for both the 16S total bacteria assay and the *Lactobacillus* assay.

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Quantitative Histology:

Table S2: Immunostaining of Inflammatory Cells*

Antibody Name	Antibody Type	Host Species	Against	Company	Catalog Number	Clone Name	Dilution	Pre-Treatment
CD1a+ (Langerhans Cell)	Monoclonal	Mouse	Human	DAKO	MS3571	010	1/50	Acetone 10 min. RT
CD35+ (Dendritic Reticulum Cell)	Monoclonal	Mouse	Human	DAKO	M0846	Ber-MAC-DRC	1/75	Acetone 10 min. RT
CD68+ (Macrophage)	Monoclonal	Mouse	Human	DAKO	M0718	EBM11	1/100	Acetone 10 min. RT
CD79α+ (B-lymphocyte)	Monoclonal	Mouse	Human	DAKO	M7050	JCB117	1/75	Acetone 10 min. RT
CD8+ (Cytotoxic T-lymphocyte)	Monoclonal	Mouse	Human	DAKO	M7103	C8/144b	1/50	Acetone 10 min. RT
NK1+ (Natural Killer Cell)	Monoclonal	Mouse	Human	DAKO	M1014	DAKO-NK1	1/100	Acetone 10 min. RT
Neutrophil Elastase (Neutrophil)	Monoclonal	Mouse	Human	DAKO	M752	NP57	1/50	Acetone 10 min. RT
CD4+ (Helper-inducer T-lymphocyte)	Monoclonal	Mouse	Human	DAKO	M0716	MT310	1/100	Acetone 10 min. RT

* Hansel stain used for Eosinophils

For both intraobserver and interobserver error assessment twenty slides were chosen at random spanning all the different inflammatory groups that were analyzed. Values with zero were omitted from the comparison and analysis of intra- and inter- observer error.

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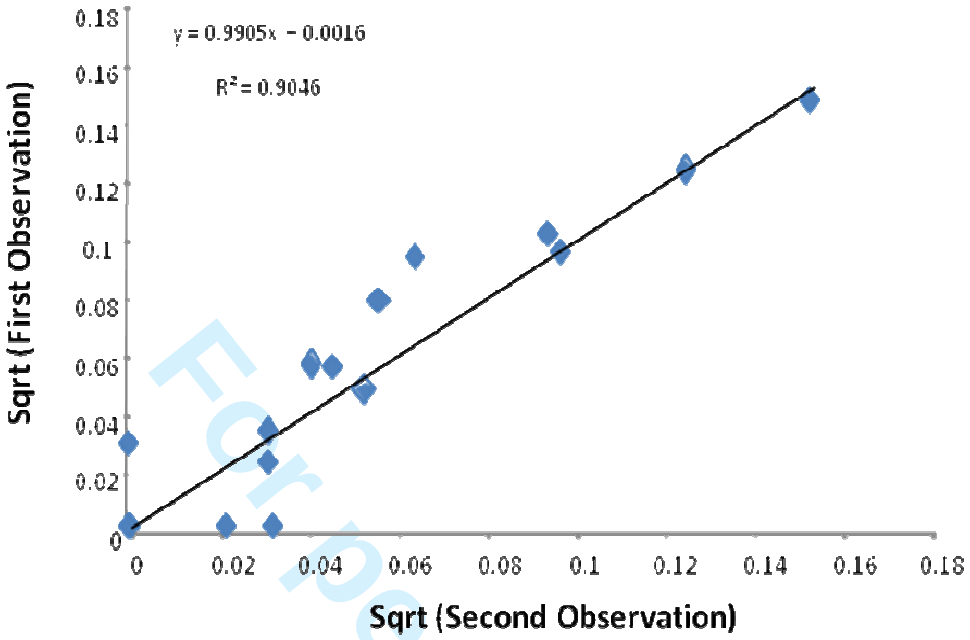

Figure S1: Intraobserver Error of Quantitative Histology Measurements of Vv in Small Airways. An R^2 of 0.9046 was observed showing good repeatability between an individual.

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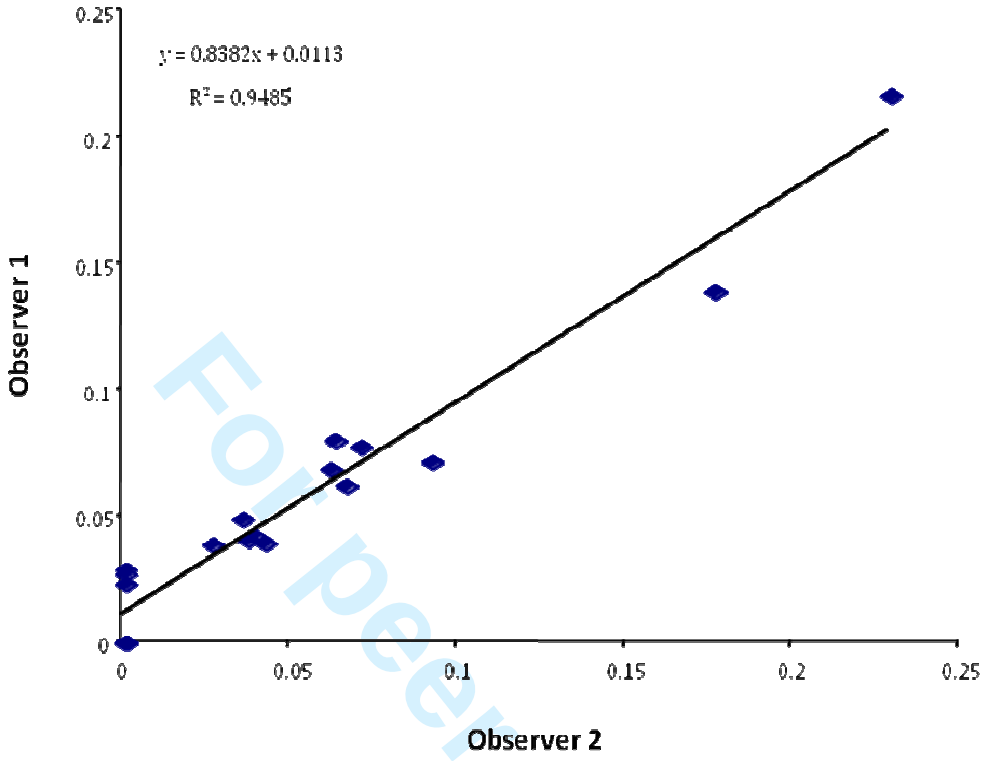

Figure S2: Interobserver Error of Quantitative Histology Measurements of Vv in Small Airways**.** An R^2 of 0.9485 was observed showing good repeatability between two different individuals.

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Results:

Table S4: Breakdown of adequate qPCR samples used for the analysis by relative location in the lung

	Top	Middle	Bottom
Control	18	21	12
GOLD 1	13	15	8
GOLD 2	18	19	12

Table S5: List of P-values for Comparisons of Total Bacteria between different Lung Locations

	Top vs. Middle	Top vs. Bottom	Middle vs. Bottom
Control	0.54	0.90	0.65
GOLD 1	0.30	0.72	0.45
GOLD 2	0.69	0.50	0.60

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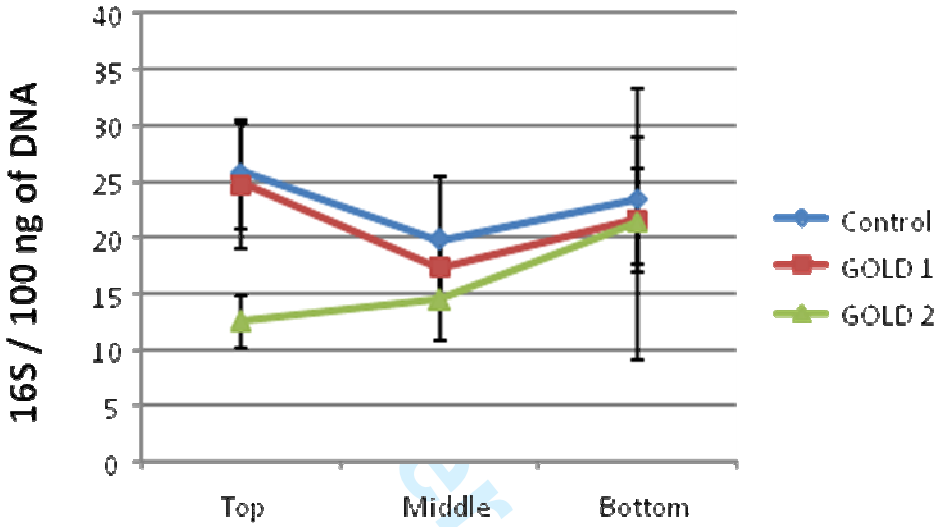

Figure S3: Total bacteria by height and GOLD stage. There is a trend for lower bacteria in the top of the lung in moderate COPD. However, no significant differences were found using ANOVA (P>0.05).

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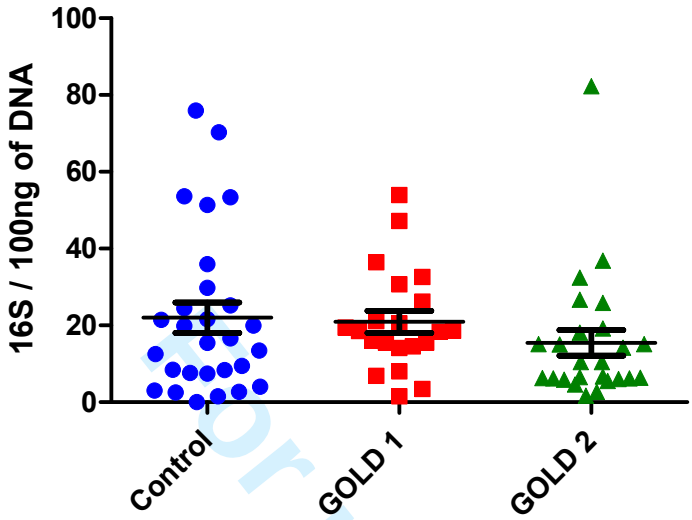

Figure S4: Average Bacterial Load per Group (error bars represent standard error). No significant difference was found in total bacteria by group (P>0.05). Controls n=28, GOLD 1 n=21, and GOLD 2 n=25.

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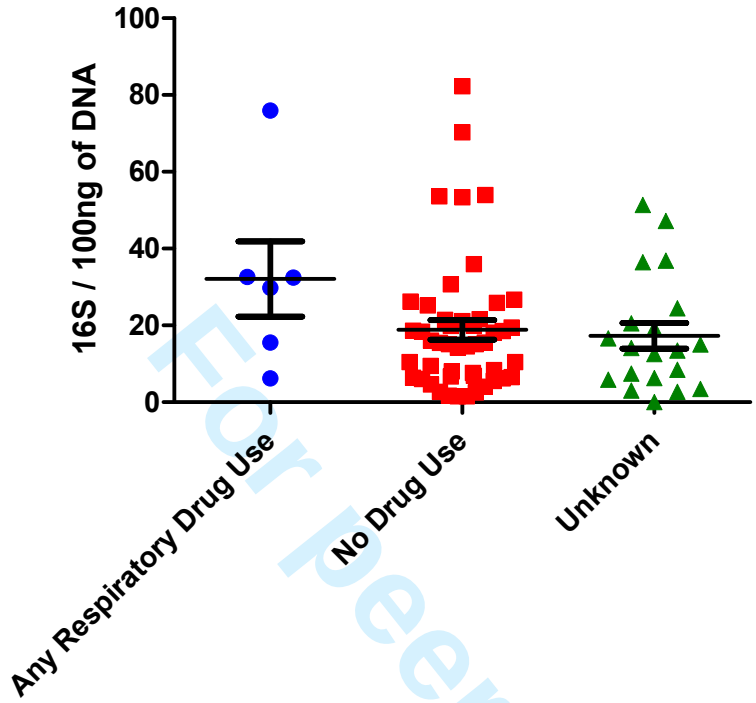

Figure S5: Total bacteria according to drug usage. No significant difference was found between the groups using ANOVA (P>0.05)

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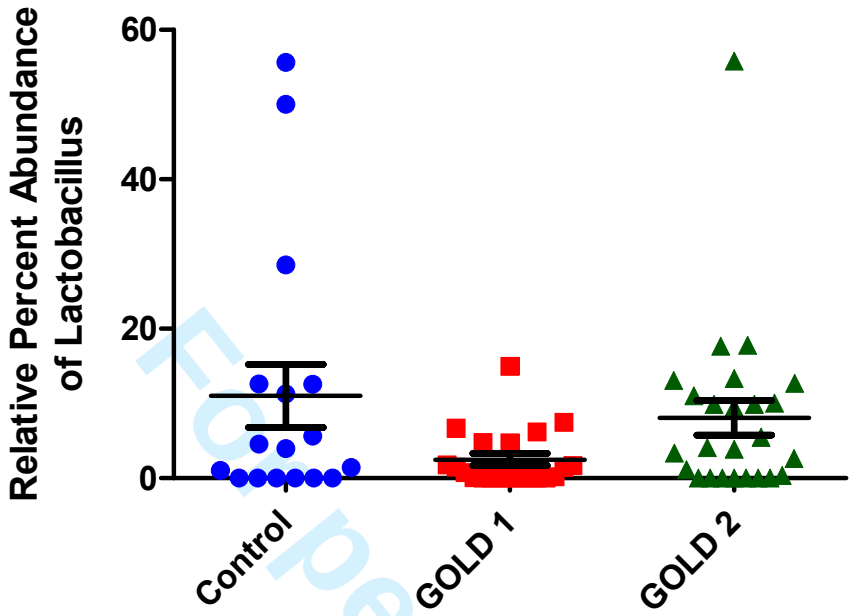

Figure S6: Average Lactobacillus abundance by sample group (error bars represent standard error). No significant difference was found between the three groups (P>0.05). Controls n=28, GOLD 1 n=21, GOLD 2 n=25.

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Table S6: Clinical characteristics separated by Lactobacillus positive or negative

	Lactobacillus Positive (mean ± SD)	Lactobacillus Negative (mean ± SD)
Age	65.1 ± 9.0	64.2 ± 10.0
Gender (M:F:Unknown)	32:19:1	15:7
FEV_1/FVC	67.6 ± 8.0	70.7 ± 10.5
FEV_1pp (percent predicted)	86.1 ± 16.6	88.6 ±16.3
Smoking History (cigarette-years)	1014.5 ± 585.6	818.9 ± 411.6

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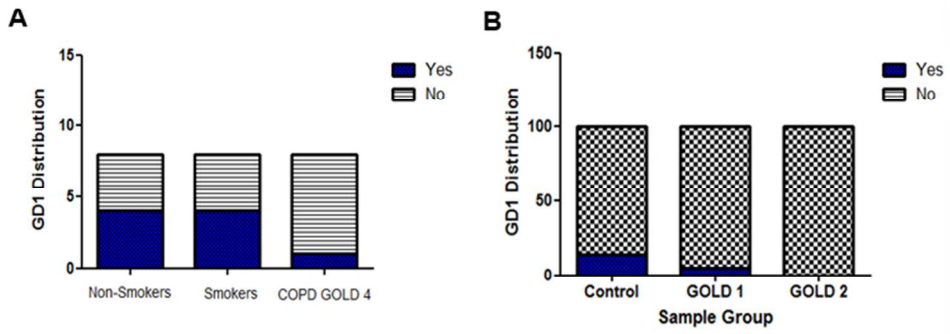

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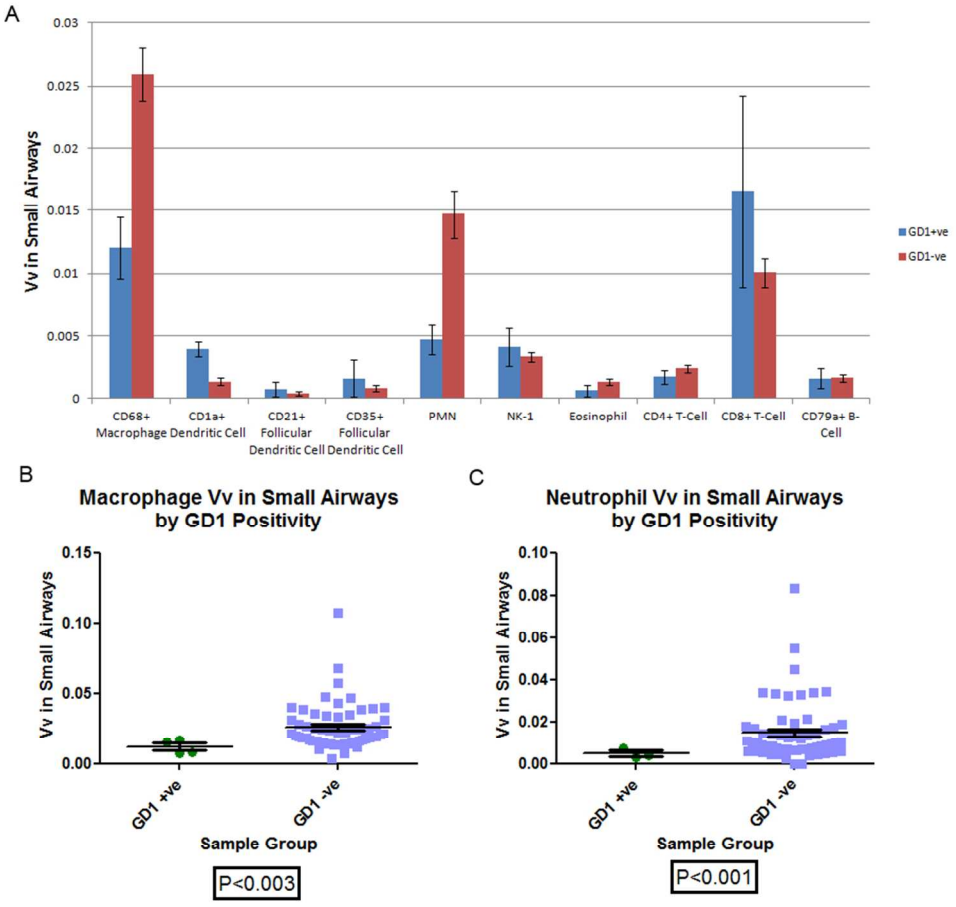

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PEER REVIEW HISTORY

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ARTICLE DETAILS

TITLE (PROVISIONAL)	Loss of GD1-positive Lactobacillus correlates with inflammation in human lungs with COPD
AUTHORS	Sze, Marc; Utokaparch, Soraya; Elliott, Mark; Hogg, James; Hegele, Richard

VERSION 1 - REVIEW

REVIEWER	Alejandro Pezzulo University of Iowa Hospitals and Clinics U.S.A
REVIEW RETURNED	15-Oct-2014

GENERAL COMMENTS	Sze et al. have submitted a simple yet elegant study in which they hypothesized that lungs of people with COPD would contain different bacterial populations than lungs of healthy people. Their context is provided by data previously published by the same group in which they demonstrated that COPD GOLD 4 lungs had higher amounts of Lactobacillus, and by studies demonstrating that GD1 positive Lactobacillus reduced inflammation in an albumin challenge model in mice. The main advantage in their approach comes from their ability to analyze lung tissue removed surgically under sterile conditions. This avoids the oropharynx/upper airway contamination bias plaguing most studies of airway microbiome. Using their combination of quantitative histology and high throughput bacterial sequencing, they found that: 1.- COPD GOLD 1/2 lungs do not contain different amounts of either total bacteria or Lactobacillus DNA. 2.- The difference in GD1 (+) Lactobacillus in COPD lungs correlates inversely with degree of inflammation. They then use their findings to conclude that GD1 (+) Lactobacillus loss may contribute to COPD pathogenesis. ***Minor Revisions*** 1.- I would suggest changing the title of the manuscript to more accurately reflect the findings. i.e., “Loss of GD1-positive Lactobacillus correlates with inflammation in human lungs with COPD” makes it easier to immediately understand the study than the current -perhaps vague- title. ABSTRACT: 2.- (line 8): Please change “the status of GD1 positivity” to “contents of GD1-positive Lactobacillus” 3.- (line 34): Please include the fact that your samples are not confounded by sampling through the mouth (a strength of your study) 4.- (line 41): A small sample size is only a problem if you require a larger sample size to achieve a certain statistical power… you may benefit from running a post-hoc power analysis to quickly list in your

	methods section how much discriminating function your study had. This is relevant only for outcomes that were not statistically significantly different in control vs. study samples. This is a minor comment that I do not think has to result in changes but would make it clearer why a small sample may be a problem. RESULTS/LEGENDS/FIGURES: 5.- My only suggestion here is to change the Y axis of the figures in Figure 1 to express " % GD1 positive" in both cases. The current form in which the data from the GOLD4 samples is shown as total number whereas the newer data is shown as percentage is confusing, as is the label for the Y axis ("distribution"). DISCUSSION: 6.- The authors have pointed out the main flaws and strengths of their study adequately and have put their results in the context of recent data. I do think that they also need to point out that it is always hard to determine whether their findings regarding GD1 are cause or consequence of lung damage, as is the case with most of the current literature analyzing microbiomes. I find their new hypothesis that reuterin may have a causal relationship with COPD inflammation particularly compelling and would perhaps emphasize this a bit more in the discussion.

REVIEWER	Christian Taube LUMC The Netherlands No competing interests declared
REVIEW RETURNED	30-Dec-2014

GENERAL COMMENTS	In the present study the authors investigate the relationship between the detection of lactobacillus in lung samples from patients with lung cancer, comparing patients with COPD (GOLD I and II) and smokers. In addition historic samples from a previous study are investigated containing non-smokers, smokers and COPD stage IV patients. Analysis of microbial colonization in the lung is of major interest and indeed the present paper offer some more insight. However, there are a couple of concerns. 1. The authors find no difference in the abundance of lactobacilli in the group of COPD patients, which is overall not high (between 2.5 and 8.7% of the groups). In addition, they find from this small number of lactobacillus positive patients only a small fraction in the smoking control group (in total 5 patients of the 74). However, the title and the discussion suggest that this factor may play a quite important role. Overall, I think these findings are interesting and it is worthwhile to describe the situation in COPD patients. However given the rarity of lactobacillus in the lung especially of the GD1 producing strain I would rather suggest to be more descriptive and stress not too much a potential clinical relevance. 2. The authors compare 5 patients without GD1 producing lactobacilli in the lung to the rest (mixture of COPD I, II and controls) in terms of inflammation. In my opinion this is not a justified comparison as the authors compare the controls (where 4 of the 5 GD1 positive patients are from) to COPD patients where we know that increase inflammation can be detected around the airways. 3. Is data available on the composition of other microorganisms in

	the lung of these patients? Is the presence/absence of lactobacillus associated with an increase/decrease in other species?

VERSION 1 – AUTHOR RESPONSE

Reviewer 1: Alejandro Pezzulo

1.- I would suggest changing the title of the manuscript to more accurately reflect the findings. i.e., "Loss of GD1-positive Lactobacillus correlates with inflammation in human lungs with COPD" makes it easier to immediately understand the study than the current -perhaps vague- title.

R1: We have changed the title of the manuscript to reflect your suggestion.

ABSTRACT:

2.- (line 8): Please change "the status of GD1 positivity" to "contents of GD1-positive Lactobacillus"

R2: Respective change has been made.

3.- (line 34): Please include the fact that your samples are not confounded by sampling through the mouth (a strength of your study)

R3: This point has been added to the strength and limitations section of the manuscript.

4.- (line 41): A small sample size is only a problem if you require a larger sample size to achieve a certain statistical power… you may benefit from running a post-hoc power analysis to quickly list in your methods section how much discriminating function your study had. This is relevant only for outcomes that were not statistically significantly different in control vs. study samples. This is a minor comment that I do not think has to result in changes but would make it clearer why a small sample may be a problem.

R4: Thank you for this comment. We have modified the abstract so that the comment about larger numbers is taken out and replaced with "…validation of these results need to be completed before…" However we prefer not to make any changes in the body of the manuscript itself.

RESULTS/LEGENDS/FIGURES:

5.- My only suggestion here is to change the Y axis of the figures in Figure 1 to express " % GD1 positive" in both cases. The current form in which the data from the GOLD4 samples is shown as total number whereas the newer data is shown as percentage is confusing, as is the label for the Y axis ("distribution").

R5: This change has been made in the revised manuscript.

6.- The authors have pointed out the main flaws and strengths of their study adequately and have put their results in the context of recent data. I do think that they also need to point out that it is always hard to determine whether their findings regarding GD1 are cause or consequence of lung damage, as is the case with most of the current literature analyzing microbiomes. I find their new hypothesis that reuterin may have a causal relationship with COPD inflammation particularly compelling and would perhaps emphasize this a bit more in the discussion.

R6: We have added a comment in the discussion about the need for potential in vitro studies to try and work out potential cause and effect (end of second to last paragraph in the discussion, Paragraph 5)

Reviewer 2: Christian Taube

1. The authors find no difference in the abundance of lactobacilli in the group of COPD patients, which is overall not high (between 2.5 and 8.7% of the groups). In addition, they find from this small number of lactobacillus positive patients only a small fraction in the smoking control group (in total 5 patients of the 74). However, the title and the discussion suggest that this factor may play a quite important role. Overall, I think these findings are interesting and it is worthwhile to describe the situation in COPD patients. However given the rarity of lactobacillus in the lung especially of the GD1 producing strain I would rather suggest to be more descriptive and stress not too much a potential clinical relevance.

R1: We have changed the title to what was suggested by reviewer 1.

2. The authors compare 5 patients without GD1 producing lactobacilli in the lung to the rest (mixture of COPD I, II and controls) in terms of inflammation. In my opinion this is not a justified comparison as the authors compare the controls (where 4 of the 5 GD1 positive patients are from) to COPD patients where we know that increase inflammation can be detected around the airways.

R2: This is a very good point and we have included in the supplement how the volume fraction measurements breakdown by GOLD grade. There is more inflammation in the GOLD 1 samples that we measured but there is no difference between control and GOLD 2 samples [Figure S7]. When we compare only the control samples by GD1 positivity the significant difference holds for the macrophage measurement [Figure S8]. In contrast, although a trend still exists, no difference was found for the PMN measurement [Figure S9]. We have added a reference to these three figures in the result section of the manuscript.

3. Is data available on the composition of other microorganisms in the lung of these patients? Is the presence/absence of lactobacillus associated with an increase/decrease in other species?

R3: At the moment data is not available on the other microorganisms but we hope to be able to eventually catalog more species specific information within these samples at a later date. The question of whether or not other species are correlated with increases or decreases of Lactobacillus is a good question that we hope to answer eventually. At the moment this question is outside the scope of this particular study.