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Monitoring T-Cell Kinetics in the Early Recovery Period of Lung Transplantation Cases by Copy Number Levels of T-Cell Receptor Excision Circle

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Abstract. *Background/Aim: Lung transplantation is a life-saving procedure for patients with end-stage lung diseases. T-Cell receptor excision circle (TREC) is circular DNA produced during T-cell receptor gene rearrangement in the thymus and indicates naive T-cell migration from the thymus. Therefore, its levels represent thymic T-cell output. Post-transplant lymphocyte kinetics correlate with graft tolerance. The aim of this study was to investigate T-lymphocyte kinetics in the early recovery period after lung transplantation. For this purpose, copy numbers of TREC were determined in patients with a lung transplant. In addition, TREC copy numbers were evaluated according to age, diagnosis and the forced expiratory volume in 1 second (FEV1) of lung transplant patients. Materials and Methods: Peripheral blood samples were taken from patients aged 23 to 59 years who underwent lung transplantation at the Thoracic Surgery Clinic, Kartal-Koşuyolu High Specialization Educational and Research Hospital. This study included peripheral blood samples from 11 lung transplant patients (comprising four with chronic obstructive pulmonary disease, three with idiopathic pulmonary fibrosis, one with cystic fibrosis, one with silicosis and two with bronchiectasis; three females in total). Samples were taken at three different timepoints: Before transplant, and 24 hours and 7 days post transplant. TREC copy numbers were analyzed with real time reverse transcriptase–polymerase chain reaction. Results: Post-transplant TREC numbers and density values were higher compared to pre-transplant values, although these differences were statistically insignificant. TREC copy numbers were found to be significantly higher in patients younger than 45 years compared to patients older than 45 years. At 24 hours after the transplant, the average TREC copy number/peripheral blood mononuclear cells of the cases with an FEV1 value of or below 50% was found to be statistically significantly higher than that of cases with an FEV1 value above 50% (p=0.046). There was no statistically significant difference in TREC copy numbers between male and female patients or by diagnostic group. Conclusion: TREC copy numbers can be evaluated as a prognostic marker for lung transplantation. There is a need for multicenter studies with more patients.*

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Lung transplantation is a life-saving procedure for those who have end-stage lung diseases. The first successful lung transplant was performed by Fritz Derom in 1971 (1). The first successful lung transplant in Turkey was performed at the Süreyyapaşa Chest Diseases and Thoracic Surgery Training and Research Hospital in 2009 (2).

Lymphocyte kinetics have an impact on the tolerance of transplanted tissue (3). The process of graft rejection or tolerance is dependent on the direct or indirect presentation of graft antigens to alloreactive T-lymphocytes (4-6). Pre-T-lymphocytes originating from the bone marrow enter the thymus *via* the blood and go through the stages of selection and maturation into T-lymphocytes. Gene rearrangement

takes place in the thymus, conferring them with the ability to recognize a wide variety of antigens.

The T-cell receptor (TCR) is expressed on the surface of T-lymphocytes and recognizes peptide antigen delivered *via* major histocompatibility complex molecules on the surface of antigen presenting cells, thereby initiates signals that enable T-cells to function as effectors. TCR undergoes gene regulation to create an active exon during the T-lymphocyte developmental stage in the thymus. In the V(D)J recombination gene editing process, a notch is opened in the double helix with the effect of different signals, and the part holding the signal end is circularly separated from the structure, this is called a signal joint T-cell receptor excision circle (sjTREC). T-Lymphocytes that have been trained in the thymus are added to the peripheral T-cell pool as naive T-lymphocytes and achieve effective function when they encounter an antigen.

The exit of the naive T-cell from the thymus is achieved through circular DNA tracking called TREC (7-10). The remaining encoded ends form a hairpin structure, which is then opened, introducing new triplet groups of nucleotides into the genome. This results in an enormous diversity of antigen repertoire. Connected segments are a coding joint, whereas separated segments are shaped as a signal joint. sjTREC exits to the peripheral blood circulation as a thymic emigrant in $CD4^+$ and $CD8^+$ T-cells (11). TREC does not replicate and becomes diluted during cell division. sjTREC is an indicator of thymic output (12-15).

The thymus shrinks after puberty and is gradually replaced by adipose tissue, however, T-cell output from the thymus continues, with thymocyte production steadily declining with aging (16-18). Data indicate that the adult thymus is also active late in life and provides support to functional T-cells in the periphery (15, 19). After treatment, the peripheral T-cell pool, which is low in immunocompromised and human immunodeficiency virus-positive patients, improves (11-13, 20). This improvement is an indication of naive T-cell output to the periphery. The same applies to patients with hematological malignancies undergoing stem cell transplantation. TREC analysis can be used to follow the recovery period (4, 21, 22).

TREC levels may be expressed using a variety of techniques including *TREC* copy numbers relative to the number of peripheral blood mononuclear cells, $CD45RA^+$ T-cells, $CD4^+$ T-cells and quantity of T-cell DNA (8, 23). The dilution impact of peripheral T-cell division complicates the interpretation of the computation of *TREC* copy numbers per 10^6 PBMCs. The use of T9REC expression per 1 ml of blood can overcome this problem. It is necessary to include the sum of lymphocytes and monocytes in this assessment (8, 11, 19). This is achieved by quantification with an appropriate TREC control gene. *TREC* measurement is compared to control genes such as chemokine receptor 5, albumin, or T-cell receptor alpha constant (TCRAC) (11, 23).

Despite being less common than those on immunodeficiencies, there have been several publications on the study of TREC levels in patients with solid organ transplants (24, 25). High *TREC* copy number in heart transplantation was related to increased lymphocyte support to the peripheral T-cell pool and rejection mechanism. While it is stated that the TREC level decreases with age in heart transplant recipients, a high TREC level is mentioned in cases of rejection (25, 26). In this study, we therefore evaluated the *TREC* copy number in two different age groups: those under 45 years old and those over 45 years old.

A study on lung transplantation and cytomegalovirus (CMV) evaluated thymic function after transplantation. Pre-transplant thymic function levels were found to be low in patients who developed CMV. There are few studies in the literature reporting the relationship between lung transplant and TREC. There are studies indicating that insufficient thymic function poses a risk for the development of CMV after transplant (27). In this regard, early monitoring of peripheral T-cell output in patients with lung transplants is important. TREC analysis is used to monitor peripheral T-cell output in patients whose immune systems have been suppressed and stem cell transplantation has been undertaken. In patients with post-transplant graft *versus* host disease, infection, the level of TREC decreases as T-cell proliferation and division in the periphery increase. T-Cell motility is not routinely monitored in solid organ transplantation. T-Cell kinetics in lung transplant patients is followed up to gain insight into the effectiveness of the treatment regimen and graft success. The aim of this study was to provide information about the early immune response in lung transplant recipients.

Additionally, we sought to see if this response was related to the forced expiratory volume in 1 second (FEV1) (28). The FEV1 value is an important parameter of respiratory function in lung transplantations. To the best of our knowledge, although many studies on the FEV1 value in patients with lung transplants have been undertaken, there is no research on the relationship between FEV1 and TREC. Considering that FEV1 values were linked to $CD4^+$ T-cell activation in smokers (29), it would be meaningful to correlate T-cell output from the thymus following transplantation. Primary graft dysfunction after lung transplantation has been reported to be more likely in patients with low pre-transplant FEV1 values (30).

In this study, *TREC* copy numbers of 11 patients with lung transplant were evaluated with real-time reverse transcriptase–polymerase chain reaction (RT-PCR) at three different timepoints. The relationships between *TREC* copy number and age, FEV1, sex and diagnosis were also investigated.

Table I. *Patient data*.

Diagnosis	Sex	Age, years	Additional illness	Patient status	Survival
Idiopathic pulmonary fibrosis	Female	57	DM	Dead	6 Months
Idiopathic pulmonary fibrosis	Male	39	DM	Dead	9 Months
Idiopathic pulmonary fibrosis	Female	53	–	Dead	7 Days
Chronic obstructive pulmonary disease	Male	49	–	Dead	1 Month
Chronic obstructive pulmonary disease	Male	51	–	Alive	–
Chronic obstructive pulmonary disease	Male	59	TBC	Dead	10 Days
Chronic obstructive pulmonary disease	Male	26		Alive	
Cystic fibrosis	Female	23	TBC	Alive	–
Bronchiectasis	Male	32	–	Alive	–
Bronchiectasis	Male	36	–	Dead	1 Months
Silicosis	Male	41	–	Dead	9 Months

Table II. *Primer sequences used in real-time quantitative reverse transcriptase–polymerase chain reaction*.

Sequence	Survival	
sjTREC	Forward primer	5'-CAC ATC CCT TTC AAC CAT GCT-3'
	Reverse primer	5'-TGC AGG TGC CTA TGC ATC A-3'
	Taqman probe	5'-FAM-ACA CCTCTG GTT TTT GTA AAG GTG CCC ACT-TAMRA-3'
TCRAC (reference standard)	Forward primer	5'-TGG CCT AAC CCT GAT CCT CTT-3'
	Reverse primer	5'-GGA TTT AGA GTC TCT CAG CTG GTA CAC-3'
	Taqman probe	5'-FAM-TCC CAC AGA TAT CCA GAA CCC TGA CCC-TAMRA-3'

sjTREC: Signal joint T-cell receptor excision circle; TCRAC: T-cell receptor alpha constant.

Materials and Methods

Multiple samples from 11 cases (eight men), aged between 23 and 59 years, were included in this study (Table I). Peripheral blood samples were obtained from patients at Kartal-Koşuyolu High Specialization Educational and Research Hospital Thoracic Surgery Clinic. Ethics Committee approval was obtained from Yeditepe University Ethics Committee (decision number 530 on 2 September 2015). *TREC* copy numbers were evaluated in 33 samples from 11 patients before transplantation and at 24 hours, and 7 days after transplantation. The blood of healthy children under 3 years of age was used to establish a reference standard. The primer and the probes had the sequences published in reference articles, and were confirmed by BLAST web site (15, 19).

The amplicon sizes of the primer sets were verified and standards with known copy numbers of the genes of interest (*TREC* and *TCRAC*) were established. DNA isolation was performed with Qiagen Mini Blood Kit (Qiagen, Hilden, Germany) from the samples separated with Ficoll-Hypaque (Life Science, Uppsala, Sweden).

Standard optimization of the method. DNA isolated from blood samples from healthy children (<3 years old) was used as a standard (19). *TCRAC* was used as the reference gene. Different temperature and primer concentrations were evaluated to determine the optimal PCR conditions. DNA ladder (Invitrogen, Life Technologies, USA) was used as reference during the optimization process. The validation process showed that an annealing temperature of 58˚C for *TREC* and 62˚C for *TCRAC*, and a primer concentration of 0.9 μl for both in the PCR reaction were optimal, in accordance with a reference study (15).

TREC and *TCRAC* standards were purified from the gel, fluorimetric measurements of concentration were made and the standards were stored at −20˚C as a stock standard for PCR. The copy numbers of the standards were calculated and serial dilutions were made in each study. During PCR, samples were prepared fresh each time from the stock standard and were studied with primers specific to *TREC* and *TCRAC* genes.

TREC and *TCRAC* standards were prepared and Qubit measurements were made: *TREC*=88 bp, *TCRAC*=80 bp.

The number of copies was calculated according to the formula below:

$$\text{Copy number} = \text{Standard DNA count} \times 6.022 \times 10^{23} / \text{base pair count} \times 1.109 \times 650$$

RT-PCR assay. RT-PCR of patients' samples and of standards were performed with primers specific to *TREC* and *TCRAC* genes given in Table II. *TREC* and *TCRAC* genes were quantified according to the standard curve.

The primary probes have the sequence used in the reference article (15) and were validated with the BLAST program (April 2016; https://blast.ncbi.nlm.nih.gov/Blast.cgi).

In this investigation, the *TREC* copy number in 1×10^6 PBMCs was calculated by dividing the mean *TREC* copy number by half the *TCRAC* copy number, since each cell has two copies, one for each chromosome. *TREC* does not replicate itself during cell division, however, *TCRAC* is passed to the newly created cell (15).

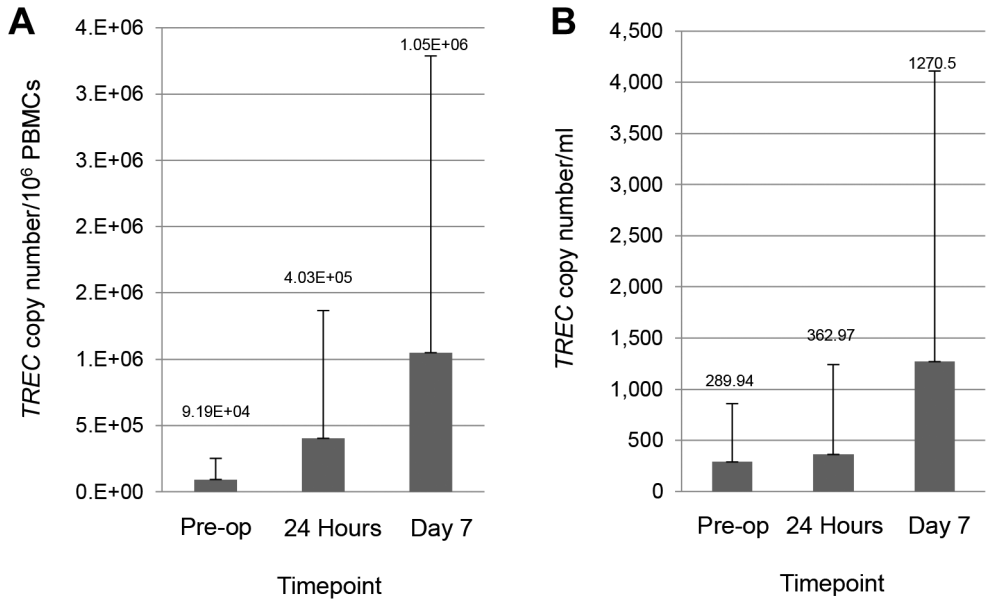

Figure 1. *T-Cell receptor excision circle (TREC) copy number per 10^6 peripheral blood mononuclear cells (PMBCs) (A) and per milliliter of whole blood (B) during the study period. The TREC copy number per 10^6 PBMCs and per milliliter of blood increased on day 7 but this was not statistically significant. Mean values and standard deviations are shown.*

By multiplying the value obtained by the number of lymphocytes plus monocytes, the number of *TREC* copies per milliliter of blood was obtained.

Statistical analysis. IBM SPSS Statistic 22 (IBM Corp., Armonk, NY, USA) program was used for statistical analysis.

The conformity of the parameters to the normal distribution was evaluated with the Shapiro Wilks test. The Mann-Whitney *U*-test was used for the comparison between two groups of data that were not normally distributed in the evaluation of *TREC* copy number. Kruskal-Wallis test was used for intergroup comparisons of non-normally distributed parameters. Wilcoxon signed-rank test was used for in-group comparisons of non-normally distributed parameters. Spearman's Rho correlation analysis was used to examine the relationships between parameters. Significance was assessed at the $p<0.05$ level.

Results

TREC copy *number*. There was no statistically significant change in the mean *TREC* copy number/PBMCs at 24 hours and 7 days compared to the pre-transplantation period ($p>0.05$) nor in the mean *TREC* copy number/PBMCs on day 7 compared to that at 24 hours ($p>0.05$). However, on day 7, the copy number was more than 10-fold compared to the pre-transplantation value (Figure 1A). There was also no statistically significant change in the mean *TREC* copy number/ml at 24 hours and day 7 compared to the pre-transplantation period ($p>0.05$) nor at 7 days compared to that at 24 hours ($p>0.05$) (Figure 1B).

Although the *TREC* copy number/PBMCs was not statistically significantly different it was found to be higher in seven out of 11 patients at 24 hours compared to pre-transplantation.

Lymphocyte count. The decrease in lymphocyte percentage at 24 hours compared to the pre-transplant period ($p=0.003$) and the increase in the lymphocyte percentage on day 7 compared to that at 24 hours were statistically significant ($p=0.041$). The decrease in the mean absolute lymphocyte number at 24 hours compared to the value in pre-transplant period was statistically significant ($p=0.004$). The results regarding lymphocyte percentage and absolute lymphocyte counts are given in Table III.

TREC copy *number and age*. We chose 45 years as a cut-off for age as it was the average age of the 11 patients. The mean *TREC* copy number/PBMCs on day 7 of the cases under the age of 45 years were found to be statistically significantly higher than that of patients aged over 45 years ($p=0.011$; Figure 2A). The mean *TREC* copy number/ml of the cases under the age of 45 years were similarly statistically significantly higher than that of cases aged over 45 years ($p=0.018$; Figure 2B).

TREC copy *numbers and FEV1 value*. The mean *TREC* copy number/PBMCs of cases with FEV1 value below 50% was statistically significantly higher at 24 hours than that

Table III. *Percentage lymphocyte count and absolute lymphocyte count in patients who underwent lung transplantation prior to transplant and at 24 hours and days after transplant.*

Timepoint		Lymphocytes (%) Mean±SD (median)	*p*-Value*	Absolute lymphocyte count ($\times 10^3/\mu l$) Mean±SD (median)	*p*-Value*
Pre transplant		19.81±8.62 (18.7)		2.05±1.11 (1.6)	
Post transplant	24 Hours	2.21±1.65 (1.5)	**0.003**[a]	0.41±0.19 (0.5)	**0.004**[a]
	7 Days	13.21±22.46 (3.3)	0.248[a]	1.08±1.55 (0.5)	0.154[a]
			0.041[b]		0.233[b]

SD: Standard deviation. *By Wilcoxon sign test *versus* [a]pre-transplant value, [b]value at 24 hours. Statistically significant *p*-values are shown in bold.

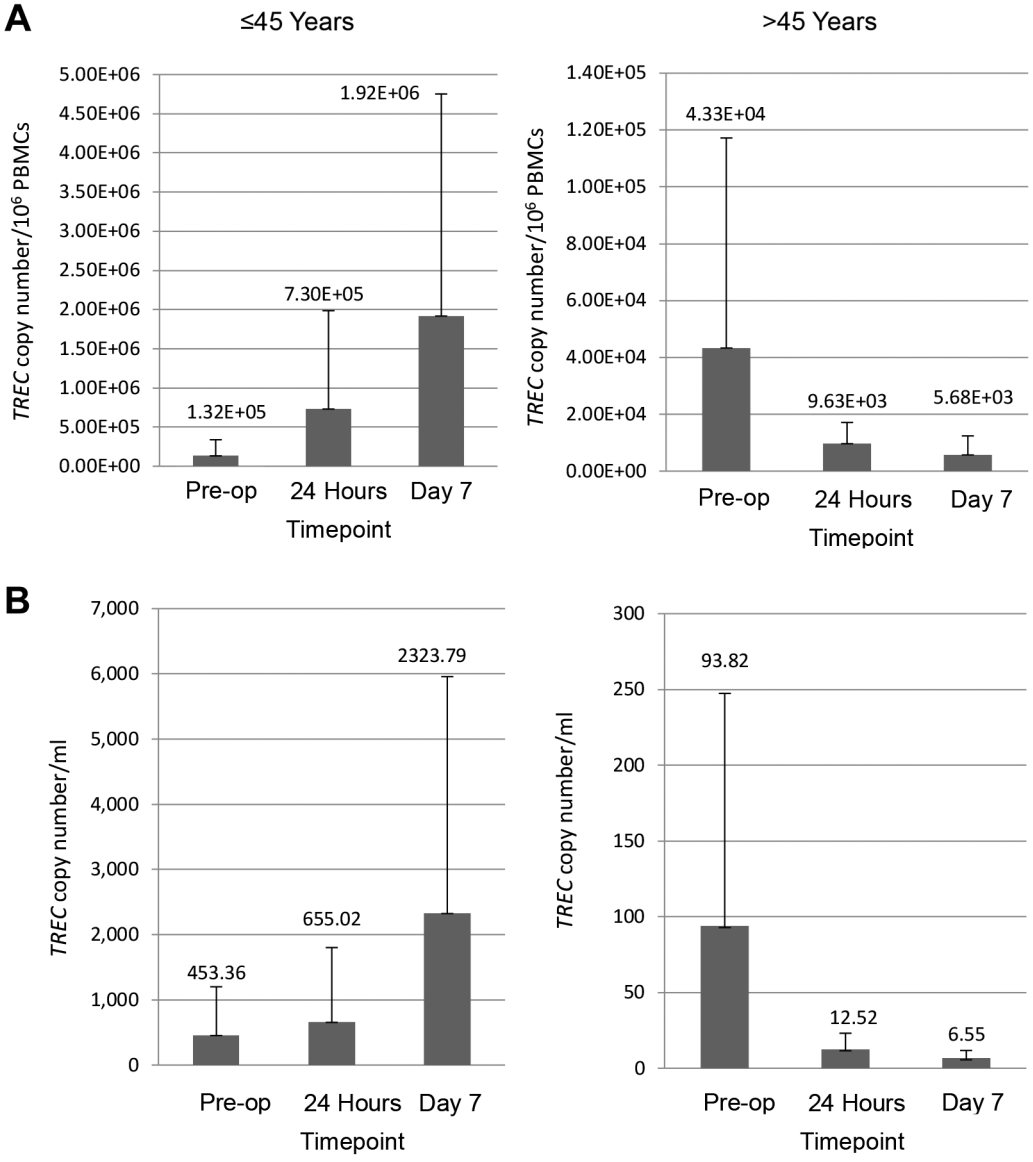

Figure 2. *T-Cell receptor excision circle (TREC) copy number per 10^6 peripheral blood mononuclear cells (PMBCs) (A) and per milliliter of whole blood (B) on day 7 after transplant. TREC copy numbers per 10^6 PBMCs (p=0.011) and per milliliter of blood (p=0.018) were statistically significantly higher in the group ≤45 years of age than in the group >45 years old. Mean values and standard deviations are shown.*

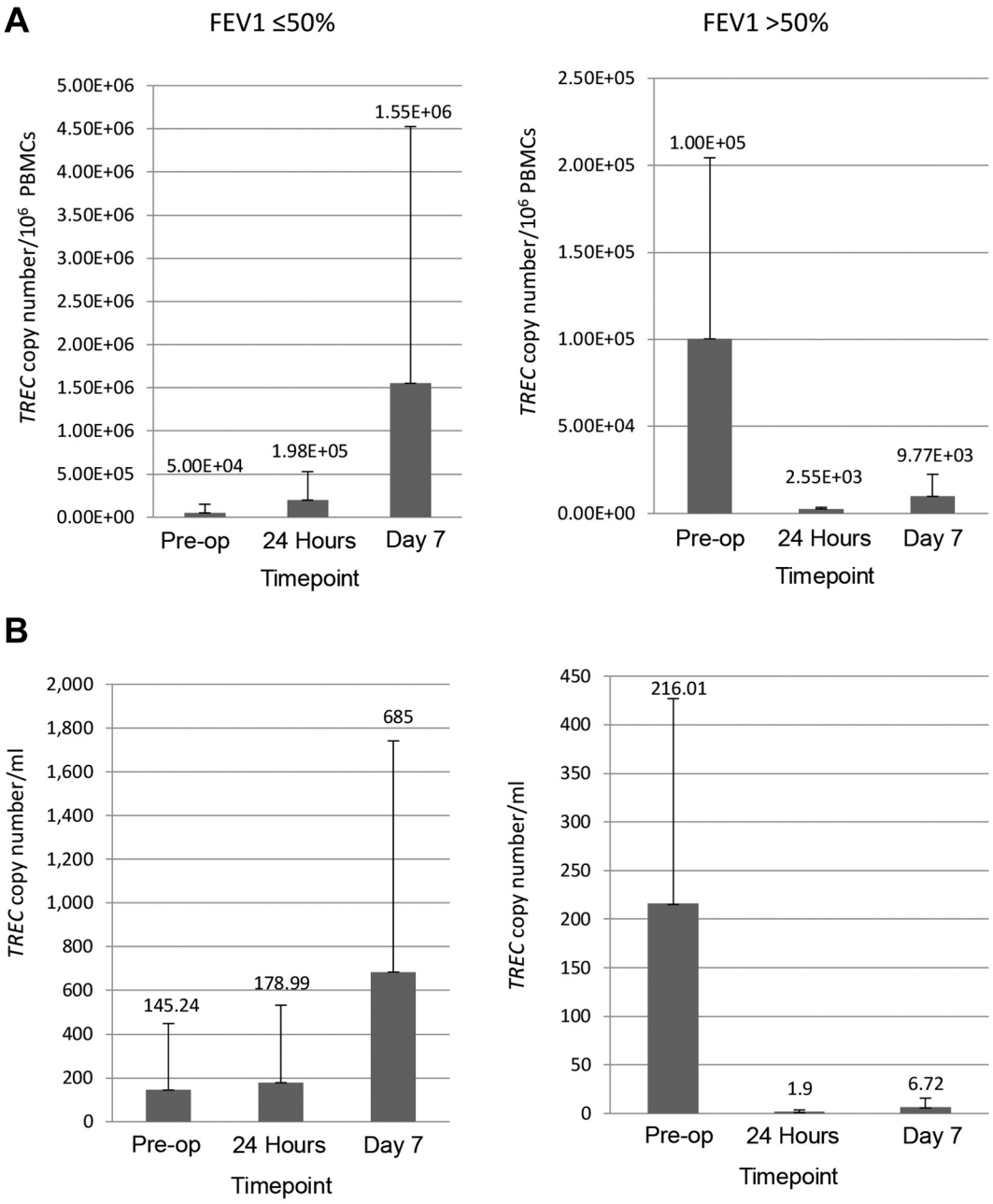

Figure 3. *T-Cell receptor excision circle (TREC) copy number per 10^6 peripheral blood mononuclear cells (PMBCs) (A) and per milliliter of whole blood (B) according to forced expiratory volume in 1 second (FEV1) during the study period. At 24 hours, TREC copy numbers per 10^6 PBMCs ($p=0.046$) and per milliliter of blood ($p=0.046$) were statistically significantly higher in the group with FEV1 ≤50% than in the group with FEV1 >50%. Mean values and standard deviations are shown.*

of patients with FEV1 above 50% (p=0.046; Figure 3A). The mean of 24 hours *TREC* copy number/ml of cases with FEV1 value of or below 50% was found to be statistically significantly higher than the mean of patients with FEV1 value above 50 (p=0.046; Figure 3B).

TREC copy *numbers and sex and diagnosis*. There were no significant difference in pre-transplant, 24-hour, 7-day *TREC* copy number/PBMCs between men and women (p>0.05); there were also no significant differences in these values when expressed as *TREC* copy numbers/ml between (p>0.05).

The patients were divided into three groups according to their diagnosis: Idiopathic pulmonary fibrosis (three patients), chronic obstructive pulmonary disease (four patients) and other diseases (silicosis, one patient; bronchiectasis, two patients; and cystic fibrosis, one patient). There was no statistically significant difference between or within groups in *TREC* copy numbers/PBMCs and *TREC* copy number/ml at 24 hours compared with pre-operatively or day 7, nor on day 7 compared with pre-operatively and 24 hours after transplantation ($p>0.05$).

Discussion

T-Lymphocytes play an important role in the response to transplanted tissue. The molecular analysis of *TREC* is performed to monitor the number of naive T-lymphocytes coming from the thymus. *TREC* copy number monitoring is employed as an indicator following therapy in diseases involving T-cell kinetics, such as immunodeficiency, hematopoietic stem cell transplantation and human immunodeficiency virus.

Several articles have been published on the analysis of TREC in solid organ transplantation (3, 24, 26). *TREC* levels reportedly decrease with age in heart transplant recipients, while high TREC levels are mentioned in cases of transplant rejection (25).

Publications about *TREC* copy numbers in patients after lung and heart transplants are limited (24, 31, 32). T-Cell responses are directly associated with the mechanism of rejection of solid organ transplants. In this sense, early monitoring of peripheral T-cell output is important in lung transplantation. TREC analysis is used to characterize peripheral T-cell output in immunocompromised patients undergoing stem cell transplantation and is considered a diagnostic marker (20, 33). T-Cell kinetics is not routinely monitored in solid organ transplantation. There is a need to support the importance of thymic output through multicenter studies with more patients.

The increase in *TREC* copy numbers after 24 hours and 7 days after transplant compared to before transplantation demonstrates that T-cell support is supplied to the peripheral T-cell pool. In addition, while the lymphocyte percentage and absolute lymphocyte values were reduced at 24 hours, the increase in the *TREC* copy number at 24 hours confirms this notion.

The reduction in mean absolute lymphocyte number and percentage at 24 hours was statistically significant ($p=0.041$). The increase in the *TREC* copy number in parallel with the increase in the lymphocyte percentage on day 7 indicates that the T-cell outflow continued after transplantation, but T-cell proliferation was not enough to reduce the *TREC* copy number. The percentage of lymphocytes was also increased on day 7 compared to that at 24 hours. The lymphocyte plus monocyte value was included when calculating the *TREC* copy number/ml. The inclusion of the lymphocyte value while calculating *TREC* copy number/ml might explain the increase in *TREC* copy number/ml on day 7 compared to that at 24 hours. It is important in this sense that the percentage of lymphocytes and the absolute number of lymphocytes decreased while the median white blood cell count increased.

The plasmid-based technique was used to generate standard curves in the vast majority of TREC studies in the literature. In this study, blood samples, in ethylene diamine tetra-acetic acid-containing tubes, from healthy children under the age of 3 years who provided blood for blood group analysis were utilized to set a standard (15).

The dilution effect of peripheral T-cell division complicated the interpretation of the TREC calculation per 10^6 PBMCs and per 1 ml of blood can overcome this problem. It is necessary to include the total of lymphocytes plus monocytes in this calculation. As stated in other studies in the literature, *TREC* copy number per millimeter or microliter is the most reliable indicator of thymic cell output (8, 34).

TREC decreases with age and it is emphasized that this decrease remains stable between the ages of 55-80 years (16). In a study using RT-PCR for quantification of sjTREC in humans, sjTREC concentration was investigated in samples from 43 healthy individuals aged 1-83 years. They showed that the TREC concentration decreased exponentially in the age range of 16 to 62 years. They also found that there was no significant difference in sj*TREC* copy number between the groups aged 1-10 and 11-40 years but there was a significant decrease between the groups aged 41-60 and 61-80 years, although there was no difference between the sexes. *TREC* is known to decline with aging due to decreasing thymus function. In another article investigating the relationship between *TREC* copy number and age, two age groups were formed with children (0-13 years) and adults (13-60 years), and it was observed that *TREC* copy numbers decreased with age in both groups (16). In our study, the fact that peripheral T-cell outflow was better in cases younger than 45 years of age compared to cases over 45 years of age confirms the literature data on effects due to age (19, 35, 36).

In solid transplants, the significance of transplant age arises. Peripheral T-lymphocyte outflow in transplant recipients also necessitates an age-related interpretation of the rejection mechanism in relation to allograft rejection. *TREC* copy number/ml increased on day 7 compared to the 24th hour in the group under 45 years of age. In addition, the lymphocyte count on day 7 increased compared to the 24th hour. This highlights the importance of adding the lymphocyte count to the calculation when calculating the *TREC* copy number. In addition, better thymic function in younger patients confirms the information that thymic naive

T-cell output will be higher than in older patients, and it can be said that the contribution of the thymus to the peripheral T-cell pool is higher in younger patients.

This study found that the mean *TREC* copy number/ml at 24 hours of patients with an FEV1 value of 50% or less was significantly higher on average than that of patients with a higher FEV1 value. The FEV1 value is an important respiratory parameter monitored in patients with lung transplants (28, 29). There are studies in the literature that associate allograft dysfunction with low FEV1 values. In our study, the *TREC* copy numbers of cases with FEV1 values below 50% were found to be high. Considering the study showing the correlation between T-cell activation and FEV1 value (29), the follow-up of T-cell exit from the thymus may be important for the follow-up of transplantation success in the early period.

There was no statistically significant difference by sex in *TREC* copy numbers by either computation techniques used ($p>0.05$). While one study on the subject reported the same finding (8), the majority state that the *TREC* level is higher in women (15, 35). Additionally, in patients aged 90 years and older, a significant decrease was detected in both sexes; however, there are also studies reporting that this difference between men and women disappears after the age of 75 years and emphasized that *TREC* concentration decreases with aging, and this decrease is stable between the ages of 55-80 years (16). Although there are studies in the literature stating that it is higher in women, no significant difference has been shown in terms of *TREC* copy number and sex (16). A study demonstrating that the *TREC* copy number declines with age found that the *TREC* copy number was higher in women than in men, however this difference was not statistically significant (35).

TREC copy numbers, in our study, were found to be higher at 24 hours and on day 7 compared to pre-transplant levels by both calculation methods, albeit not statistically significant. The *TREC* copy numbers in the group under 45 years of age were found to be statistically significantly higher than the group over the age of 45 by both calculation methods. According to both calculation methods; *TREC* copy numbers were found to be statistically significantly higher in cases with FEV1 value of 50% or less compared to the group with a value more than 50%.

Working with larger groups of patients with lung transplants will be more meaningful in terms of revealing the change of thymic output by sex and associating it with graft tolerance. Furthermore, conducting a study in groups with a higher number of patients undergoing lung transplantation owing to diverse lung diseases will shed light on whether the thymic output changes depending on the diagnosis. This study was a preliminary study to understand whether thymic output represents a marker to be used in the follow-up of transplant success, but more research in multi-center studies including different parameters related to graft rejection is needed.

Conflicts of Interest

The Authors report no conflicts of interest concerning the materials or methods used in this study or the findings reported in this article.

Authors' Contributions

Fatma Tuba Akdeniz and Zeynep Akbulut: Project design, conducting laboratory processes, sharing the results with other researchers, statistical analysis, article writing. Mustafa Vayvada: Specimen collection, transplantation, clinical stage and scientific opinion. Merih Kalamanoğlu Balcı: Project design, patient selection, providing patient data, reviewing draft article, providing relevant references. Ali Yeğinsu: Design of the project, provision of patient samples, article writing and review. Gülderen Yanıkkaya Demirel: Project design, planning, conducting and supervising laboratory studies, writing and reviewing articles. Cemal Asım Kutlu: Design of the project, provision of patient samples, article writing and review.

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