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Association of Vitamin D and LL-37 levels in Bronchial Alveolar Lavage Fluid with Acute Cellular Rejection, Lung Infection and Chronic Rejection in Lung Transplant Recipients: A Pilot Case-Control Study

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ABSTRACT

Association of Vitamin D and LL-37 levels in Bronchial Alveolar Lavage Fluid with Acute Cellular Rejection, Lung Infection and Chronic Rejection in Lung Transplant Recipients: A Pilot Case-Control Study

By Jenny Han,MD

More than 9,000 people in the US are living with a transplanted lung and that number is consistently increasing. Despite immunosuppressive therapy, 55% of lung transplant recipients have acute cellular rejection within the first year of transplant. Acute cellular rejection is an important risk factor for developing chronic lung rejection, also known as bronchiolitis obliterans syndrome (BOS). Acute cellular rejection has also been found concomitantly in lung transplant recipients who have various types of bacterial and viral respiratory infections, which often leads to chronic lung rejection. Approximately half of the lung transplant recipients develop chronic rejection, which limits survival to a median of 5 years after lung transplantation. Previous studies have shown that lung transplant patients who have lower levels of vitamin D have more episodes of acute cellular rejection and increase risk of lung infection compared to patients with normal vitamin D. Therefore, vitamin D therapy in lung transplant recipients is a potential pathway to reduce acute cellular rejection, lung infection and possibly chronic lung rejection.

This is a retrospective case-control pilot study to elucidate the potential association of active vitamin D and LL-37 concentration in bronchial alveolar lavage fluid with acute cellular transplant rejection, lung infection and chronic rejection. This is the first study to report levels of LL-37 in BALF in lung transplant patients. We found a weak correlation between vitamin D and LL-37, ρ=0.27. Results of the study revealed no significant association between levels of vitamin D and LL-37 in BALF with acute cellular rejection, lung infection, chronic rejection or survival However, a unique finding to this study was that females had lower mean difference of LL-37 in BALF (-1.56ng/mL) compared to males and this was statistical significant, p=0.03.

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TABLE OF CONTENTS

INTRODUCTION

More than 9,000 people in the US are living with a transplanted lung and that number is consistently increasing. As of June 2011, 1,830 lung transplants were performed; the largest number of lung transplants ever in one year. [1] Despite immunosuppressive therapy, 55% of lung transplant recipients have acute cellular rejection within the first year of transplant.[2] Acute cellular rejection is an important risk factor for developing chronic lung rejection, also known as bronchiolitis obliterans syndrome (BOS). Acute cellular rejection has also been found concomitantly in lung transplant recipients who have various types of bacterial and viral respiratory infections, which often leads to chronic lung rejection.[3-6] Approximately half of the lung transplant recipients develop chronic rejection, which limits survival to a median of 5 years after lung transplantation.[2] Furthermore, lung transplant has the highest rate of rejection compared to other solid organ transplants.[2] Vitamin D therapy in lung transplant recipients is a potential intervention to reduce acute cellular rejection, lung infection and possibly chronic lung rejection.

25 hydroxyvitamin D is transported into cells (such as monocytes and macrophages) and is converted into the active form of 1, 25 hydroxyvitamin D by 1α-hydroxylase. The active 1, 25 hydroxyvitamin D is bound to the vitamin D receptor and transported into the nucleus where it up regulates mRNA transcription of an anti-microbial peptide known as cathelicidin. The only cathelicidin found in humans is LL-37.[5] The up regulation of this antimicrobial peptide stimulates pulmonary macrophages and respiratory epithelium to phagocytize pathogens. LL-37 has antimicrobial activity against Gram-positive, Gram-negative bacteria, fungi and viruses. [7,8] In monocytes and

macrophages, pathogens bind to toll like receptor 1 and 2 and stimulate 1α-hydroxylase to convert 25 hydroxyvitamin D into the active 1, 25 hydroxyvitamin D form to up regulate LL-37 synthesis. [9] Therefore, vitamin D through LL-37 has a direct effect on the host immune response. There has been no previously reported literature regarding measurement of LL-37 in bronchoalveolar lavage fluid (BALF) in lung transplant recipients.

There is a possibility that vitamin D can have a direct impact on clinical outcomes in lung transplant recipients by minimizing their acute cellular rejection episodes that can then decrease the risk of developing chronic rejection and death. This has not been studied thus far and can be an integral cost-effective therapy that can easily be applied to improve the lung transplant recipient's host response and lung function. The proposed causal pathway is that vitamin D depletion is associated with acute rejection based on previously reported studies in lung transplant that will lead to chronic rejection. Therefore, the aim of this study is to evaluate if there is an association with vitamin D levels and risk of developing acute cellular lung rejection, lung infection and chronic rejection.

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BACKGROUND

In recent years, there have been multiple studies revealing the association of vitamin D deficiency and the impact on multiple diseases, particularly bone health. Vitamin D has traditionally been known to assist in bone homeostasis and calcium metabolism by UV sunlight absorption, which converts pre-vitamin D in the skin, and then hydroxylases in the liver and then the kidney to assist in bone health by increasing calcium bone resorption. [10] Vitamin D is largely acquired through the sun and through dietary intake, therefore, vitamin D can have seasonal variation and several studies have reported this phenomenon on the effects of the host immune status. [11] There have been numerous studies now that reveal vitamin D has a hormonal mechanism since vitamin D receptors are found on multiple cells in various organs. Therefore, vitamin D is associated with several other disease states that have affected the host immune response and pulmonary systems.

Vitamin D has been demonstrated in the treatment of lung infections, such as tuberculosis and influenza. An interesting case-fatality study during the 1918-1919 influenza pandemic revealed an inverse association with UVB irradiance and the rate of influenza and influenza complicated with pneumonia, which were more prominent in the winter.[12] This study hypothesized that vitamin D exposure or lack of may have a direct role in the incidence of influenza and influenza complicated with pneumonia. A double-blind randomized study conducted in school age children involved vitamin D supplementation of 1,200 IU/day for approximately four months compared to placebo; and found that children who took vitamin D supplementation had less incidence of

influenza compare to children who took placebo, RR 0.58 95% (CI 0.34,0.99) p=0.04. [13] Another prospective study conducted in the U.S. obtained serial vitamin D levels in healthy adults during the fall and winter and found that levels of vitamin D serum concentration >38 ng/ml or higher, significantly reduced the incidence of acute viral respiratory tract infections by 2.7 times, p=0.02.[14]

In regards to tuberculosis, there have been several reported studies revealing that vitamin D deficiency is associated with active tuberculosis. A small study found 95 patients with active tuberculosis had a prevalence of vitamin D insufficiency (<30 ng/ml) of 85%. [15] In a case-control study of Asians, an undetectable level of vitamin D was associated with a higher risk of having tuberculosis. [16]Additionally, most promising was a study by Selvaraj that found treating tuberculosis patients with vitamin D allowed patients to clear their sputum of tuberculosis faster than those who were not treated with vitamin D. [17]

Specifically in regards to the lungs, there has been growing evidence that vitamin D insufficiency is associated with decreased lung function in forced expiratory volume in 1 second (FEV1) in patients with chronic obstructive pulmonary disease. [18,19] In adult asthma population, higher vitamin D levels were associated with an increase in lung function of FEV1 of 22.7 ml (±9) for each nanogram per milliliter increase in vitamin D, p=0.02.[20] Likewise in the autoimmune interstitial lung disease cohort study of 118 patients, this study revealed an association of vitamin D insufficiency with reduced lung function and diffusion capacity, p=0.15 and p=0.004 respectively. [21]

There is much less reported literature on LL-37, which is an anti-microbial peptide. Vitamin D up regulates mRNA synthesis of this anti-microbial peptide in the

nucleus of macrophages and monocytes. This anti-microbial peptide participates directly in killing pathogens. [9] This has been shown *in vivo* with vitamin D mediated innate immunity through LL-37 against mycobacteria. [22] In patients infected with mycobacterium tuberculosis, higher serum levels of LL-37 correlated with positive acid-fast bacilli in sputum.[15] In mice, LL-37 demonstrated anti-viral activity in the lung. [8] Furthermore, LL-37 was found to be able to transmigrate over the plasma membrane of Candida Albicans, which induced phase separation in the plasma membrane. [23] These studies highlight the direct anti-microbial effects that LL-37 has to various pathogens. Therefore, LL-37 is an immediate downstream effect from vitamin D.

Based on previous studies revealing the strong association between serum concentrations of vitamin D with lower lung volumes and its association with disease severity in asthma, interstitial lung disease and COPD, low vitamin D status in lung transplant recipients could have a significant impact on lung health. [20,24-26] Verleden demonstrated in their cohort study that 47% (48/102) of lung transplant recipients were vitamin D deficient (<20 ng/mL). Forced expiratory volume in one second was lower in the deficient group compared to the normal group, p=0.019 and in multivariate analysis, there was an association between FEV1 and vitamin D levels, p=0.021. [27] Additionally, patients with lower levels of vitamin D had more episodes of acute cellular rejection compared to patients with normal vitamin D, p=0.0038.[24] Also, a study by Lowery revealed an association between vitamin D deficiency and increase risk of lung infection and acute cellular rejection in lung transplant recipients. [27] There were more acute cellular rejection episodes in the vitamin D deficient group compared to the non-deficient group, p=0.02. Furthermore, there were more infections in the deficient group compared

to the non-deficient group, p=0.006. There was nearly a fivefold higher mortality rate after one year in the deficient group compared to the non-deficient group, incident rate ratio of 4.79, (CI 1.06,21.63) p=0.04. Therefore, the exposure of interest is whether vitamin D and LL-37 is associated with the outcomes of acute cellular rejection, lung infection and chronic lung rejection in lung transplant recipients.

METHODS

Aim 1: To perform a retrospective pair matched case-control study in adult lung transplant recipients comparing vitamin D and LL-37 in bronchial alveolar lavage fluid from cases (acute cellular lung rejection) compared to controls (no rejection).

Hypotheses: Lung transplant recipients who have lower levels of vitamin D and LL-37 in bronchial alveolar lavage fluid (BALF) are at higher risk for acute lung rejection.

Aim 2: Cohort study of 36 lung transplant recipients designated to estimate the association between vitamin D/LL-37 and the risk of lung infection.

Hypotheses: Lung transplant recipients who have lower levels of vitamin D and LL-37 in BALF are at higher risk for lung infection.

Aim 3: Cohort study of 36 lung transplant recipients designated to estimate the association of vitamin D/LL-37 and the risk of developing chronic lung rejection.

Hypothesis: Lung transplant recipients who have lower levels of vitamin D and LL-37 in BALF are at higher risk for chronic lung rejection.

Study Design:

To address if low levels of vitamin D and LL-37 is associated with increased risk of acute cellular rejection, a pair matched case-control pilot study was conducted comparing 18 lung transplant patients with acute cellular rejection to 18 lung transplant patients who do not have acute cellular rejection. The cases and controls were matched on two factors; the time post-transplant to acute cellular rejection and the underlying diagnosis for lung transplantation. To address the question of whether low levels of vitamin D and LL-37 were associated with an increased risk of lung infection, a cohort study was conducted using the 18 cases and 18 controls from the case-control study

population as the underlying cohort. This same cohort of 36 was used to address the question of whether low levels of vitamin D and LL-37 were associated with an increased risk of developing chronic lung rejection.

Sources of Data:

Previously banked BALF samples were obtained from a lung transplant recipient cohort enrolled in a prior IRB approved research study from 2007-2010. 36 lung transplant recipients were selected from a cohort of 85 lung transplant patients from a previously existing lung transplant database. Each subject had previously obtained BALF that was collected serially during routine lung transplant surveillance. Subjects with a known history of acute cellar rejection were selected and matched to subjects without acute cellular rejection according to their underlying lung diagnosis and by number of days after lung transplant. Acute cellular rejection was defined according to standard International Society for Heart and Lung Transplant guidelines. [28]

Samples were analyzed at the pre-rejection time point when the subject was first asymptomatic (for example when the patient had no clinical signs or symptoms of rejection) as well as the post-rejection time point when the subject's symptom had resolved and was first asymptomatic. Matched controls had their BALF analyzed at the corresponding day's post-transplant and pre-rejection time points. **(Figure 1)**

Data Collection:

The following data was collected during the course of the study: demographic data of lung transplant recipient, date of lung transplantation, pre-transplant serum

vitamin D, presence of lung infection, chronic lung transplant rejection status, death, and vitamin D and LL-37 levels in BALF.

Primary outcomes:

The exposures of interest are vitamin D levels and LL-37 levels in BALF. The primary outcomes are acute cellular rejection (Aim 1), lung infection (Aim 2) and chronic rejection (Aim 3). Covariates of age, gender and race were selected, because these are common factors that can affect the primary outcomes and may be associates with vitamin D levels or LL-37 levels in BALF. There were no missing values; however, three LL-37 levels were lower than the detectable limits of the assay and were not included in the analysis.

Analytic Plan:

Descriptive analysis was performed on the exposure of interest, vitamin D and LL-37. Vitamin D was analyzed by season, since seasonal variation is widely known to impact vitamin D levels. For Aim 1, the purpose is to perform a retrospective pair matched case-control study in adult lung transplant recipients comparing vitamin D and LL-37 in BALF from 18 cases (acute cellular lung rejection) compared to 18 controls (no rejection), using Mann-Whitney Wilcoxon nonparametric test. A conditional logistic regression model with Vitamin D and LL-37 levels and other covariates for instance; age, race, gender and season were compared by case status (acute rejection versus no rejection); controlling for the paired matching factor of time to acute rejection after transplant and the underlying lung diagnosis. To estimate the association between vitamin D and LL-37 in BALF, Spearman correlation was performed because vitamin D and LL-

37 levels in BALF are not normally distributed. Chi-square test and Fisher exact test were used to compare categorical variables. To evaluate if there were any seasonal variations, age or gender association with vitamin D levels, a linear regression model of vitamin D levels with these covariates were performed. Additionally, linear regression was performed to evaluate if there were any predictors of vitamin D or LL-37 levels

For Aim 2, a cohort study of 36 lung transplant recipients were designated to estimate the association between vitamin D and LL-37 and the risk of lung infection, using Mann-Whitney Wilcoxon nonparametric test and bivariate and multivariate logistic regressions. Other covariates in the model included race, gender, pre-rejection vitamin D levels and pre-transplant serum vitamin D levels. The same statistical analysis was performed for Aim 3, except the outcome was chronic rejection compared to no chronic rejection. Vitamin D and LL-37 levels were compared across various groups; age, race, gender and season and based on whether or not there was acute cellular rejection. Chi-square test and Fisher exact test were used to compare categorical variables. Additionally, linear regression was performed to evaluate if there were any predictors of vitamin D or LL-37 levels.

For Aim 3, Kaplan-Meier survival analysis was performed to compare vitamin D levels >=12 ng/mL compared to vitamin D levels <12 ng/mL on the outcome of time to chronic lung rejection. Vitamin D level of 12 ng/mL was categorized since this was the median found in BALF. Lastly, a Cox proportional hazards model was performed to evaluate if continuous vitamin D levels in BALF increased the hazard ratio of developing chronic rejection. *P* values of < 0.05 were considered statistically significant. All analyses were done with SAS/STAT Version 9.3 (SAS Institute, Cary, NC).

RESULTS

A descriptive analysis of the levels of Vitamin D and LL-37 varied by age, race and gender were performed. The median levels of vitamin D and LL-37 were similar across groups. **(Table 1a)** The only notable difference was LL-37 levels were lower in females compared to males (0.08 ng/mL, 0.24ng/mL respectively, p=0.06), which would become significant in later analysis. **(Table 1b)** To assess if there was seasonal variation of vitamin D, we found that although there were slightly lower levels of vitamin D during the fall, there were actually higher levels of vitamin D in the winter. **(Table 1c)**

To assess if there were significant covariates that were associated with the levels of vitamin D and LL-37 a linear regression was performed. Blacks resulted in a higher level of vitamin D compared to white, [mean difference in level, 5.35 (CI 0.38, 10.33) p=0.04]. **(Table 1d)** Also as noted previously, being female was associated with a lower level of LL-37 in BALF. Females had lower mean difference of LL-37 in BALF (-1.56ng/mL) compared to males and this was statistical significant, p=0.03. **(Table 1e)** This association was found independent of vitamin D levels in BALF.

Since levels of LL-37 in BALF have not been reported, Spearman correlation was performed to evaluate if vitamin D levels in BALF correlated with LL-37 in BALF. This revealed a weak correlation between vitamin D and LL-37, ρ=0.27. **(Table 2)**

For Aim 1, descriptive analysis for categorical and continuous variables comparing cases (patients with acute cellular rejection) to controls (no acute rejection) was conducted. **(Table 3a, 3b)** Vitamin D levels were compared between cases and controls and revealed that pre-transplant serum vitamin D levels were low in both groups. **(Table 3c)** Normal serum vitamin D levels are >30ng/ml and the median were 22 ng/ml

and 22.5 ng/ml for cases and controls, respectively. There was no difference between the levels of vitamin D and LL-37 in BALF during acute cellular rejection between cases and controls. **(Table 3c, 3d)** Vitamin D has been shown to have seasonal variations and analyses were performed to evaluate if vitamin D levels varied by season. There was no seasonal variation with levels of vitamin D by group. **(Table 3e)**

The primary analysis for Aim 1 was a conditional logistic regression to estimate the association of vitamin D and LL-37 levels in BALF with acute cellular rejection based on the pair-matched factor, and there was no meaningful difference between cases and controls adjusting for age and gender. (**Table 4a, 4b)** Since pre-rejection vitamin D and LL-37 levels in BALF were also analyzed, conditional regression was performed with pre-rejection levels to see if the levels prior to acute rejection had an association with outcome of acute cellular rejection. **Table 4c** reveals no significant association with pre-rejection levels. The same findings were true with pre-rejection LL-37 levels in BALF.

For Aim 2, **Table 5a and 5b** show descriptive analyses of categorical and continuous variables comparing transplant patients with lung infection to those with no infection. This demonstrated that both groups were similar and they were accurately matched. The only difference was lung transplant recipients who did not have lung infection had lower levels of vitamin D compared to those who did have lung infection, p=0.04. (Table 5a) Bivariate and multivariate logistic regression was performed to estimate the association of vitamin D and LL-37 levels in BALF with lung infection. **(Table 5c, 5d, 5e)** There was no association detected.

For Aim 3, Kaplan-Meier survival analysis was used to compare patients with vitamin D levels >=12 ng/mL and vitamin D levels <12 ng/mL on the outcome of time to chronic lung rejection. Vitamin D level of 12 ng/mL was categorized since this was the median found in BALF. (Figure 2, 3) There was no survival benefit detected. Lastly, the results of a Cox Proportional hazard ratio revealed no increased risk for chronic rejection based on level of pre-transplant serum vitamin D, vitamin D and LL-37 levels in BALF during acute cellular rejection, [HR 0.98, CI(0.92,1.04), HR 1.05, CI (0.98,1.13), HR 1.17, CI (0.95,1.43), respectively. **(Table 6a, 6b)**

DISCUSSION

The descriptive analysis of vitamin D and LL-37 BALF levels by age, race and gender did not reveal any significant findings. Vitamin D levels were higher in blacks compared to whites, which is different from previously reported studies. Typically, the melatonin in darker skin blocks UV absorption and prevents conversion in the skin into pre-vitamin D for hydroxylation to the active form of vitamin D. Therefore, this finding is contrary to previous reports of vitamin D levels by race. It also more intriguing to understand the significance of this finding because within the lung transplant recipient population the donor recipient and organ donor may be of different race. Therefore, it is possible that an African-American lung recipient received a Caucasian lung. We do not have the information of the lung donor's race in this study but may be something to consider in future organ transplant research. Therefore, it is unclear how to interpret these findings.

Unique to this study was the association between female gender and lower levels of LL-37 that has never been previously reported. In the descriptive analysis of LL-37 levels by age, race and gender, there was signaling that being female had lower levels of LL-37 independent of the vitamin D levels. In further analysis, this association was statistical significant. This could be a chance finding however, throughout different modeling analysis this trend was consistent. Perhaps being female may increase the risk of lung infection that needs further validation.

Seasonal variation did not have an impact in this patient population. Vitamin D levels were actually higher in the winter and lower in the spring and fall. This is likely

due to the fact that lung transplant recipients are not the healthiest patients and still reside indoors most of the time. Regardless of the reason, this shows that vitamin D was not impacted in anyway by seasonal variation.

Since this is the first study to report levels of LL-37 in BALF, we were curious to see if vitamin D levels in BALF correlated with the downstream effect of LL-37. We demonstrated a modest association of vitamin D levels and LL-37 in BALF (ρ=0.27). In the descriptive analysis of aim 1, we found that pre-transplant serum vitamin D levels were low in both groups, the median 22 ng/ml and 22.5ng/ml for cases and controls, respectively. It is possible that due to low levels of vitamin D, no meaningful association was found between levels of vitamin D and LL-37 and the primary outcome in our conditional logistic regression model. A potential limitation is that three levels of LL-37 were undetectable and this could cause bias; however, two of the levels were from cases and one from a control so it was not all in one group. This study did confirm prior studies that lung transplant recipients are invariably vitamin D depleted both before and after transplantation.

For Aim 2, the descriptive analysis revealed that lung transplant recipients that did not have lung infection had lower levels of vitamin D. 54% of patients without lung infection had levels less than 20 ng/ml, which is defined as deficient. This would suggest that vitamin D levels did not impact lung infection status. However, a limitation to this result is that the pre-transplant serum vitamin D level varied in the time course prior to transplantation. The serum level could have been drawn months prior the actual date of the transplant. The exact level of serum vitamin D on the day of transplant is unknown. It is possible that if the levels were deficient these patients may have been more likely to be

on supplementation prior to transplant. For Aim 3, there was no survival benefit with higher levels of vitamin D and no improvement of time to chronic rejection with higher levels of vitamin D.

There are several possibilities to why this study was negative. First, the study could be negative because there is truly no association between vitamin D levels, LL-37 and the outcome on acute cellular rejection, lung infection and chronic rejection. Secondly, the small cohort size could mean that the study was underpowered to detect a meaningful association. Thirdly, the levels of vitamin D in the majority of patients were low to start and therefore, could have blunted any meaningful difference that could have been detected. Lastly, there is an unmeasured but potentially important confounder which is immunosuppression. The constant change in immunosuppression could have greatly altered the vitamin D and LL-37 levels since they are part of the innate immune response. Based on the study results, measuring vitamin D and LL-37 levels in the BALF of lung transplant recipients is not clinically useful at this time.

This study suggests that there may be signaling or influence between gender and LL-37 in BALF during acute cellular rejection that has not been described before. Perhaps, women are at higher risk of obtaining lung infection compared to men. This study was not powered for this comparison, but would be a potential area of future research. This study adds to the lung transplant literature by failing to find an association between vitamin D and LL-37 with relevant lung transplant outcomes (acute rejection, lung infection, chronic rejection). This study also reports the novel findings of LL-37 in BALF associated with gender. Using this study as a pre-intervention study, the next step would be a prospective vitamin D replacement lung transplant cohort study to evaluate if

levels of vitamin D and LL-37 in lung fluid are associated with acute cellular rejection, lung infection and chronic rejection.

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Figure 1. Schema of the Matching Factor for Cases compared to Control

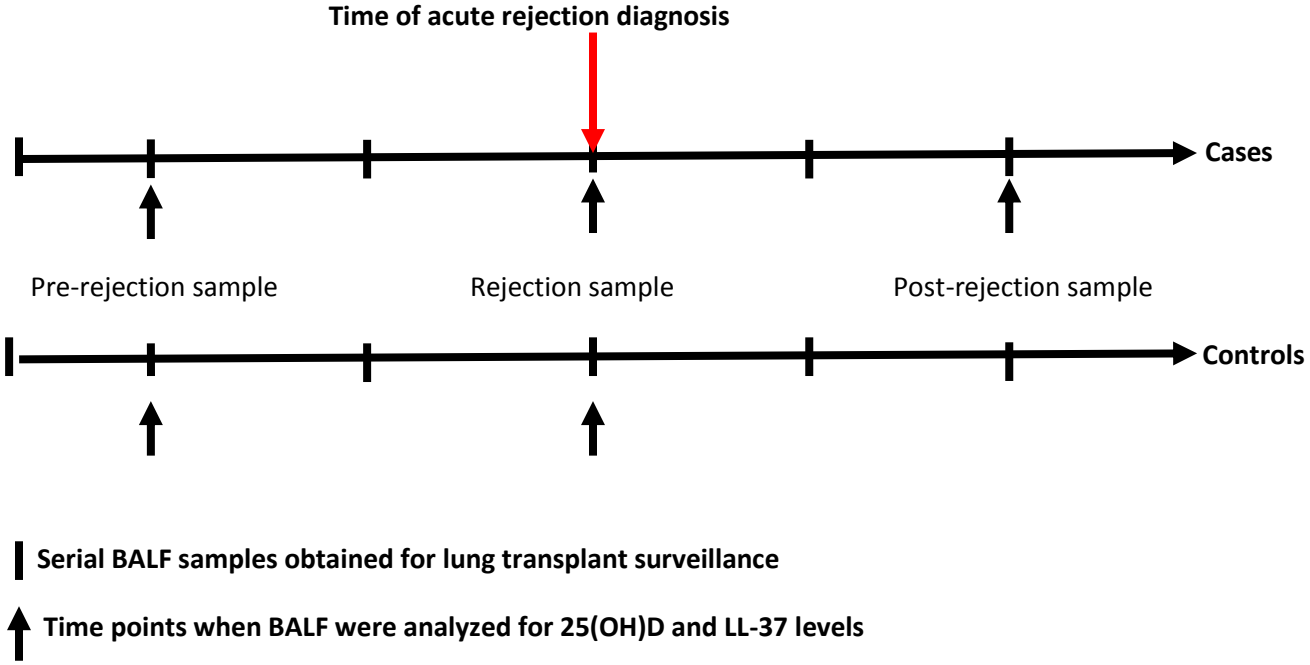

Table 1a. 25(OH)D Level in BALF by Different Groups-Median ng/mL

Risk Factors		N	Median	Q1-Q3	P-Value
Age	<=50	28	12.35	9.40-13.95	0.92
	>50	7	12.60	11.60-13.00	
Race	Black	7	12.70	12.40-27.20	0.36
	White	28	12.00	10.00-13.50	
Gender	Female	14	12.45	10.70-13.30	0.63
	Male	21	12.70	10.20-13.50	

BALF=Bronchial Alveolar Lavage Fluid

Table 1b. LL-37 Levels in BALF by Different Groups-Median ng/mL

Risk Factors		N	Median	Q1-Q3	P-Value
Age	<=50	28	0.11	0.04-1.29	0.79
	>50	8	0.11	0.05-0.32	
Race	Black	7	0.11	0.09-6.37	0.36
	White	29	0.11	0.04-0.48	
Gender	Female	15	0.08	0.04-0.24	0.06
	Male	21	0.24	0.06-2.25	

BALF=Bronchial Alveolar Lavage Fluid

Table 1c. 25(OH)D levels in BALF by seasons (N=36) ng/mL

Season	N	25(OH)D Median	Q1-Q3	P-value
Fall	8	10.10	8.7-12.9	0.15
Winter	11	13.2	11.6-19.9	
Spring	7	11.7	6-13.9	
Summer	10	12.7	11.8-12.9	

BALF=Bronchial Alveolar Lavage Fluid

Table 1d. Linear Regression with 25(OH)D Levels in BALF During Acute Cellular Rejection by Gender, Age and Race (ng/mL) N=36

Risk Factors		Mean Difference in 25(OH)D levels (ng/mL)	95% CI	P-Value
Gender	Female vs Male	-2.51	(-6.57,1.55)	0.22
Age	per year	0.009	(-0.23, 0.25)	0.94
Race	**Black vs. White**	**5.35**	**(0.38,10.33)**	**0.04***

* *P* values of < 0.05 were considered statistically significant

Table 1e. Linear Regression with LL-37 in BALF During Acute Cellular Rejection by Gender, Age and Race N=33

Risk Factors		Mean Difference in LL-37 Levels (ng/mL)	95% CI	P-Value
Gender	**Female vs. Male**	**-1.56**	**(-2.98,-0.14)**	***0.03**
Age	Per year	0.03	(-0.06,0.11)	0.50
Race	Black vs. White	1.55	(-0.21,3.31)	0.08

* *P* values of <0 .05 were considered statistically significant

Table 2. Correlation of 25(OH)D and Acute Rejection LL-37 in BALF ng/mL during Acute Cellular Rejection

	Spearman Correlation Coefficient (N=33)	P-Value
25(OH)D in and LL-37 levels in BALF during Acute Cellular Rejection	0.27	0.12

BALF=Bronchial Alveolar Lavage Fluid

Table 3a. Descriptive Statistics of Categorical Variables for Each Group (N=36)

	Case N=18 (%)	Control N=18 (%)	N (%)	P-Value
-Black	2 (11)	5(27.8)	7	0.40
-White	16 (88.9)	13 (72.2)	29	
Female	6 (33.3)	9 (50)	15	0.31
Lung Infection	3 (16.7)	7 (38.9)	10	0.14
Pre-transplant Lung Disease (Matched Factor)				
-CF	1	1	2 (6)	
-IPF	6	6	12 (33)	
-COPD	10	10	20 (55)	
-Sarcoid	1	1	2 (6)	

P=values are based on chi-square test or Fisher Exact Test.
CF=Cystic Fibrosis; IPF=Idiopathic Pulmonary Fibrosis; COPD (chronic obstructive lung disease)

Table 3b. Descriptive Statistics of Continuous Variable for Each Group (N=36)

Risk Factors	Cases Median (Q1-Q3)	Control Median (Q1-Q3)	P-value
Age (years)	59.5 (52-60)	58 (53-61)	0.92
Acute Rejection Days Post-Transplant	53.5 (42-90) N=18	57.5 (48-92) N=18	0.66

Table 3c. 25(OH)D levels for Each Group (N=36) ng/mL

Risk Factors	Cases Median (Q1-Q3) 25(OH)D	Control Median (Q1-Q3) 25(OH)D	P-value
Pre-Transplant Serum 25(OH)D Levels ng/mL (normal >30ng/mL)	22 (14-28) N=17	22.5 (17-27) N=18	0.50
Pre-Rejection 25(OH)D in BALF (ng/mL)	10.45 (9.8-13.5) N=10	12.85 (10.75-15.20) N=16	0.19
25(OH)D in BALF during Acute Cellular Rejection (ng/mL)	12.3 (8.9-13.5) N=18	12.6 (11.4-13.9) N=18	0.51

BALF=Bronchial Alveolar Lavage Fluid

Table 3d. LL-37 Levels in BALF for Each Group ng/mL

Risk Factors	Cases Median (Q1-Q3)	Control Median (Q1-Q3)	P-value*
Pre-Rejection LL-37 in BALF ng/mL	0.89 (0.10-5.10) N=10	0.36 (0.13-0.73) N=17	0.26
LL-37 in BALF during Acute Cellular Rejection (ng/mL)	0.17 (0.03-1.42) N=18	0.09 (0.04-0.29) N=18	0.50

BALF=Bronchial Alveolar Lavage Fluid

Table 3e. 25(OH)D level in BALF by Season and Group (n=36)

Season	Case		Control		P-value
	Median	(Q1-Q3)	Median	(Q1-Q3)	
Spring+Summer	12.05	(10.4-13.3)	13.2	(10.7-13.5)	0.27
Fall+Winter	12.7	(11.7-15.3)	11.8	(8.9-12.8)	0.78

Table 4a. Conditional Logistic Regression Model for 25(OH)D in BALF During Acute Cellular Rejection Adjusting for Age and Gender (N=36)

Risk Factors		Odds Ratio	95% CI	P-Value
25(OH)D in BALF during acute cellular rejection	Per ng/mL	0.96	(0.86, 1.08)	0.48
Age	Per year	0.94	(0.80, 1.10)	0.42
Gender	Female vs. Male	0.42	(0.08, 2.24)	0.31

BALF=Bronchial Alveolar Lavage Fluid

Table 4b. Conditional Logistic Regression Model for LL-37 in BALF During Acute Cellular Rejection Adjusting for Age and Gender (N=33)

Risk Factors		Odds Ratio	95% CL	P-Value
Acute Rejection LL-37 in BALF during acute cellular rejection	Per ng/mL	1.00	(0.71, 1.42)	0.98
Age	Per year	0.95	(0.81, 1.11)	0.51
Gender	Female vs. Male	0.38	(0.07, 2.00)	0.26

BALF=Bronchial Alveolar Lavage Fluid

Table 4c. Conditional Logistic Regression Model for Pre-Rejection 25(OH)D Adjusting Age, Race (N=36)

Risk Factors		Odds Ratio	95% CL	P-Value
Pre-rejection 25(OH)D in BALF	Per ng/mL	0.73	(0.47, 1.14)	0.16
Age	Per year	0.89	(0.79, 1.14)	0.52

BALF=Bronchial Alveolar Lavage Fluid

Table 5a. Descriptive Statistics of Categorical Variables for Lung Infection vs. No Infection (N=36)

Risk Factors		Lung Infection		Total	P-value
		Yes n=10 (%)	No n=26 (%)		
Race	Black	2 (20)	5 (19)	7	0.99
	White	8 (80)	21 (81)	29	
Gender	Female	6 (60)	9 (35)	15	0.26
	Male	4 (40)	17 (65)	21	
Lung Diagnosis Prior to Transplant	CF	0 (0)	2 (8)	2	0.30
	IPF	1 (10)	7 (27)	8	
	Emphysema	8 (80)	12 (46)	20	
	Pulmonary Fibrosis	0 (0)	4 (15)	4	
	Sarcoidosis	1 (10)	1 (4)	2	
Pre-Transplant serum 25(OH)D	**Deficient**	**1 (10)**	**14 (54)**	**15**	**0.04***
	Insufficient	6 (60)	6 (23)	12	

Normal	2 (20)	6 (23)	8

* *P* values of < 0.05 were considered statistically significant

Table 5b. Descriptive Statistics of Continuous Variables for Lung Infection vs. No Infection - Median (N=36)

Risk Factors	Yes (n=10)		No (n=26)		P-value
	N	Median (Q1-Q3)	N	Median (Q1-Q3)	
Acute Rejection Days Post Transplant	10	60.5 (53-92)	26	54 (36-90)	0.47
LL-37 in BALF (ng/mL) during acute cellular rejection	10	0.13 (0.05-1.16)	26	0.11 (0.04-0.48)	0.65
25(OH)D in BALF (ng/mL) during acute cellular rejection	9	10.4 (8.4-12.6)	26	12.75 (11.4-13.9)	0.14
Pre-rejection LL-37 in BALF (ng/mL)	7	0.36 (0.24-3.11)	20	0.41 (0.1-1.04)	0.52
Pre-rejection 25(OH)D in BALF (ng/mL)	6	13.95 (11.3-17.4)	20	10.8 (10-13.7)	0.07
Pre-Transplant serum 25(OH)D Level (ng/mL)	**9**	**26 (26-28)**	**26**	**19 (14-26)**	**0.04***
Age (years)	10	59 (52-60)	26	58.5 (53-61)	0.66

** P values of < 0.05 were considered statistically significant*
BALF=Bronchial Alveolar Lavage Fluid

Table 5c. Cohort Univariate Logistic Regression Model for Lung Infection vs. No Infection (N=36)

Risk Factors	Odds Ratio	95% CI	P-Value
LL-37 in BALF during acute cellular rejection (per ng/mL)	1.07	(0.78,1.46)	0.69
25(OH)D in BALF during acute cellular rejection (per ng/mL)	1.00	(0.88,1.13)	0.98
Race (Black vs. White)	0.75	(0.10,5.66)	0.78
Gender (Female vs. Male)	2.83	(0.63,12.71)	0.17
Pre-Rejection LL-37 (per ng/mL)	1.25	(0.83,1.88)	0.28
Pre-Rejection 25(OH)D (per ng/mL)	1.20	(0.96,1.49)	0.11
Pre-transplant Serum 25(OH)D (per ng/mL)	1.07	(1.00,1.15)	0.05
Age (per year)	1.00	(0.92,1.10)	0.91

BALF=Bronchial Alveolar Lavage Fluid

Table 5d. Multivariate Logistic Regression Model for Lung infection vs. No infection, Adjusting for 25(OH)D , Gender, Age (N=36)

Risk Factors	Odds Ratio	95% CI	P-Value
25(OH)D in BALF during acute cellular rejection	1.01	(0.89,1.16)	0.83
Gender (Female vs. Male)	2.46	(0.50,12.14)	0.27
Age (per year)	1.03	(0.92,1.15)	0.65

BALF=Bronchial Alveolar Lavage Fluid

Table 5e. Multivariate Logistic Regression Model for Lung infection vs. No infection Adjusting for LL-37, Gender, Age (N=33)

Risk Factors	Odds Ratio	95% CI	P-Value
LL-37 in BALF during acute cellular rejection	1.23	(0.84,1.81)	0.29
Gender (Female vs. Male)	4.26	(0.73,24.85)	0.11
Age (per year)	1.00	(0.91,1.11)	0.96

BALF=Bronchial Alveolar Lavage Fluid

Figure 2. Kaplan-Meier Curves of Time to Chronic Rejection for 25(OH)D BALF ≥12 ng/mL or <12ng/mL (P-Value=0.60)

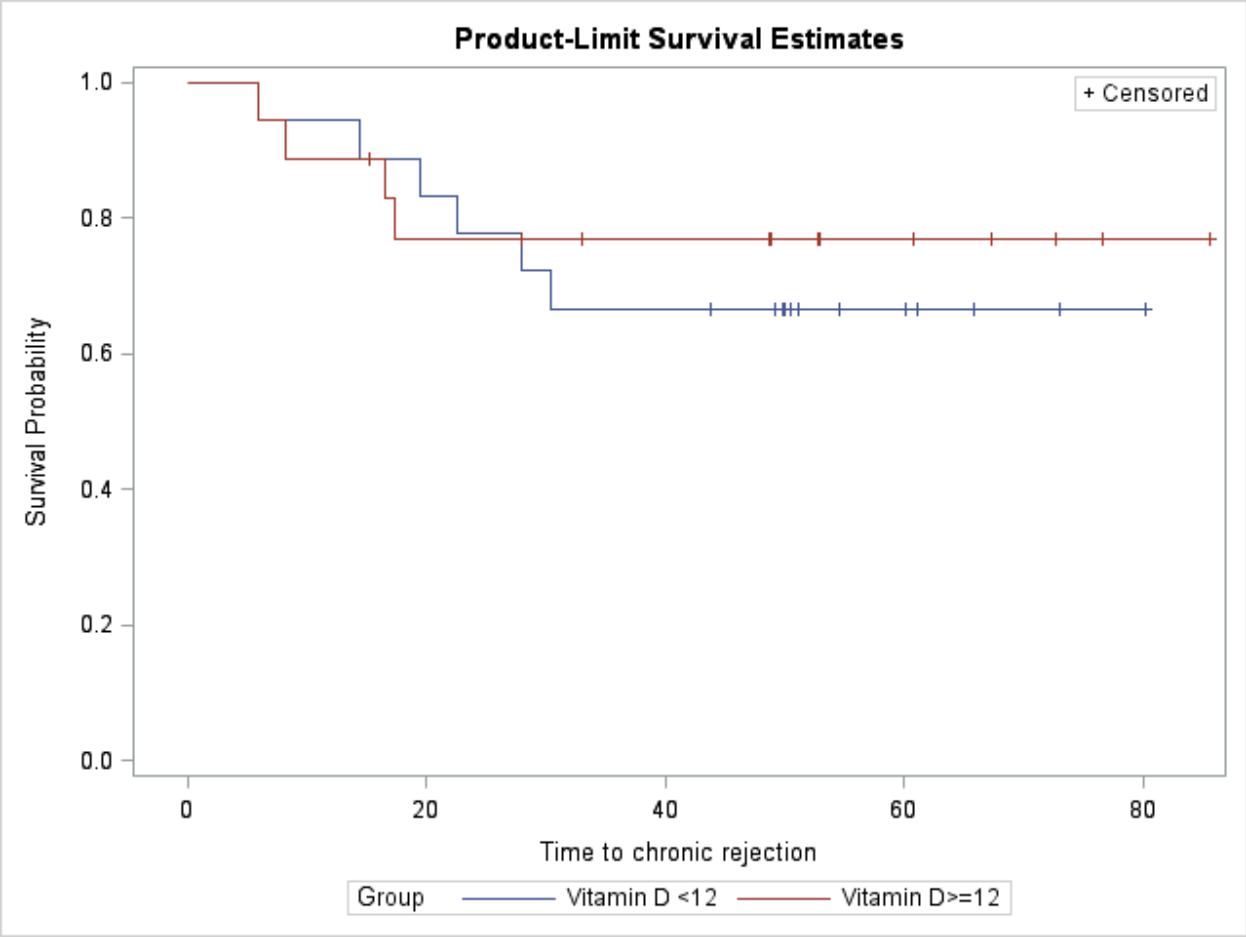

Figure 3. Kaplan-Meier Curves of Time to Death for 25(OH)D BALF ≥12ng/mL or <12ng/mL (P-Value=0.80)

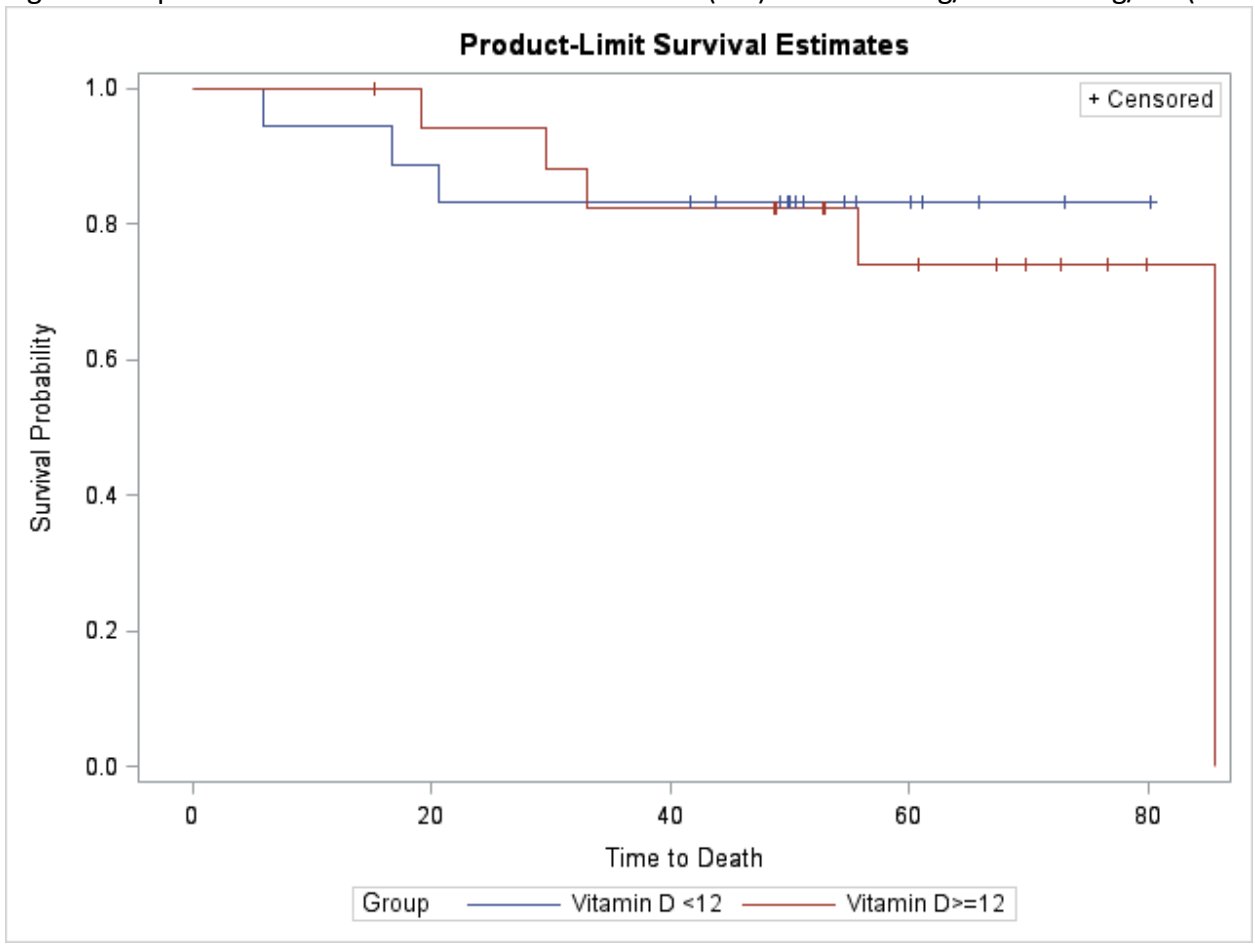

Table 6a. Univariate Cox Proportional Model for Chronic Rejection

Risk Factors	N	Hazard Ratio	95% CI	P-Value
25(OH)D in BALF during acute cellular rejection (ng/mL)	36	1.05	(0.98, 1.13)	0.17
LL-37 in BALF during acute cellular rejection (ng/mL)	33	1.17	(0.95,1.43)	0.14
Pre-Transplant Serum 25(OH)D (ng/mL)	35	0.98	(0.92,1.04)	0.41

BALF=Bronchial Alveolar Lavage Fluid

Table 6b. Univariate Cox Proportional Model for Death

Risk Factors	N	Hazard Ratio	95% CI	P-Value
25(OH)D in BALF during acute cellular rejection(ng/mL)	36	0.98	(0.85, 1.12)	0.76
LL-37 in BALF during acute cellular rejection (ng/mL)	33	1.14	(0.89,1.46)	0.31
Pre-Transplant Serum 25(OH)D (ng/mL)	35	0.97	(0.90,1.05)	0.39

BALF=Bronchial Alveolar Lavage Fluid