# Clinical relevance of thymic and bone marrow outputs in multiple sclerosis patients treated with alemtuzumab

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## ARTICLE INFO

## ABSTRACT

Thymic and bone marrow outputs were evaluated in 13 sequential samples of 68 multiple sclerosis patients who initiated alemtuzumab and were clinically followed for 48 months. Three months after alemtuzumab infusions, the levels of new T lymphocytes were significantly reduced, but progressively increased reaching the highest values at 36 months, indicating the remarkable capacity of thymic function recovery. Newly produced B cells exceeded baseline levels as early as 3 months after alemtuzumab initiation. Heterogeneous patterns of new T- and B-cell recovery were identified, but without associations with age, sex, previous therapies, development of secondary autoimmunity or infections, and disease re-emergence.

Trial registration version 2.0–27/01/2016.

## 1. Introduction

Alemtuzumab is a humanized monoclonal antibody administered intravenously which has been approved for adults with highly active relapsing remitting multiple sclerosis (RRMS) (Ruck et al., 2015; Kasarello and Mirowska-Guzel, 2021), based on findings that it reduces relapse rates more than IFN-β in treatment-naïve patients with active disease and in patients who had recently relapsed while taking a standard disease-modifying therapy (Cohen et al., 2012; Coles et al., 2012). It targets CD52, a cell surface glycoprotein expressed at high levels on T and B lymphocytes (Hale et al., 1990), cells which play a central role in multiple sclerosis (MS) pathogenesis (Murúa et al., 2022).

The mechanism by which alemtuzumab exerts its therapeutic effects in MS is not completely elucidated, but it may involve immunosuppression through antibody- and complement-dependent cytolysis (Xia et al., 1993; Hu et al., 2009), followed by a subsequent beneficial reconstitution of the immune system cells, which is usually achieved over several months or years (Hill-Cawthorne et al., 2012). However, the recovery from the profound lymphocytopenia observed after each alemtuzumab cycle, is different in T- and B-cell compartments (Cossburn et al., 2013) and, therefore, the clinical efficacy of the treatment may reflect the sum of interactions among different subsets to provide more effective control of multiple pro-inflammatory effector cell types. While alemtuzumab is not known to have a significant impact on lymphocyte precursors (Mavromatis and Cheson, 2003), it has been proposed that the post-alemtuzumab T-cell lymphopenia can be related to therapy effect on CD52-expressing thymocytes (Minagar et al., 2010) or to a disease-specific impairment of lymphocyte homeostasis (Jones et al., 2010). These effects could exacerbate the typical reduced thymic output of newly produced T lymphocytes (Hug et al., 2003; Chiarini et al., 2010), which is one of T-cell compartment defects observed in MS patients (Thewissen et al., 2005). Accordingly, the post-alemtuzumab total CD8 and CD4 T-cell recovery is only partial, being achieved by 30% and 21% of treated patients, respectively, with the total T-lymphocyte count that reach the normal lower limit after a single treatment cycle in 36 months (Hill-Cawthorne et al., 2012). In patients receiving two alemtuzumab administrations, the percentage of CD4 T cells returned to basal levels after 48 months and the recovery was characterized by a significant increase in regulatory T-cell percentage and function at 24 months, when compared to baseline (Rolla et al., 2022). However, the bias in favor of regulatory lymphocyte subsets is not restricted to the T-cell compartment, but also involves robust increases in the percentages of regulatory B cells, as well as Natural Killer cell subsets and monocytes (Gilmore et al., 2020; Kashani et al., 2021).

The post-alemtuzumab B-lymphocyte repopulation is much faster than that of T lymphocytes, with a B-cell compartment that is largely naïve (Heidt et al., 2012; Thompson et al., 2010) and with a B-cell number that exceeds the pre-treatment levels after 7–8 months (Hill-Cawthorne et al., 2012). This can be due to the attempt of B cells to maintain homeostasis by filling the "empty space" left by T lymphocytes (Beutner and MacDonald, 1998).

Although the temporal pattern of post-alemtuzumab repopulation is considered a fundamental mechanism leading to durable efficacy in absence of ongoing treatment (Gilmore et al., 2020), it could also correlate with the principal safety concern of this therapy. As such, the frequent thyroid secondary autoimmunity, the less common immune thrombocytopenic purpura and the rare Goodpasture's renal syndrome (Coles et al., 2012) occur at a median of 32 months after first alemtuzumab course and may develop when B-cell reconstitution dominates over T-cell recovery (Baker et al., 2017). However, there is a marked discordance in the data of T- vs B-lymphocyte reconstitution kinetics measured by flow cytometry (Wiendl et al., 2019; Gilmore et al., 2020; Walo-Delgado et al., 2021).

An alternative method to measure T- and B-cell recovery is the quantification of T-cell receptor excision circles (TRECs) and K-deleting recombination excision circles (KRECs). TRECs are circular fragments of DNA created during the process of lymphocyte differentiation, when T-cell receptor (TCR) rearrangements occur within the thymus. TRECs are considered useful markers of thymic output because they are almost entirely of thymic origin, are stable, do not degrade over time, and do not replicate when a cell divides (Douek et al., 1998). Analogously, KRECs, which are formed randomly during Ig kappa light chain rearrangement and are not duplicated during B-cell proliferation (van Zelm et al., 2007), are considered a marker of bone marrow output. Simultaneous TREC and KREC analyses have been widely applied in different clinical settings to evaluate the production of new T and B cells (Sottini et al., 2010; Kwok et al., 2020).

Here, a duplex digital PCR (dPCR) assay, that measures TRECs and KRECs in dried blood collected on nylon swabs (Tessitore et al., 2017), was used to quantify newly produced T and B cells in patients that initiated alemtuzumab therapy and were clinically followed for 48 months. The aims were to verify when effective immune recovery starts, which were the kinetics and extent of B- and T-cell outputs from production sites, and if there was an association with secondary autoimmunity, infections or re-emergent MS.

## 2. Methods

### 2.1. Patients and controls

For this prospective longitudinal multicenter study, 77 patients (age 18 to 55 years) with a diagnosis of RRMS, who initiate treatment with LEMTRADA® in respect of the approved Summary of Product Characteristics (SmPC) and according to the reimbursement of Italian Medicines Agency (AIFA) were consecutively enrolled in 11 Italian MS centers. Exclusion criteria for enrollments were the presence of any progressive form of MS and any of the contraindications noted in the SmPC. The two alemtuzumab courses were administered at the beginning of the study and after 12 months. Peripheral blood samples were obtained before alemtuzumab initiation (T0), and then at 3 (T3), 6 (T6), 9 (T9), 12 (T12), 15 (T15), 18 (T18), 21 (T21), 24 (T24), 27 (T27), 30 (T30), 33 (T33), and 36 (T36) months, concomitantly with clinical examinations and together with routine blood tests required for patient follow-up.

Since the premature immunosenescence onset has been demonstrated in MS patients (Dema et al., 2021), we included in the analysis two groups of healthy controls (HC) a group of 227 age-matched subjects (a-mHC; age range 18–55 years) and a group of 97 older "immunosenescent" individuals (oHC; age range 56–91 years). The HC were either habitual blood donors or individuals who have been tested for routine diagnostic examinations at the ASST Spedali Civili, the main hospital of Brescia (Italy).

### 2.2. Ethics statement

The study was approved by local Ethic Committees (protocol NP 2263), was conducted according to the criteria set by the declaration of Helsinki ethical principles, and all patients signed a written informed consent before participating in the study.

### 2.3. Quantification of TRECs and KRECs by dPCR performed on dried blood

The number of TRECs and KRECs was quantified by dPCR (QuantStudio™ 3D Digital PCR System, Applied Biosystems) using about 500 ng of DNA extracted from whole blood collected in ethylenediaminetetraacetic acid tubes and absorbed on flocked swab (FS 4N6FLOQSwab code 4504C Genetics, Copan, Brescia, Italy). Their quantities have been expressed per mL of blood according to the formula already reported (Tessitore et al., 2017).

## 2.4. Statistical analysis

To evaluate the impact of age and sex on TREC and KREC changes over the different time-points, a univariate ANOVA was performed setting sex and age categories as independent variables and TRECs and KRECs/mL as dependent variables.

To test whether mean difference of TREC and KREC values at baseline between the two HC groups and the enrolled patients was statistically significant the unpaired *t*-test was employed.

To follow the recovery progression, the ratio of TREC and KREC levels at specific time points (T3, T12, T15, T24, and T36) and the baseline (T0) was calculated as fold change increase ($> 1$) or decrease ($< 1$) of new T- and B-cell production along the study.

To understand and follow the TREC and KREC fold changes along the time, Kaplan Meier curves were plotted considering the TREC and KREC values along the whole alemtuzumab trial from T0 to T36 (1095 days). The full-recovery (fold change $\geq 1$) and the partial recovery (fold change $\geq 0.5$) were set as events to be analyzed.

BlueSky Statistics 10 (GA) and Prism GraphPad (9.0) software were used for statistical analysis.

## 3. Results

### 3.1. Study population

The clinical follow-up lasted 48 months and visits were performed at all time points. The last biological samples were obtained at 36 months since the first infusion of alemtuzumab and blood samples were drawn by the MS center nurses only after the neurologist had verified patient compliance.

One patient dropped-out immediately before the first blood drawing, one upon performing the first blood sampling at the pre-therapy time-point, and a third before the second therapy cycle. One patient received rituximab before the second course of alemtuzumab, hence was excluded from the study.

In addition, no blood samples were available at T33 or T36 for 5 patients, and as a result, the total number of patients included in this study was 68.

The complete protocol sample-set, consisting of 13 samples per patient, was obtained only from 24 patients out of 68, with the highest number of missing samples occurring simultaneously with the first Coronavirus Disease 2019 (COVID-19) pandemic wave in Italy. Similarly, also clinical records were incomplete due to COVID-19.

A marked imbalance in sex towards a female predominance was evident (Table 1). Disease activity [relapse or/and magnetic resonance imaging (MRI) activity] in the previous year was recorded in 97.1% of patients. Most of the patients had relapses in the 12 months prior the study inclusion. Among them, 29 displayed 1 relapse, 18 had 2 relapses, 6 and 3 patients had 4 and 5 relapses, respectively; one had 6 relapses. The majority have been treated with at least one disease modifying therapy prior to alemtuzumab and 20.6% were treatment-naïve. Natalizumab, fingolimod and interferons were the most common MS medications given to the patients before alemtuzumab initiation.

### 3.2. Clinical characteristics of alemtuzumab-treated patients

While the median Expanded Disability Status Scale (EDSS) was stable during the follow-up, in 10/59 (17%) and 15/61 (25%) patients EDSS worsened after 2 and 4 years of therapy, respectively (supplementary Table 1). During the treatment, residual MRI activity was observed in 23/64 (36%) patients in the first year, 8/53 (15%) in the second one, 9/46 (20%) in the third year, and in 10/48 (21%) patients in the fourth year. A relapse was observed in 13/66 (20%), 6/64 (9%), 5/61 (8%) and 7/61 (11%), in the first, second, third and fourth year after the alemtuzumab courses, respectively (supplementary Table 1).

Among all 68 patients, 21 (31%) developed a secondary autoimmune disease during the follow-up (supplementary Table 1). Time of occurrence of a secondary autoimmune disease was 23 (range 20–34) months. Infective complications (see supplementary Table 1) occurred in 12/68 (18%) patients.

### 3.3. Levels of TRECs and KRECs before alemtuzumab initiation

Before starting alemtuzumab therapy, the mean levels of TRECs were significantly lower than that of a-mHC (unpaired *t*-test with Welch's correction, $p = 0.0008$), but higher than oHC ($p = 0.0007$; Fig. 1). On the other hand, the basal level of KRECs of MS patients was only slightly higher than that of HC, independently to the HC age ($p = 0.156$ for a-mHC and 0.065 for oHC).

Pre-therapy TREC and KREC values did not differ in males and females, though, as expected, the regression analysis showed a correlation of TRECs with age in MS patients (data not shown). Similarly, there were no differences depending on the last therapy used before the first alemtuzumab course even if the patients received natalizumab or fingolimod, which are known to respectively increase (for the mobilization of precursors) and decrease the number of circulating lymphocytes, including new lymphocytes (Kaufmann et al., 2018; Paghera et al., 2020).

One possible explanation is that the influence of the two drugs on TREC and KREC levels could have been reversed during the washout period. Additionally, TREC and KREC values at baseline could be a result of the number (which ranged from 0 to 8; see Table 1) and length of previous therapies as well as the length of the washout period.

### 3.4. Kinetics of new T- and B-cell output in alemtuzumab-treated MS patients

The kinetics of TREC and KREC depletion and repopulation during the two alemtuzumab courses are shown in Fig. 2.

The same analysis, performed by calculating the values of the two markers per µg of DNA (Supplementary Fig. 1), demonstrated comparable trends of new T- and B-cell recovery. Therefore, TREC and KREC number did not reflect the different number of peripheral blood cells.

Statistical analysis was performed comparing the data at the following time points pre-therapy basal level (T0); early post-first alemtuzumab course (T3); late post-first alemtuzumab course/pre-second alemtuzumab course (T12); early post-second alemtuzumab course (T15); post-second alemtuzumab course (T24); and late post-second alemtuzumab course (T36). Three months after the first infusion (T3), the mean level of TRECs was significantly reduced ($p < 0.001$). Then, the repopulation of new T cells started increasing almost to the pre-therapy levels at T12 ($p < 0.001$ vs T0; Fig. 2, left panel). Although the mean value of TRECs at 3 months after the second alemtuzumab course (T15) was slightly higher than that observed at 3 months after the first course ($p = NS$), this number was significantly lower than that of the pre-therapy status ($p = 0.0001$). Then, the repopulation of new T cells progressively proceeded exceeding the mean of all other time points at T36 ($p < 0.0001$ vs T0, T12, T15, and T24). Although we cannot know whether this TREC overproduction could have occurred with a single infusion of alemtuzumab, our data indicated that it has required a long time since, at 24 months of therapy, the level of TRECs was comparable to that of pre-therapy ($p = NS$).

The kinetics of KREC repopulation (Fig. 2, right) was different from that observed for TRECs because starting 3 months after the first infusion, the KREC levels always exceeded the basal one being significantly higher at T12, T24, and T36 ($p = 0.0001$ T12 and $p < 0.001$ T24 and T36).

A characteristic that clearly emerged from these data was the highly heterogeneous values obtained for the two markers at all time points of the follow-up. However, the extreme inter-individual variation in both TRECs and KRECs was observed also in HC (Fig. 1).

**Table 1**
Baseline patients' characteristics.

| Patients' characteristics | |
|---|---|
| Males, n (%) | 23/77 (29.9) |
| Females, n (%) | 54/77 (70.1) |
| Age, mean ± SD, years | 34.1 ± 8.1 |
| Patients included in the study | |
| Males, n (%) | 21/68 (30.9) |
| Females, n (%) | 47/68 (69.1) |
| Age at baseline, mean ± SD, years | 35 ± 8.2 |
| EDSS at baseline, mean ± SD | 2.59 ± 1.3 |
| Disease duration, mean ± SD, years | 8.3 ± 6.5 |
| Number of patients with relapses n (%) | 57 (83.8) |
| ARR in the year before alemtuzumab therapy, mean ± SD | 1.5 ± 1.1 |
| Patients with MRI activity*, n (%) | 56 (82.3) |
| Patients with disease activity, n (%) | 66 (97.1) |
| Number of previous therapies, median (range) | 2 (0–8) |
| Last previous therapy, n (%) | |
| No therapies | 14 (20.6) |
| Interferons | 9 (13.2) |
| Glatiramer acetate | 3 (4.4) |
| Teriflunomide | 1 (1.5) |
| Dimethyl fumarate | 3 (4.4) |
| Fingolimod | 18 (26.4) |
| Natalizumab | 19 (27.9) |
| Cyclophosphamide | 1 (1.5) |
| Autoimmunity at baseline, n (%) | 3 (4.4) |

ARR: annualized relapse rate; EDSS: Expanded Disability Status Scale; MRI: magnetic resonance imaging; n: number

* New or enlarged lesions in T2-derived sequences and/or new T1-gadolinium-enhancing lesions.

### 3.5. Extent of TREC and KREC production that follows alemtuzumab therapy in MS patients

The extent of the depletion of newly produced T and B lymphocytes for each individual patient was measured as fold change variation calculated as TREC and KREC changes between T3 and T15 vs T0. Similarly, values obtained at T12 and T24 vs T0 were used to assess the extent of early immune recovery, while those found at T36 vs T0 were used to detect the long-term effect on T- and B-cell immune reconstitution. A fold change > 1 is considered as an "increased trend"
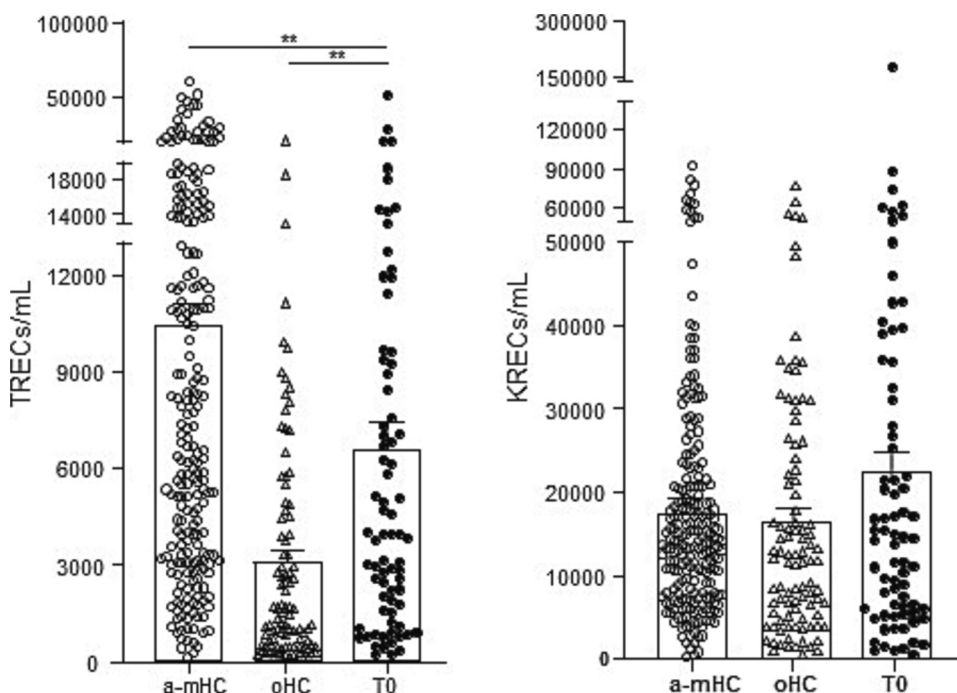
indicating TREC or KREC values exceeding those of pre-therapy; a fold change < 1 is considered as a "decreased trend" describing instead TREC or KREC values lower than those at T0.

All patients except one (98.5%) and 61 patients (89.7%) showed a decreased trend of TRECs at 3 months from the first (T3) and second alemtuzumab course (T15), respectively (Fig. 3). On the other hand, at the same time points, a decreased trend of KRECs was observed only in 13 (19.1%) and 17 (25%) patients, indicating that, in most of them, the recovery of new B-cell production was much faster than that of new T cells.

At both T12 and T24, 48.5% of patients showed an increased trend of TRECs, with mean values exceeding those of pre-therapy; at T36, 72.1% of patients had a fold change > 1 (Fig. 3). At all time-points, most patients had a fold change of KRECs > 1, meaning that they have KRECs above the basal levels.

However, the extent of immune recovery at T36 was extremely broad with a fold change equal to 1.10 to 85.16 for TRECs and from 1.01 to 118.4 for KRECs. The highest fold change values were found in the same patient in whom TRECs increased from 344/mL at T0 to 29,271/mL at T36 and KRECs from 574/mL to 67,980/mL.

Among patients with a decreased trend at 36 months, fold change values < 1 were more contained and varies from 0.90 to 0.05 for TRECs and between 0.97 and 0.02 for KRECs. At T36, only three patients had both TREC and KREC levels lower than those found before therapy initiation.

### 3.6. Probability of T- and B-cell recovery after alemtuzumab therapy

The probability that thymic and bone marrow output could increase at least half (50%) compared with the initial status (T0; event "recovery of 50%") along the 36 months of the follow-up (1095 days) was 94.3% (surv. 0.0569) for TRECs and 98.9% (surv. 0.0109) for KRECs (Fig. 4). The probability that T and B cells could reach a 100% of recovery along the 36 months of the follow-up (1095 days; event "recovery of 100%") compared with the initial status (T0) was 82.2% (surv. 0.1781) for TRECs and 97.1% (surv. 0.0289) for KRECs (Fig. 4).



**Fig. 1.** Comparison between the levels of TRECs and KRECs in all enrolled MS patients analyzed before alemtuzumab therapy (T0) and in age-matched (a-mHC) and older (oHC) healthy controls.
Box plots correspond to mean and standard deviation (SD).
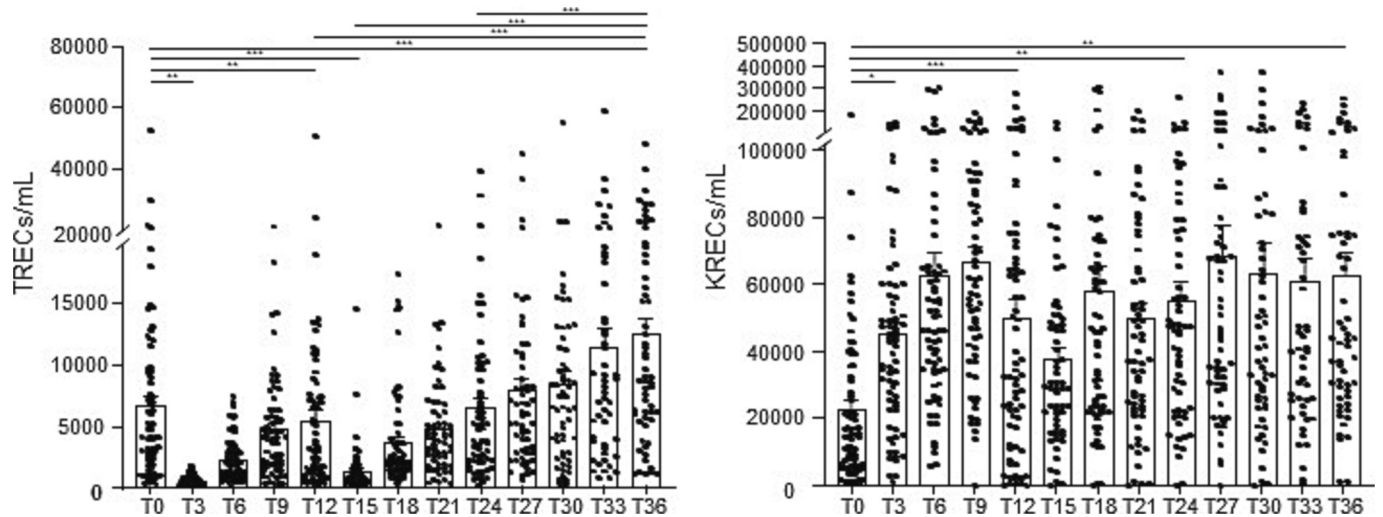** $p < 0.001$.

**Fig. 2.** Kinetics of TREC and KREC depletion and repopulation, shown as TRECs/mL and KRECs/mL. Box plots correspond to mean and standard deviation (SD). * $p < 0.05$, ** $p < 0.001$; *** $p \leq 0.0001$.
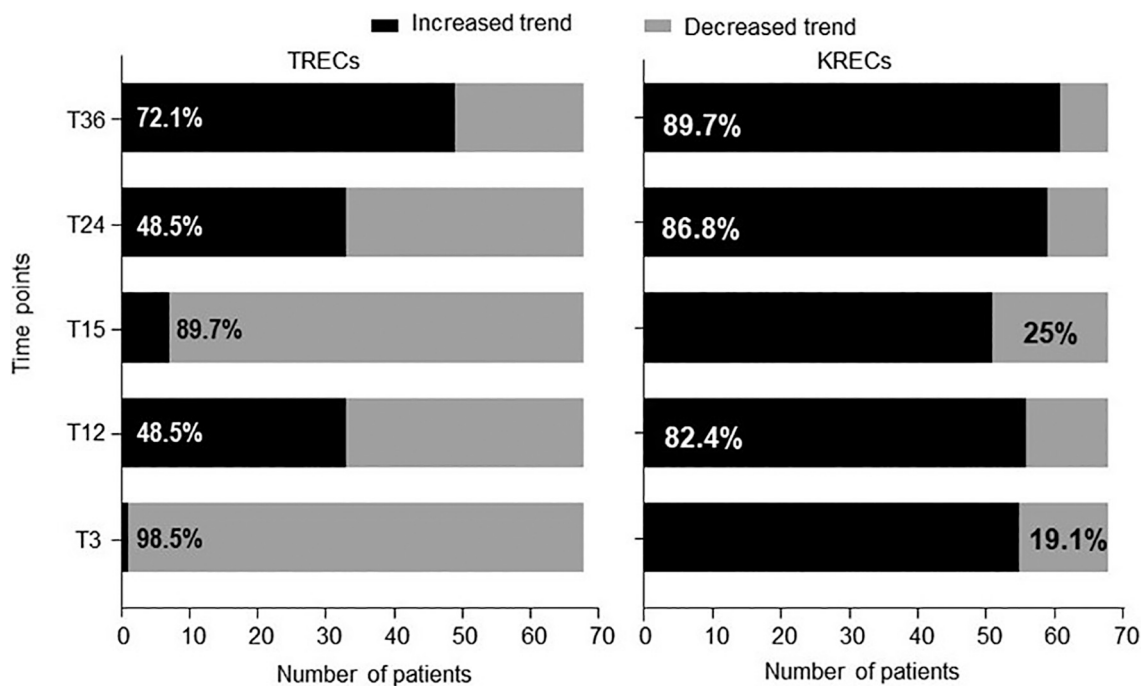


**Fig. 3.** Percentage of patients showing a fold change increase (black bars and white numbers) and decrease (gray bars and black numbers) of TRECs and KRECs.

### 3.7. Alemtuzumab-induced immunotypes

According to their different patterns of immune recovery, alemtuzumab-treated patients were classified in 5 groups, defined as immunotypes (from A to E in the Fig. 5).

The 5 immunotypes were the following A) patients that showed no or very limited increase of the TREC and KREC values ($N = 10$); B) patients with a similar pattern of TREC and KREC recovery ($N = 27$); C) patients with an increasing number of TRECs, but just at the latest time points ($N = 14$); D) patients with a preferential, but limited increase of KRECs ($N = 7$); and E) patients with an overstated increase of KRECs over TRECs ($N = 10$); two representative examples were reported for each immunotype in the corresponding panels of Fig. 5.

### 3.8. Relation between post-alemtuzumab thymic and bone marrow recovery with basal levels of TRECs and KRECs, sex, age, and previous treatments

To investigate a possible influence of pre-therapy levels of TRECs and KRECs with the extent of recovery, the means of TRECs and KRECs at the baseline was used as cut-offs and patients were sorted according to these cut-offs. At T0, patients with TRECs below the cut-off were 46; and 42 out 46 (91.3%) showed an increase of TRECs at T36. Among the 22 patients with TRECs above the cut-off, 10 (45.5%) had a TRECs increase at T36. Patients with KRECs below the cut-off were 42 and in all of them KRECs increased at T36; 19 of the remaining 26 (73%) patients with KRECs above cut-off showed increased KRECs at T36. Therefore, thymic and bone marrow reconstitution was only marginally influenced by the number of TRECs and KRECs that characterizes the pre-therapy period.
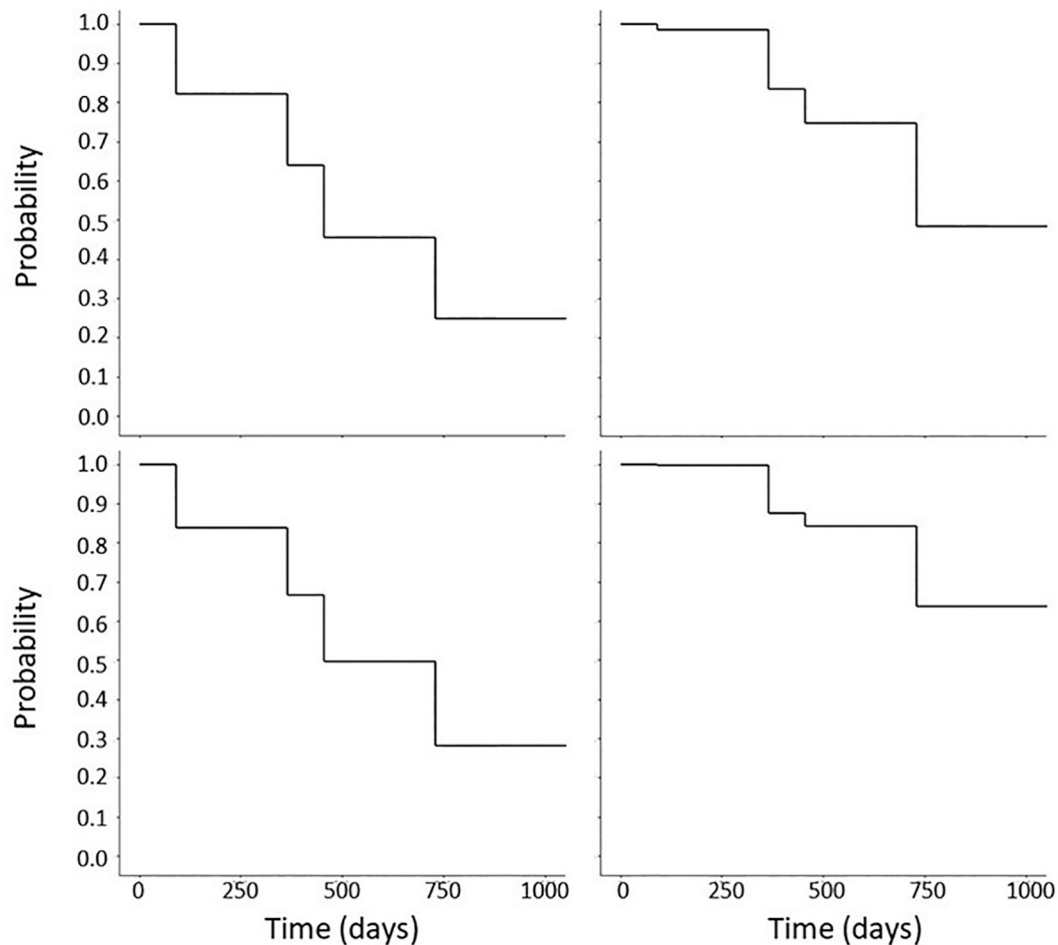
**Fig. 4.** Kaplan Meier survival curves reporting the probability of a 50% (upper plots) and 100% (lower plots) recovery for TRECs (left) and KRECs (right).

The univariate ANOVA analysis, performed to evaluate if age and sex could have an impact on TREC and KREC changes over the different time-points, showed that TREC and KREC levels were not different in males and females at the various time-points of the study (data not shown).

In addition, to investigate the effect of age on TREC and KREC level's modifications during the study period, we divided patients in two age categories, younger (18–35 years old) and older (36–55 years old), both comprising a similar number of patients (33 and 35 subjects, respectively). Although younger individuals always showed a higher mean number of TRECs than older subjects at all time points, the differences between them were not significant. Similarly, no age-related differences were observed for KRECs (data not shown), though, after 36 months of therapy, KREC values reached the highest levels in older individuals (76,337/mL vs 41,758/mL in younger subjects), despite a similar number of TRECs in the same group of patients. This suggests a defect of lymphocyte homeostasis in older subjects.

All these analyses indicate that neither sex nor age could influence the extent and type of immune recovery in alemtuzumab-treated MS patients.

Similarly, although there was not a clear association between the number of previous therapies and the different patterns of immune recovery, patients with immunotype A (lack of immune recovery of both T and B compartments) were those treated with a higher number of previous therapies. Likewise, we did not find any association between the duration of MS disease and the number of previous therapies in the different types of immune recovery behaviors. However, it is of note that patients with impaired (immunotype A) or disproportional immune

recovery of new B cells vs new T cells (immunotype D and E) preferentially received natalizumab before alemtuzumab therapy.

### 3.9. Relationship between post-alemtuzumab thymic or bone marrow recovery and development of secondary autoimmunity, infective complications or re-emergent MS activity

Differences of TRECs and KRECs at baseline or along the whole study period were not observed neither in patients who developed secondary autoimmunity events nor in those who did not (data not shown). However, since only 21 patients developed an autoimmune disease during follow-up, and, given also the profound and heterogenous changes in the levels of TRECs and KRECs upon reconstitution in individual patients, the presence of secondary autoimmunity was evaluated in the 5 alemtuzumab-induced immunotypes. No relation was found between autoimmune reactions and immunotypes as confirmed by these findings 4/10 (40%) patients of immunotype A, 6/27 (22%) of immunotype B, 5/14 (36%) of immunotype C, 2/7 (29%) of immunotype D, and 3/10 (30%) of immunotype E, developed secondary autoimmunity. Thereby, it seems that secondary autoimmunity is not related to disproportional T- and B-cell recovery, as previously hypothesized.

Furthermore, there was no relationship between TREC and KREC levels and the development of infectious complications because infections were observed in 2/10 (20%) patients of immunotype A, 3/27 (11%) of immunotype B, 5/14 (36%) of immunotype C, 2/7 (29%) of immunotype D, and 0/10 (0%) of immunotype E. Surprisingly, it has not been detected a greater number of infections even in patients with defects of both thymic and bone marrow outputs (immunotype A).
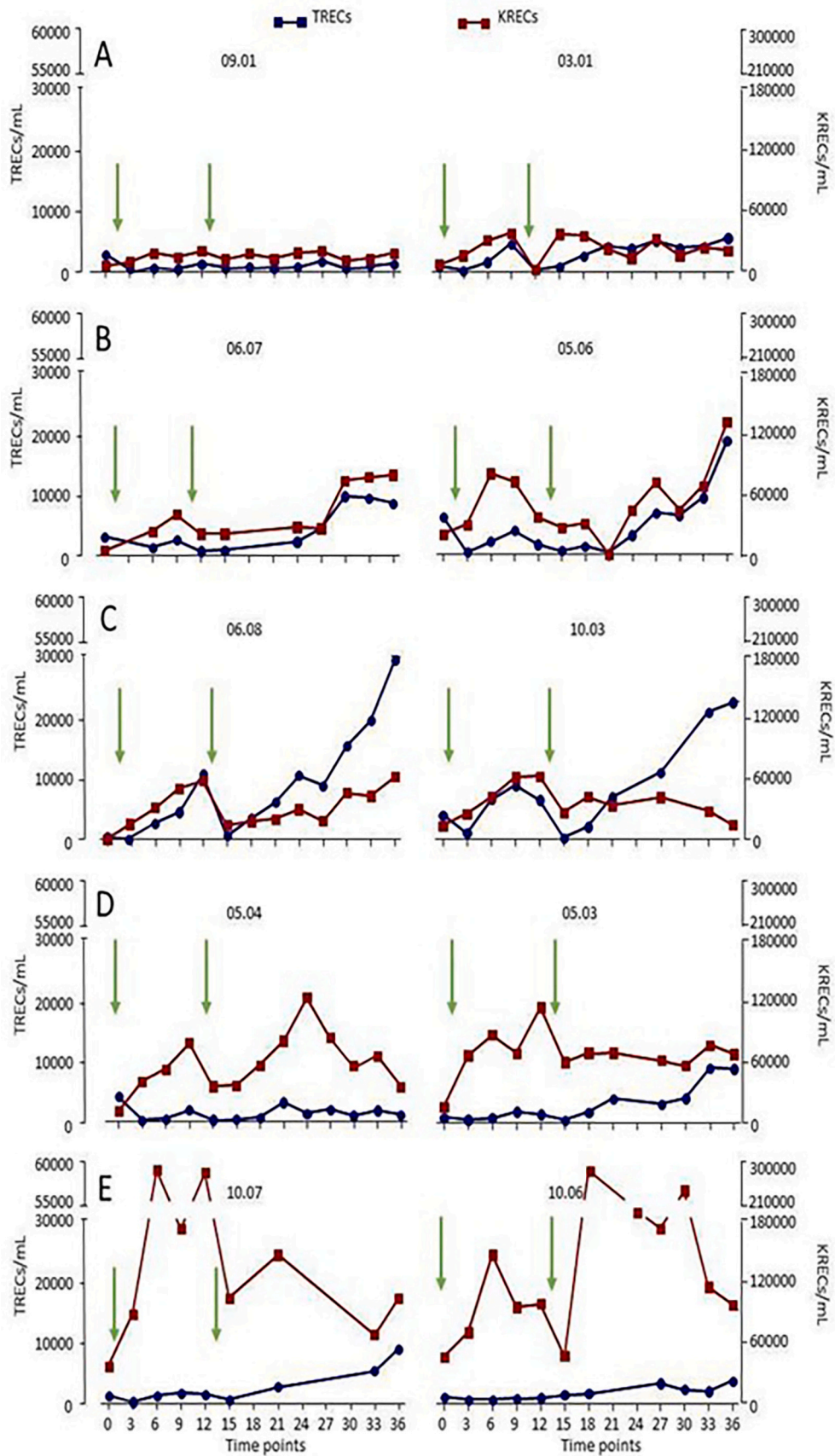
**Fig. 5.** Different patterns of immune recovery based on TREC and KREC quantification. Arrows indicate alemtuzumab courses, dots TRECs/mL and squares KRECs/mL. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

At any time-point, there was not relationship among disease activity, measured by MRI, and different alemtuzumab-induced immunotypes.

## 4. Discussion

The most relevant result that emerges from this study is the ability of the thymus of MS patients to recover its functionality, so that at 36 months of alemtuzumab therapy, the level of TRECs exceeds that of the pre-therapy in most of the patients.

Even though MS is considered an immunosenescence model (Dema et al., 2021, these data suggest that MS patients' thymus may still have thymopoietic potential. This agrees with the recent evidence obtained in elderly subjects, indicating that although aging significantly compromises thymus functionality, this organ maintains a proportion of functional cortical and medullary regions and displays an active thymopoiesis (Velardi et al., 2021). In addition, now it is known that age-related involution of thymic tissue is not that dramatic as reported in earlier studies, and residual thymic tissue can be visible by computed tomography scan in subjects over 70 years of age (Drabkin et al., 2018).

Therefore, alemtuzumab-treated MS patients with immunotype B and C can resemble the long-lived individuals, in whom the higher production of new T cells, and the subsequent increased TCR diversity, may represent hallmarks of longevity (Britanova et al., 2014).

In alemtuzumab-treated MS patients, the induction of new T-cell production is highly variable and may have different implications. In all subjects, the profound lymphopenia, characterizing the first months following alemtuzumab courses can lead to the homeostatic expansion of residual non-depleted T cells (Amoriello et al., 2021) with the aim to maintain the total size of the T-cell pool at a near-constant level (Hickman and Turka, 2005). Accordingly, it has been demonstrated that following each cycle of alemtuzumab there is an oligoclonal expansion of T cells with low TCR diversity compared to baseline, which was more pronounced in CD8 cells (Jones et al., 2013). This early repopulation of T cells, promoted by homeostatic proliferation of cells rather than by newly generated cells from the thymus, suggests that thymopoiesis is not directly affected by alemtuzumab treatment. However, we found that after several months since alemtuzumab initiation, the thymus of most patients, especially those of immunotypes B and C, starts to export newly generated T cells harboring TRECs. A similar recovery of TREC production was also described in MS patients treated with autologous hematopoietic stem cell transplantation (HSCT), in whom thymic reactivation could take place also in adult individuals. After autologous HSCT, dominant TCR clones present in CD4 T cells before treatment were undetectable following the reconstitution, and patients largely developed new T-cell repertoires (Muraro et al., 2014). Therefore, the long-lasting generation of new T cells displaying broader TCR repertoires, leading to a renewal of the immune system and to an immune tolerance restoration, appears to be a crucial step for a successful of both HSCT and alemtuzumab therapies of MS.

Depending on unknown causes, in some subjects treated with alemtuzumab, such as those of immunotype A of our cohort, the reactivation of thymic function is absent or limited, which may lead to the impaired T-cell reconstitution potentially associated with an increased risk of infections or disease reactivation. This, however, was not observed in our patients, perhaps because those included in immunotype A were very few.

The extent and rapidity of new B-cell recovery after alemtuzumab is difficult to be explained, especially because, compared with our understanding of T-cell reconstitution, less information is available regarding the kinetics of B-cell recovery after immunological insults or manipulations as well as on the factors regulating this process. It is known that the production of B-cell precursors in the bone marrow is significantly decreased in aged humans (Guerrettaz et al., 2008). Moreover, B-cell numbers generally return to normal levels within 12 months after HSCT, though up to 2 years are necessary for complete the B-cell compartment recovery (Ogonek et al., 2016). Even when the total number of B cells recovers, their functionality can remain compromised (Velardi et al., 2021). Probably several factors such as conditioning regimens, total body irradiation, corticosteroids and graft versus host disease negatively affect B-cell reconstitution after immunological injuries (Velardi et al., 2021.

It is important to underline that also B-cell-targeting therapies significantly delay B-cell recovery because B-cell counts of patients with lymphoma receiving the anti-CD20 monoclonal antibody rituximab 1–12 months before HSCT, reached values comparable to controls only 24 months after transplant (Buser et al., 2008). Naïve B-cell repopulation of alemtuzumab-treated MS patients is much faster and is completed in about 6 months (Baker et al., 2017). Accordingly, we observed a rapid and substantial increase in the amount of newly produced B lymphocytes in immunotypes C and D patients, perhaps in the patent to fill the "empty space" left by T cells, that recovered only several mounts later.

An optimal B-cell recovery is important not only for protective antibody generation and efficient antigen presentation, but also for a proper immune tolerance. Accordingly, the alemtuzumab induced hyper-repopulation of B cells at 6–12 months of therapy has been proposed as a substrate for the secondary B-cell autoimmunity associated with this drug (Baker et al., 2017; von Kutzleben et al., 2017). However, more recent data indicated that patterns of repopulation were not associated with secondary autoimmunity (Wiendl et al., 2019; Gilmore et al., 2020). As such, also our study showed that even in immunotype E patients, characterized by a very disproportional new B-cell increase over T-cell recovery, no significantly more secondary autoimmunity events were developed. This result may be biased by the fact that the percentage of our patients who developed secondary autoimmunity throughout the entire follow-up (29%) was lower than that reported in the literature (38%) (Coles et al., 2017).

Another observation that emerges from our results is the high heterogeneity of TRECs and KRECs levels found at the baseline, in the extent of immune reconstitution, and in the pattern of immune recovery in different patients. However, it is becoming increasingly evident that genetic and environmental factors, hormones, food, sex, access to care, conditions of well-being or poverty, as well as the country socio-political situations could affect the shape of immune system and generate an enormous intra- and inter-individual heterogeneity for multiple immunological parameters even in healthy individuals (Brodin and Davis, 2017; Duffy, 2020).

However, we cannot forget that the anomalous values we have seen for individual time fluctuations may be also due to sampling and analytical variability in our relatively small cohort of patients as well as any other potentially source of inconstancy.

## 5. Conclusions

Even though the thymus is exquisitely sensitive to acute and chronic insults, we found that in MS patients it also has a remarkable capacity to recover its function and that alemtuzumab therapy can reverse thymic involution.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jneuroim.2023.578170.

**Declaration of Competing Interest**

The authors declare no conflicts of interest with the manuscript. The funding source had no influence on the study design, collection, analysis, and data interpretation. The funding source was not involved in the decision to publish.

## Data availability

Data will be made available on request.

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