Clinical significance of T cell receptor excision circle (TREC) quantitation after allogenic HSCT

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INTRODUCTION

Allogenic hematopoietic stem cell transplantation (allo-HSCT) is widely used as a mode of treatment in a variety of benign and malignant disorders. Despite being lifesaving in some situations, it is not without severe drawbacks, such as failure of engraftment, graft-versus-host disease (GvHD), relapse, and profound and long-lasting immunodeficiency with fatal infections [1].

Reconstitution of the different lymphocyte populations and myeloid cells is an important event after allo-HSCT, routinely tested with absolute lymphocyte and lymphocyte subset counts, as well as antibody titers. The thymus has an important role in long-term reconstitution which may provide a chance of targeting it therapeutically [2]. T cell reconstitution occurs either by peripheral expansion of donor and recipient T cells that survived conditioning, or by de novo production of naive T cells in the recipient thymus. This T cell repertoire is vital for the development of a strong adaptive immune response against pathogens and tumors, without leading to GvHD [3-5].

T cell receptor excision circles (TRECs) are proposed to be quantitative markers of thymic output which is not yet routine in transplantation procedures [6]. TRECs are circular DNA by-products generated from double-stranded intervening sequences during the V(D)J recombination process that joins the TCR gene segments. TRECs seem to be stable throughout the life of a T-lymphocyte. The population of TRECs is diluted by cell proliferation. At the double-positive αβ-TCR/CD3 stage of thymocyte development, most TCR-α gene loci first undergo a rearrangement that deletes much
of the TCR-δ gene locus, which is located between clusters of Vα and Jα segments. This rearrangement forms a signal joint (sj) between the δRec segment and the downstream Jα segment. sjTREC is the segment that contains the deleted Dδ, Jα, and Cδ segments [7].

Studies on the clinical utility of TRECs were initiated by screening programs for severe combined immunodeficiency (SCID) [8]. This was followed by research on the role of TREC measurement in various diseases and infections like T cell lymphoma, and HIV and retroviral infections [9-12].

The role of TREC quantification has evolved in both HSCT and solid organ transplantation. Some researchers have reported that pre-transplant TREC predicts acute rejection in renal transplant patients [10]. Others reported increased TREC levels during rejection episodes of cardiac transplants [13].

In the context of HSCT, studies were done in different settings and time points, and correlated with outcomes [14-16]. However, correlations between TREC levels and HSCT outcomes remain to be elucidated. Given the simplicity of the test and the provisional value in the evaluation of different outcomes of transplant, we aimed at analyzing the role of TREC quantification in a number of allogenic HSCT transplant recipients. We compared TREC levels to their age-matched sibling healthy donors, to different parameters, and to different transplant outcomes. We focused on early single-point measurements to emphasize the role of the test in predicting outcomes which, in turn, may facilitate therapeutic interventions.

**MATERIALS AND METHODS**

**Patients’ data**

The study was conducted on 200 subjects, 100 patients receiving allogenic HSCT from an HLA-identical sibling and 100 donors taken as controls. The cases were collected from both BMT units in Alexandria and Nasser Institute, Cairo over a period of two years.

**TREC analysis**

DNA extraction was done using ABIOpure extraction kit (Cat No: M501DP100, Alliance Bio Inc., Bothell, WA, USA). In some patients, T cells were separated by rosette selection technique (StemCell Technologies, Vancouver, BC, Canada) and DNA extracted. Samples were collected from controls once and compared to both pretransplant and day 28 samples from patients.

Detection of TREC values was done by real time PCR using standard curve method for target gene amplification. Primers and probes were supplied by Applied Biosystems (ThermoFisher Scientific, California, CA, USA) with the following sequences: CACATCCCTTTCAACCATGCT (forward primer); GCCAGCTGCAGGGTTTAGG (reverse primer); and FAM-ACACCTCTGGTTTTTGTAAAGGTGCCCACT-TAMRA (TaqMan probe). The PCR mixture contained 10 μL of mastermix (containing 0.125 μL; Ampli Taq, 2.5 μL; Buffer, 1.75 μL of 50 mM Mg, 0.5 μL of 10 mM dNTP), 1 μL of 12.5 μM of each forward and reverse primer and 1 μL of 5 μM probe, 5 μL of template DNA (or standard plasmid dilution), and 3 μL water. The experiment was done on Stratagene thermocycler (ThermoFisher Scientific) with cycles as follows: 95°C for 5 min to activate the Platinum Taq, followed by 50 cycles of 95°C for 30 s and 60°C for 1 min. The standard curve was constructed using a plasmid generously provided by Dr. Daniel Douek, Vaccine Research Center, National Institute of Allergy and Infectious Diseases, Bethesda. The concentration of the plasmid as determined by its absorbance was 1.4 mg/mL. Following the instructions of the providing laboratory 994 μL water was added to 6 μL plasmid to yield 1 mL of 2×10⁹ plasmid copies/μL. The standard curve was constructed with the following serial dilutions: 2×10⁷, 2×10⁶, 2×10⁵, 2×10⁴, 2×10³, 2×10², 20, and 2 plasmid copies/μL. The curve efficiency was 76.8%, Rsq was 0.948, and Y intercept (slope) was -4.039 (Fig. 1).

TRECs were calculated as TREC copies/μg DNA, and TRECs/mL. TREC copies/mL blood were calculated using the following equation:

\[
\text{Total DNA (μg) in 300 μL WB DNA (μg) amplified in RQ-PCR} \times \left( \frac{\text{No. of TREC}}{300} \right)
\]

Where (WB — whole blood; RQ-PCR — real time quantitative PCR; No. of TREC is absolute value derived from RQ-PCR standard curve) [16].

**GvHD prophylaxis**

GvHD prophylaxis in patients was done using cyclosporine A from day 1 till 1 year after transplantation, as guided by trough levels. In addition, methotrexate was administered...
on days 1, 3, 6, 11, and 15. For B thalassemia patients, methotrexate was replaced with steroids from day 7 to +4.

**CD34 dose and source of stem cells**

All cases received mobilized peripheral blood stem cells as a source of the graft. The mean and median dose of CD34+ cells was 9.1±5.7×10^6/kg BW (range of 3.4–36.5×10^6 cells/kg BW) and 8.55×10^6/kg BW, respectively.

**Statistical analysis**

Collected data were analyzed using IBM SPSS software version 20.0 (IBM Corp, Armonk, NY, USA). Qualitative data were described using number and percent. Quantitative data were described using range, mean, standard deviation, and median values. Significance of the obtained results was judged at the 5% level.

### Table 1. Patient characteristics.

|                                 | Patients (N=100) | Control subjects (N=100) | Test of significance | P   |
|---------------------------------|------------------|--------------------------|----------------------|-----|
| Age (mean±SD, yr)               | 20.5 (2.5–48.0)  | 22.0 (3.0–44.0)          | U=1242               | 0.956|
| Benign/malignant                | 26/74            | NA                       | -                    | -   |
| Diagnosis                       |                  |                          |                      |     |
| ALL                             | 18%              |                          |                      |     |
| AML                             | 44%              |                          |                      |     |
| Biphenotyping acute leukemia    | 2%               |                          |                      |     |
| Myelodysplastic syndromes       | 6%               |                          |                      |     |
| Beta thalasemia major           | 14%              |                          |                      |     |
| Aplastic anemia                 | 8%               |                          |                      |     |
| Paroxysmal nocturnal hemoglobinuria | 2%          |                          |                      |     |
| Chronic myeloid leukemia         | 4%               |                          |                      |     |
| Niemann pick disease            | 2%               |                          |                      |     |
| Conditioning regimen            |                  |                          |                      |     |
| FLU/BU                          | 16%              |                          |                      |     |
| BU/CY                           | 40%              |                          |                      |     |
| BU/CY/ATG                       | 14%              |                          |                      |     |
| CY/TBI                          | 16%              |                          |                      |     |
| FLU/CY                          | 10%              |                          |                      |     |
| FLU/ALK                         | 4%               |                          |                      |     |
| Sex matched/mismatched          | 56/44            | NA                       | -                    | -   |
| Neutrophil engraftment (days)   | 15 (10–27)       |                          |                      |     |
| Platelet engraftment (days)     | 14 (5–33)        |                          |                      |     |
| GvHD (yes/no)                   | 30/70            |                          |                      |     |
| Acute                           | 8                |                          |                      |     |
| Chronic                         | 22               |                          |                      |     |
| Relapse (yes/no)                | 10/64            |                          |                      |     |
| Infections (pos/neg)            | 26/74            |                          |                      |     |
| Survival (alive/dead)           | 72/28            |                          |                      |     |
| TREC (day 28) copies/μg DNA (N=100) | 28.3 (0-2215)     | 31.3 (1.4-1384.0)        | U=1210.5             | 0.785|
| TREC/ml (day 28) (N=100)        | 2984.5 (0.0-399400.0) | 5723.0 (114.5-571483.3)  | 990.5               | 0.074|
| TREC copies/ml (N=30)           | 51.4 (0–448)     |                          | NA                   |     |
| TREC copies/ml (N=30)           | 534 (0–5888)     |                          |                      |     |
| Pretransplant TREC (N=34)       | 43.6 (0–6359.0)  | 111.0 (1.41–517.50)      |                      |     |

**RESULTS**

**Patient characteristics**

Two hundred subjects were involved in this study—100 patients undergoing allogenic HSCT and 100 age matched controls who were their donors. Patients’ ages ranged from 2.5 to 48 years with a mean of 20.5 years, (72% adults and 28% pediatric patients). Twenty-six and 74% of cases were transplanted for benign and malignant diseases, respectively. Additionally, 56 and 44% of cases were sex matched and mismatched, respectively. Follow-up of patients was done for a median of 2 years. Outcomes were measured as days to neutrophil and platelet engraftment, development of infections, and GvHD, as well as survival rates during the follow-up period. Neutrophil engraftment occurred at a median of 15 days (range, 10–27) and failed in one patient, while platelet engraftment occurred at a median of 14 days (range, 5–33) and failed in three patients. Twenty-six percent of patients developed infections, while 30% developed GvHD (10 of them acute GvHD and 20 chronic GvHD). Out of
the 74 malignant cases, 10 relapsed (13.5%). Twenty-eight percent of patients died during the follow-up period due to infections, relapse, and failure of engraftment (Table 1).

**Day 28 whole blood TREC**

TRECs were measured at a single point in all patients—day 28 post-transplant.

Median TREC levels was 28.3 copies/μg DNA in patients (range, 0–2215) versus 31.3 copies/μg DNA in control samples (range, 1.4–1384), while median TREC/mL blood was 2984.5 (0.0–399400.0) in patients versus 5723 (114.5–571483.3) in control samples. Therefore, no significant difference in TREC copies/μg DNA at day 28 was detected between patients and controls (P=0.785). TREC levels/mL blood in patients was significantly lower than that in control (P=0.074).

**Day 28 TREC levels and patient characteristics**

There was a negative correlation between patients’ age and both TRECs and TRECs/mL (P=0.002 and 0.003, respectively). We further tested this correlation in healthy controls and found a similar decrease in TREC levels with age in healthy controls (P=0.001) (Fig. 2). No difference was detected between males and females regarding TREC and TRECs/mL levels. (P=0.334 and 0.633, respectively). There was a significant difference between TREC copies/μg DNA at day 28 between patients with malignant diseases (21.0; range was 0–2215.0) and benign diseases (108.2; range was 0–1997.0) (P=0.045). Similarly, TRECs/mL value was 12178.9 (0.0–399400.0) and 2730.0 (0.0–370600.0) in benign and malignant diseases, respectively (P=0.054) (Fig. 3). Among the 38 patients aged below 18 years, 22 were transplanted for benign diseases versus 6/62 in cases above 18 years, but median levels of TRECs in benign diseases were higher in both age groups as compared to those in malignant diseases within the same age group.

**Day 28 TREC levels and patient outcomes**

There was no correlation between TRECs and TRECs/mL and days to neutrophil and platelet engraftment. There was no significant difference in TREC levels between patients who developed infections and GvHD. However, levels of TRECs at day 28 were lower in patients who later developed infections, and higher in those who developed GvHD. Median TREC level values were 52.9 copies/μg DNA (range, 0–2215.0) in patients who developed GvHD versus 23.4 copies/μg DNA (0–1853.0) in those who didn’t (P=0.330). Additionally, median values were 23.4 copies/μg DNA (0–2215.0) in patients with infections versus 35.0 copies/μg DNA (0–1997.0) in patients without infections (P=0.626). Infectious complications were cytomegalovirus (CMV) reactivation in six patients, Herpes simplex virus in two, and severe bacterial sepsis and pneumonia in 18 patients. We could not study TREC levels in patients according to the type of infections due to the small number of patients in each group.

We compared TREC levels at day 28 between patients who showed a relapse (N=10) and other patients who suffered malignant diseases but did not show a relapse (N=64). Patients who showed a relapse had lower median levels though with no significant difference [21.0 (0–175.2) vs. 44.8 (0–2215.0) copies/μg DNA]. No correlation was detected between TREC levels and survival during the follow-up period (Fig. 4).

We further divided our cases into low or high levels according to the median TREC levels, which were 28.3 and 2894.5 for TRECs and TRECs/mL, respectively. We found a statistically significant difference between TREC levels in patients with benign and malignant diseases—75.6% of malignant patients had low TREC levels, whereas 24.4% had high TREC levels (P=0.02). Additionally, none of the patients with high TRECs/mL showed a relapse, while 15% of patients with low TRECs/mL showed a relapse (P=0.009). No other differences were detected between the patients with low and high TREC levels (Table 2).

**Multivariate analysis**

Multivariate analysis was conducted to study the different variables in correlation to TREC levels. Multivariate analysis showed significance with age (P=0.026) and relapse (P=0.0...
-0.037) and an approaching significance regarding survival ($P=0.075$).

**T cell TREC**

TREC**s were measured from DNA isolated from T cells at day 28 in 30 cases. Measurement of TREC**s from T cell DNA did not seem to add any value to the test. T cell TREC**s were 51.4 copies/µg DNA (0–448) while T cell TREC/mL was 534 copies/mL blood (0–5888). T cell TREC**s did not correlate with whole blood TREC**s in these thirty cases (rs=0.064 and $P=0.820$ for TREC**s, and rs=0.075 and $P=0.791$ for TREC**s/mL).

**DISCUSSION**

TREC**s have been studied as markers of immune reconstitution following HSCT. However, the role of this test as a predictor of outcome in HSCT remains to be elucidated. The aim of our work was to study TREC levels at a single point post-transplantation, correlate it with different patient parameters, and test whether the levels would predict outcomes of transplant. The study was conducted on 200 sub-
jects—100 patients undergoing HSCT from an HLA identical sibling and their donors were taken as controls.

We chose the measurement at day 28 for sampling feasibility, as this is the point when samples are withdrawn for chimerism testing. Additionally, this time point is early, so their role in prediction of outcomes could also be studied. Initially, we had collected the samples at engraftment, but this would have varied from patient to patient. Previously, studies have used many different points for measurement of TRECs, from day 15 to one-year post-transplant [17, 18].

At day 28, there was no statistically significant difference in TRECs/μg DNA levels between the patients and controls. Alternatively, the TREC copies/mL values were lower in patients than in controls, with a P-value approaching significance (0.07). Previous studies have not measured TREC levels before three months post-transplant, nor have they reported the full recovery of TRECs before 3–9 months [17-19]. Considering our data regarding TREC/μg DNA, it seems that patients reached the same level of TRECs as their controls. One possible reason for this relatively rapid recovery of TRECs is that all our patients had a completely matched sibling donor. In a study by Fu et al. [19] TREC recovery was the most rapid in patients with a matched sibling donor compared to haploidentical sibling or matched unrelated donors. We should rely more on TRECs/mL blood as the number of TRECs/mL is independent of cell count, cell death, and dilution by peripheral expansion of naive T cells [16]. In a study by Ringhoffer et al. [20] the effect of cell proliferation causing dilution of TRECs was overcome by considering the ratio of sjTREC and βTREC.

In the current study the three factors that affected TREC levels post-transplant were age, nature of the disease, ie, whether benign or malignant, and TREC levels pre-transplant.

In accordance with published data, TREC had a strong negative correlation with age in controls and patients at day 28. Age has also been inversely correlated with thymopoiesis. This has been reported in normal individuals and in many clinical situations, including recipients of high-dose chemotherapy, HSCT recipients, and patients infected with human immunodeficiency virus [21]. This is attributed to the thymic involution occurring with age and contributes to superior outcomes of HSCT in younger patients [22, 23]. This indicates another reason for the patient and control TREC levels being comparable. Controls were patient siblings, mostly in the same age category. Since age affects TREC levels the most, it seems reasonable that the difference between patients and controls would not be significant.

Patients transplanted for malignant diseases showed significantly lower TREC levels than benign diseases. One study states that thymopoiesis was markedly reduced both in newly diagnosed and chemotherapy-treated patients with acute lymphocyte leukemia. Others have reported decreased TREC numbers and poorer thymic function following treatment in patients with hematological diseases [1, 24-26]. Moreover, a recent study has shown that TBI used in conditioning of some of malignant cases compromises cortical and medullary thymic epithelial cells (TEC), a critical population for thymic renewal and thymopoiesis [27].

Pre-transplant thymic function is perhaps one of the least studied possible effects on TREC levels. One study used pre-transplant TREC levels as a prognostic factor for transplant outcome. They found that patients with high TREC levels have better OS, and decreased incidence of severe GvHD and opportunistic infections. Another study found similar results, with a correlation between high levels of sjTRECs before HSCT and a reduced risk of death, mainly due to a reduction in the incidence of relapse. Unfortunately, in both studies pre-transplant TREC levels were not correlated to TREC levels post HSCT or other thymic activity parameters [28, 29]. In our study pre-transplant TRECs correlated with post-transplant values, though none of them correlated with clinical outcomes.

We did not find any correlation between day 28 levels of TRECs and either development of infection or GvHD. However, we observed that patients who developed infections had lower TRECs and those who developed GvHD had higher TRECs at this time point. A number of studies have referred to decreased levels of TRECs at the onset of acute GvHD and during chronic GvHD. They have gone further to include the thymus as one of the organs involved in GvHD damage [30-32]. None have, however, used this single point measurement to predict occurrence of GvHD. None of our patients had actually developed GvHD at the time of sampling. We assume that increased level of TRECs in patients is one mechanism of host naive T cell proliferation, leading to GvHD. Similarly, a study conducted in renal transplant patients reported increased levels of recent thymic emigrants (RTE) before renal graft rejection [33]. Skert et al. [34] reported that the level of TRECs was increased at day 28 in patients who developed chronic GvHD, among many other changes in immunological variables. This would further support our data and may urge an extension of this single point measurement study on larger numbers of patients.

Although statistically insignificant, we found lower levels of TRECs in patients who developed infections. This is contradictory to most of the published data, which report significantly lower levels of TRECs in patients who develop infections [35]. In a study by da Rocha et al. [36] a 3-fold lower risk of developing severe infections was observed in those patients who had effective sjTREC+ T-cell recovery at 6 months, and a 9-fold lower risk at 12 months. In another study, lower TREC levels at 9 months were related to CMV episodes, but not to other infections [36]. We did not segregate based on types of infection in our study.

None of the patients exhibiting high TREC levels/mL showed a relapse, while 15% of patients with low TREC/mL showed a relapse and the result was statistically significant (P=0.009). A study by Uzunel et al. [37] reported that in acute myeloid leukemia (AML) patients, low TREC level 2 months post-transplantation was correlated to high relapse incidence at 5 years, while in patients with chronic leukemia and myelodysplastic syndrome (MDS), high TREC levels were correlated with improved relapse-free survival. This,
together with our results, strongly suggests the utility of TREC levels to predict relapse. Sairafi et al. [38] also support the use of TREC measurement as part of the standard repertoire of immunological monitoring after autologous stem-cell transplantation (ASCT).

The published data on TRECs in the field of transplantation show great variation. This is probably due to many factors. One of them is the variability in method design, since some use the absolute quantification of TREC, while others use relative quantification by the delta-CT method. Another is the quantification of TREC in different subpopulations, like CD3+, CD4+ or CD8+ T cells. In addition, TREC results have been expressed in different ways, such as TREC/cell count, TREC/mL or μL of blood, or even TREC/μg of DNA. sjTREC levels are also influenced by many factors, such as longevity of naïve T cells, peripheral expansion or apoptosis of T cells, and intracellular degradation [14, 16].

We aimed at studying whole TREC levels at a single point and to detect its utility as a cost-effective test. Limitations of the study include heterogeneity of the patients, though this is the case in many studies involving HSCT. Another limitation is that different T cell subset counts were not recorded and we did not follow up TREC levels at different time points, but we aimed at detecting the utility of a simple and cost-effective test and its predictive value at a single point.

We present some preliminary data in this study that should be further investigated. We aim at extending this research to larger groups of HSCT recipients and stratifying them into subgroups according to the different patient-related and transplant-related variables. The simplicity of the test and the predictive value would make it a likely candidate for routine testing post-transplantation.

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The study protocol was approved by the ethics committee at Alexandria Faculty of Medicine and informed consent was obtained from all patients or caregivers of the included children, since participation was voluntary.

Authors’ Disclosures of Potential Conflicts of Interest

No potential conflicts of interest relevant to this article were reported.

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