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T cell receptor excision circles (TREC) and recent thymic migrant cells in specific immunotherapy and respiratory allergy to *Dermatophagoides pteronyssinus*

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Key words
TREC, CD31 cells, asthma, rhinitis, specific immunotherapy, immunotherapy mechanism, allergy mechanism, Dermatophagoides pteronyssinus, nasal challenge test, lymphocytes

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Summary
Introduction. *T cell receptor excision circles (TREC) on CD31+ T cells are related to recent thymic emigrant cells (RTEs). The involvement of the functional thymic tissue occurs early in the IgE-mediated allergic reaction, and in response to specific immunotherapy (SIT). Aim. Evaluation of specific immunotherapy effects on TREC number in peripheral T cells in patients allergic to Dermatophagoides pteronyssinus (Dpt).* ***Method.*** *85 respiratory allergic patients (both genders), 41 of them (Group II) under maintenance treatment to Dpt SIT (21 sublingual-SLIT, and 20 subcutaneous-SCIT), were selected. The allergic patients (Group I) without specific treatment were submitted to an allergen challenge test (22 nasal and 22 conjunctival). Peripheral cell analysis was performed immediately before treatment and 60 or 240 minutes after allergenic extract administration. TREC quantification was performed in CD4+CD31+ and CD8+CD31+. The results were expressed per 100.000 cells related to RTEs. Samples from 10 healthy individuals (Control - Group III) were obtained with the same method.* ***Results.*** *The value of TRECs on RTEs was constant in control groups. For Group I patients (nasal or conjunctival test), TREC quantification in CD31+ T cells showed relevant individual changes, even in the patients tested earlier (60 minutes), and statistical significant at 240 minutes. Both SCIT and SLIT had also demonstrated enormous individual changes, particularly on TRECs/CD4+CD31+ cells assay. Basal values in Group III were significantly higher than those observed in active patients groups.* ***Conclusion.*** *Thymic functional activity is earlier involved in the allergic reaction and SIT. IgE-mediated allergy is able to induce RTEs in the periphery, particularly TRECs/CD4+CD31+ cells. Both SLIT and SCIT showed reduced RETs in the periphery, probably due to maturation of regulatory T cells. Our results suggest a crucial role of the functional thymic tissue on the central mechanism of this therapy.*

Introduction

The systemic nature of allergies by the IgE mechanism is a well establish theory, extensively supported in the literature (1).

The underlying immune mechanism requires the involvement of multiple immuno-inflammatory cells and also biological mediators. Furthermore, the pathologic effects that result from a specific response to allergens will, necessarily, result in the involvement of central immune organs (2, 3).

In fact, the specific immunotherapy either by subcutaneous (SCIT) or sublingual (SLT) routes represent, at the moment, the only alternatives of treatment that allow the modification of the natural history of the disease (5-8).

The mechanisms by which the immunologic tolerance is developed, despite extensive study, are not unequivocally established (9,10).

An important study by Senti G et al. in which the therapeutic allergen extract was administrated directly on the lymphatic ganglions, demonstrated its efficacy in a reduced period of treatment and supported the requirement of the central immune structures in the stimulation of the central immune mechanisms (11).

The IgE-mediated allergic reaction dictates a premature systemic effect, which will develop simultaneously to immunoinflammatory mechanisms at the local of allergen exposure.

In vivo studies showed that, in parallel to the inflammatory activity at the site where the allergic stimulation took place, there is also involvement of adjacent lymph ganglion structures in parallel to the recirculation of circulating cells. These circulating cells penetrate the structures related to the central immune system, namely bone marrow and thymic functional tissue (12).

The focus of the inflammatory activity in anatomic areas reported to the central immune system assumes not only a systemic effect of the IgE-mediated allergy, but also a prompt and central involvement of the immune system.

In the same way, we were able to observe that a specific immunotherapy (SCIT or SLIT) determines the early involvement of these structures, which have potential implications on the immunomodulation mechanism (13,14).

The initial response to the therapeutic administration of the specific allergens occurs early in time, and the systemic effect is faster than the local inflammatory activity.

There are no significant differences, in terms of magnitude of the response, at the level of the central immune organs for different types of extract and route of administration.

However, one can admit that there is a distinct local mechanism of induction between a SLIT and a SCIT, despite having identical systemic effects. This mechanism likely depends on the phenotypic heterogeneity of the dendritic cells (DCs) present at the sublingual mucosa and in the subcutaneous tissue.

In fact, if the work developed by JA Denburg et al. is unquestionable regarding to the contribution of the bone marrow to the development of an allergic reaction, the involvement and persistence of the thymic activity throughout life is harder to demonstrate, at least in a clear and direct way.

Our group was able to show this type of activity, in the IgE-mediated allergic response and in the response that result from the administration of specific immunotherapy. These results were independent of the route of administration, either SLIT or SCIT (12,13) and, for this last one, independently of the type of extract (aqueous, *depot* or polymerized).

The fact that the thymus involutes with age and is functional only early in life has been traditionally assumed (15). However several data have demonstrated that thymic function persists in elderly individuals. The human peripheral T-cell pool begins to be established early in fetal development, after the developing thymocytes cross through the thymic microenvironment and migration of mature thymocytes into peripheral sites, such as lymph nodes and spleen. The thymus is the unique lymphoid organ responsible for the generation of self-tolerant and competent naïve T cells, as well as of self antigen-specific natural regulatory T cells (17).

The absolute numbers of naïve T cells in young and elderly humans are relatively stable, and the $CD4^+$ T-cell immunity is maintained in adults. $CD4^+$ cells can proliferate post-thymically while retaining their naïve phenotype and functional characteristics (18). Currently two distinct naïve cell subsets are considered: one quiescent subset highly enriched in recent thymic emigrants (RTEs; freshly emigrated from the thymus) and a second subset comprising naïve $CD4^+$ T cells that have proliferated in the periphery (19).

The surface molecule CD31 (platelet endothelial cell adhesion molecule-1: PECAM-1) can be used to distinguish $CD31^+$ thymic-naïve $CD4^+$ T cells in the peripheral blood of healthy humans (18).

Besides using $CD4^+CD31^+$ to characterize RTEs, the use of the T cell receptor excision circles (TRECs) is an additional interesting marker (18-20). TRECs are stable cytoplasmic DNA episomes formed during T cell receptor rearrangement in α/β Th cells (19). When the T-cell receptor is formed, a signal-joint TREC is produced during the rearrangement of the T cell receptor α gene segment in approximately two-thirds of αβ T-cells. The δ gene is located within the T-cell receptor gene locus (20). As TRECs are not replicated during mitosis they are diluted out during cell divisions, which includes priming of RTEs to become memory Th cells, but also homeostatic cell division of naïve Th cells (18).

Healthy individuals show only a slight variation in their TREC levels over time, supporting that their thymus is working at a constant level (20). TRECs are detected on RTEs $CD4^+$ and $CD8^+$, but also T-$CD45RA^+$ and T-$CD45RO^+$ and peripheral mononuclear blood cells (PMBCs) (18).

The analysis of both CD31 and TREC is by definition a marker of development related to thymus function, and

the peripheral concentration could be used to estimate organ production and immune reconstitution (18).
In previous studies we have demonstrated that in parallel to the immediate response that results from an IgE-dependent allergenic mechanism, the cellular response occurs early in the process, and the involvement of the central immune organs is crucial since the beginning of the reaction. Furthermore, the dynamics of the specific immunotherapy mechanism with allergens is also itself premature and translated by the activity of the structures reported to the central immune system.
The main purpose of this study was to evaluate the production of RTEs cells. In particular we were interested in the RTEs cells that are able to support the crucial relevance of the mechanisms of the thymic tissue in the allergic reaction and in the induction of the immune tolerance subjacent to SIT.

Methods

Patients and healthy controls

Patients with respiratory allergies to *Dermatophagoides pteronyssinus* (Dpt), with moderate persistent controlled bronchial asthma (21) associated to persistent moderate/severe (22) rhinitis were selected. These patients were followed up in the Immunoallergology Department at the Coimbra University Hospital.
All adult patients, from both genders, have filled in and signed an Informed Consent, and the study was approved by the Ethics Committee of the Hospital.
All patients underwent the challenge between January and March 2009, a period of low natural exposure to mites in Portugal.
None of the patients presented any other pathology, besides allergic disease, namely inflammatory or infectious diseases, pregnancy or mental impairment.
There was no pharmacological medication in place, besides the one required for the allergic disease, with the exception of oral anticonception medication, for the case of some women.
The control group was selected from a population of healthy individuals, which are potential organ donors, with no manifestations of allergic disease.
The following groups of patients were studied:

- Group I: 44 patients with respiratory allergic disease and no prior treatment with SIT, to conduct the allergen-specific challenge test with Dpt (nasal or conjunctival).
- Group II: 41 patients with respiratory allergic disease, performing a maintenance treatment with SIT (SCIT or SLIT) for at least 1 year. It was also a requirement that the clinic efficacy was demonstrated, which could be accessed by the complete remission of the symptoms, and no need of preventive and/or rescue anti-allergic medication. It was also taken into account the favorable evolution of the laboratory parameters, namely reduction of the skin reactivity to the allergen assessed by the cutaneous Prick tests and reduction in the levels of the serum specific IgE, relatively to the beginning of the treatment. All the therapeutic extracts were from Bial/Aristegui (Bilbao, Spain): SLIT (aqueous, 0.97 μg/ml of Der p 1 and Der p 2, in the dosage of 5 drops) and SCIT (polymerized, 1.95 μg/ml of Der p 1 and Der p 2, corresponding to the subcutaneous injection of 0.5cc). All extracts were administrated under strict hospital vigilance. The day of the administration coincided with what was scheduled in the maintenance therapeutic plan (monthly for SCIT and 3 times per week for SLIT).
- Group III: 10 healthy individuals. Patients with allergic disease were asked to interrupt the treatment with systemic and/or local anti-histaminics for 3 days, with topical corticosteroids and systemic leukotriene receptor antagonists in the 8 days prior to the study.

Specific nasal and conjunctival challenge tests

An aqueous extract of Dpt with a 5 mg/ml concentration (23 μg/ml of *Der p* 1, Bial/Aristegui, Bilbao, Spain) was used. Skin Prick tests with 1/1, 1/10, 1/100 and 1/1000 dilutions, as well as positive and negative controls, were performed in all patients according to the standard procedure. The concentration used in the specific challenge was the minimum that induced a prick test wheal equal to the one induced by histamine. Interestingly, the concentration corresponded to the 1/10 dilution in all patients. All the procedures were performed in the morning and after an adaptation to room temperature for 30 minutes.
Nasal challenge test was performed with unilateral nasal application of 2 consecutive puffs (total volume of 160 μl) of the Dpt extract to the less congested nostril, using a nasal applicator spraying and patients were asked not to inhale during the allergen spraying. Conjunctival challenge test consisted in unilateral ocular application of 1 drop (50 μl) of the Dpt extract in the inferior and external quadrant of the bulbar conjunctiva.
Nasal and eye symptoms were recorded each minute until five minutes after specific provocation tests, using a clini-

cal score system to assess and to ensure the positivity of the response (23,24).

Allergenic therapeutic extract

The allergenic extracts administered to all the patients were from Bial/Aristegui (Bilbao, Spain). These Dpt extracts were glutaraldehyde modified in a volume of 0.5cc subcutaneous or aqueous extract administrated sublingually (5 drops), according standard methods (5,8). All the patients were submitted to the maintenance dose according to the individual schedule.

Laboratorial procedures

Blood samples

Peripheral blood samples (30 ml) were collected into heparin-coated tubes and PAXgene Blood RNA Tubes (Qiagen®) in a quiet and relaxed environment, at controlled and constant temperature.
For all patients and controls a blood draw was performed in two time points. In T0, before the provocation test or before the administration of specific immunotherapy (both subcutaneous or sublingual) and a second time point, either at 60 minutes (T60) or 240 minutes (T240) after the diagnostic or therapeutic procedure.
For the healthy individuals from Group III three blood draws were performed on the same day, with similar intervals as compared to the active groups in study.

T-cell subset separation

$CD4^+$ and $CD8^+$ T cells were subsequently isolated from PBMC by positive immunoselection using Dynal $CD4^+$ beads or $CD8^+$ beads according to the manufacturer's guidelines (Dynal, Oslo, Norway).

Magnetic Activated Cell Sorting

$CD4^+$ and $CD8^+$ T cells were enriched to high purity (98%) from PBMCs or CMBCs by magnetic separation using releasable CD4-MultiSort-Microbeads (Miltenyi Biotec). After release of the $CD4^+$ or $CD8^+$-MultiSort-Microbeads the T cells were depleted with CD45RO-Microbeads (Miltenyi Biotec) to obtain pure $CD45RA^+CD45RO^-$T cells. These cells were then separated in a third magnetic separation step into $CD31^+$ and $CD31^-CD45RA^+$ These subpopulations were stained with anti-CD31-FITC (Becton Dickinson) and subsequent labeling with anti-FITC-Microbeads (Miltenyi Biotec).

Flow cytometry and immunofluorescence staining of tissue sections

Cell populations were studied by four-color flow cytometry (FACSCanto, FACSDiva software; Becton Dickinson, San Jose, CA, USA) using fluorochrome- labeled mAbs to CD3, CD4, CD8, CD19, CD31, CD45RA and CD45RO (Pharmingen, San Diego, CA, USA).

Quantification of TRECs by Real-time PCR

Quantification of TREC in sorted $CD4/31^-$, $CD4/31^+$, $CD8/31^-$ and $CD8/31^+$ cells was done by real-time quantitative PCR with the 5±±-nuclease (TaqMan) assay in ABI 7900 system (Perkin-Elmer, Norwalk, CT, USA). Cells were sorted by use of these monoclonal antibodies combination: CD3-PE (Beckman Coulter), CD4-APC (Beckman Coulter), CD8-PercP Cy5.5 (Becton Dickinson), CD31-FITC (BD Pharmingen, San Diego, USA). DNA was extracted by cell lysis with 5 µl proteinase K (100 µg/ml) at least for 1 hour at 56°C, followed by incubation at 95°C for 15 min. Real time-PCR was done on 5 µl of cell lysate (±50000 cells) with the primers: F-5'-CACATCCCTTTCAACCATGCT and R-5'-GCCAGCTGCAGGGTTTAGG, and probe 5'-[6FAM]ACACCTCTGGTTTTTGTA AAGGTGC CCACT[TAM] (Sigma-Aldrich, USA). PCR reactions contained 0.125 µl Applied Taq polymerase, 3.5 µl $MgCl_2$ 25 mM, 0.5 µl dNTPs 10 mM, 1 µl each primer 12.5 µM, 1 µl probe 5 µM, 0.25 µl of BD636 reference (Megabases) in a final volume of 25 µl. The PCR reaction was set to 95°C for 5 minutes, followed by 40 cycles at 95°C for 30 sec and 60°C for 1 min. A standard curve was plotted using 5 µl of standard with 10^3, 10^4 and 10^5 molecules of plasmid, and numbers of TRECs in samples was calculated using SDS 2.0 software (Perkin-Elmer, Norwalk, CT, USA).

Statistical analysis

The concentrations were expressed as average ± standard deviation (SD). Wilcoxon signed Rank test (paired samples) was used to compare the determinations in different times. Kruskal-Wallis one-way analysis of variance and Mann Whitney U test were used to test for differences between the different groups. In all cases $p<0.05$ was considered significant.

Table 1 - Characteristics of the patients and healthy controls

	Group I				Group II				Group III
	Nasal		Conjunctival		SCIT		SLIT		
	T0-T60	T0-T240	T0-T60	T0-T240	T0-T60	T0-T240	T0-T60	T0-T240	
n	12	10	11	11	10	10	10	11	10
F/M	6/6	6/4	8/3	7/4	6/4	64	91	8/3	6/4
Age (Y)	32.5±8.7	25.3±11.7	28.36±5.0	27.9±6.8	31.9±7.5	35.8±11.7	30.4±9.6	28.3±10.3	29.5±6.4
Evol (Y)	13.4±4.5	12.7±5.2	10.7±7.1	11.9±4.9	12.1±4.3	13.1±6.1	9.9±5.5	10.6±6.7	
sIgE-1	31.24±12.5	32.5±13.8	37.2±28.1	41.2±17.8	12.4±9.2	13.2±12.7	18.4±15.5	17.4±14.2	
sIgE-2					17.8±15.8	22.7±19.5	21.6±14.6	22.9±16.5	

n: number, F: Female; M: Male; Y: Years; Evol: Disease Evolution in Years; sIgE-1: specific IgE to Dpt immediately before the treatment; sIgE-2: specific IgE to Dpt at the time of the study. IgE results expressed in KU/L.

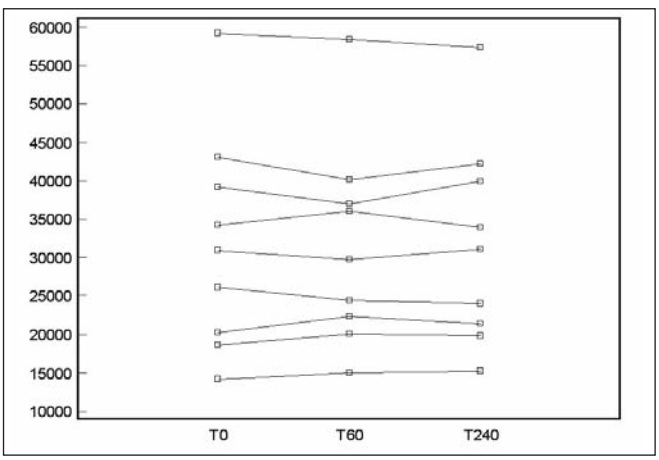
Results

Demographic and clinical characteristics of the patients and healthy controls are shown in Table 1.

All patients submitted to the specific allergic reaction presented clinical positive scores. There were no local or systemic adverse side effects in patients submitted to SIT. There were no significant clinical differences between the patients of either groups or subgroups.

In relation to the total number of $CD4^+$ e $CD8^+$ cells, there was no significant difference in patients submitted to the specific challenge tests (nasal and conjunctival) or to specific immunotherapy (SLIT or SCIT) between both time points analysed.

Figure 1 - Number of TRECs per 100.000 CD4 + CD31 + cells in healthy individuals (Group III). The results shown are for each patient, both baseline and data obtained 60 and 240 minutes after the first blood sample.



In the control group, there was no difference in the determination, for each individual, of the number of TRECs in 100.000 $CD4^+CD31^+$ (Fig. 1) or $CD8^+CD31^+$ cells.

In some samples, particularly in the group treated with SCIT and monitored 1 hour after the administration, it was not possible to obtain reliable results due to technical difficulties in the DNA sampling and isolation.

In allergic patients submitted to challenge test (Group I) the variation in the number of TRECs in peripheric lymphocytes are quite evident. The nasal test causes a more expressive response (TRECs/$CD4^+CD31^+$) than the induction by the conjunctival challenge test, especially in the group analysed by the second time 4 hours later (Fig. 2).

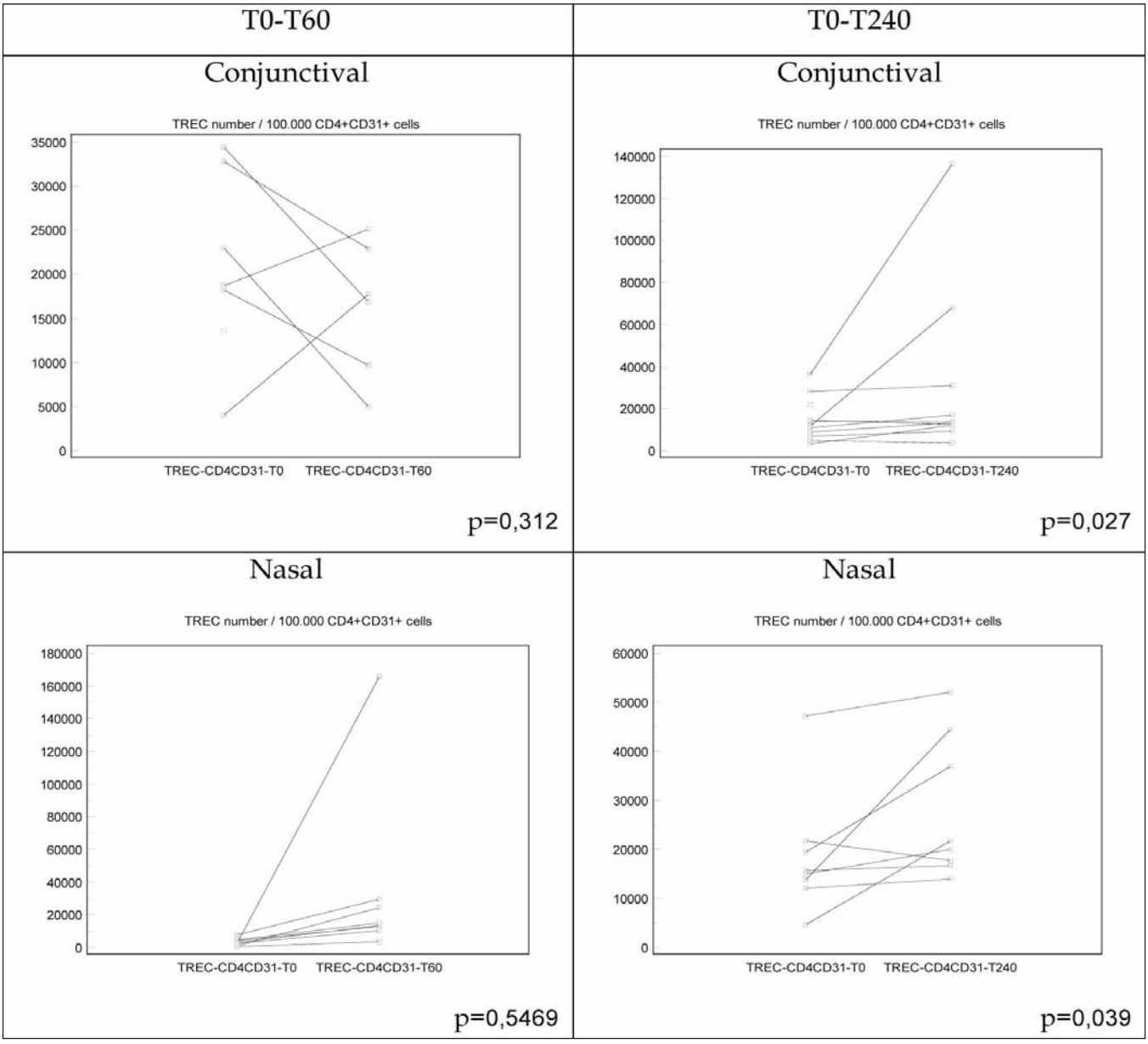
Despite some divergent results, the number of TRECs in $CD4^+CD31^+$ cells seems to be lower with SIT, particularly in the group studied 4 hours after the administration of the therapeutical extract (Fig. 3).

Regarding the number of TRECs in the $CD8^+CD31^+$ population, there were changes induced both by the challenge test and the therapeutic effect (Figs. 4, 5). The results obtained in these cells were more inconsistent than the ones obtained with $CD4^+CD31^+$ cells. Although the evaluation of the dynamics of the individual response to the allergen or to the treatment is the most relevant feature, it might also be interesting to analyze the average basal levels of TRECs/100.000 $CD4^+CD31^+$ cells obtained in the three groups in this study.

In patients in maintenance treatment, with excellent clinical efficacy, the average value, despite slightly higher, has no statistic significance (p=0.32) when compared to allergic patients not subjected to specific treatment (Fig. 6).

Both nasal and conjunctival challenge test resulted in clinically positive allergic reaction. Similarly, both SCIT and

Figure 2 - Number of TRECs per 100.000 CD4 + CD31 + cells in patients submitted to allergen challenge test (nasal and conjunctival). The results shown are for each patient, both baseline and data obtained 60 and 240 minutes after the first blood sample.



SLIT demonstrated an excellent clinical response. The results obtained, particularly regarding the number of TRECs/100.000 $CD4^+CD31^+$ cells, showed important differences in the time point analysed, when compared to healthy individuals.

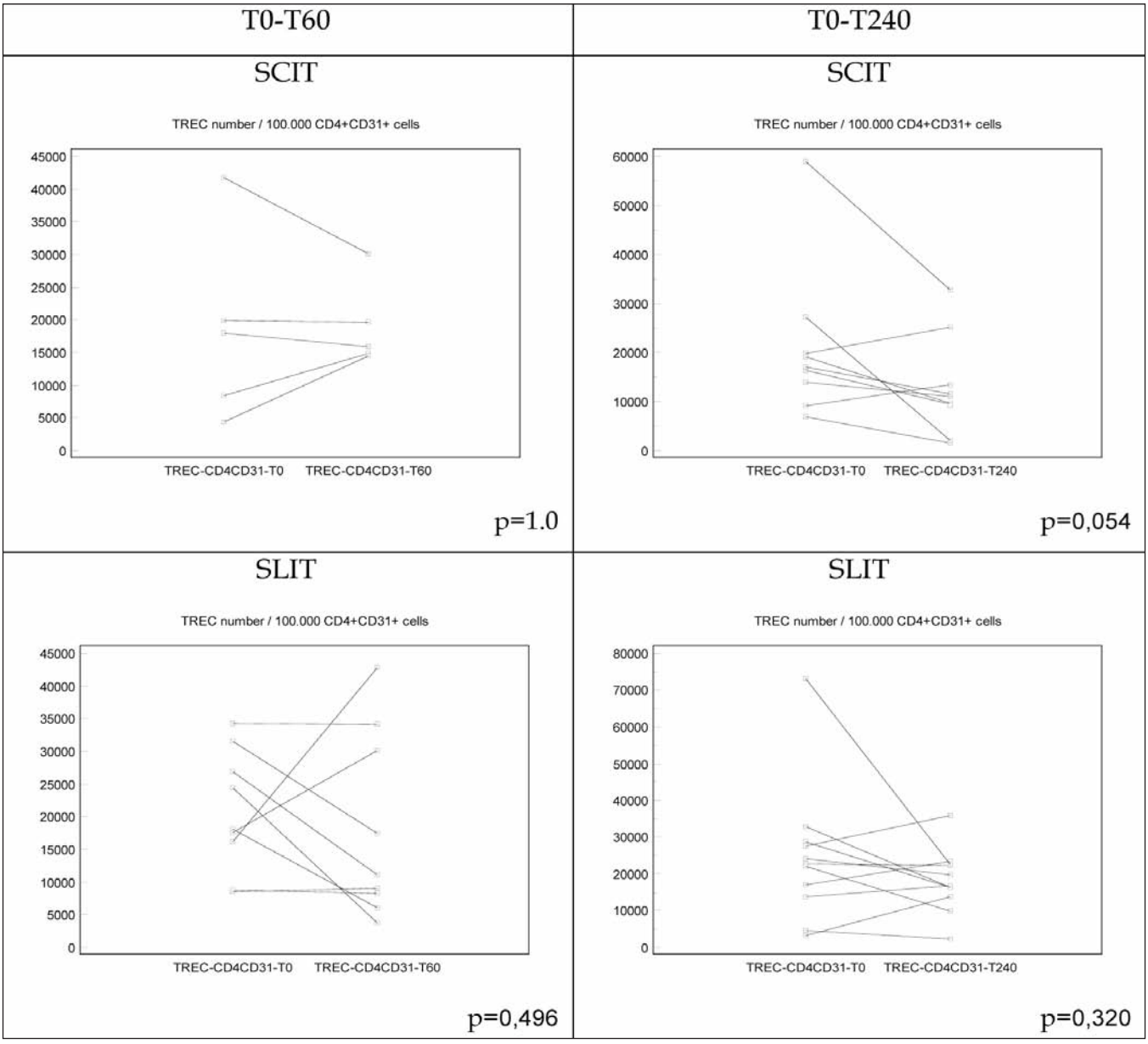
When the average values for all patients submitted to a specific allergic reaction are compared to the results obtained to patients submitted to SIT, despite no statistical significance, an elevation in the allergic response is observed (Fig. 7).

Discussion

The determination of thymic output by quantification of TRECs is an interesting investigation line in transplantation, infection, autoimmunity or immunodeficiency disorders (25). To our knowledge, the RTEs assessment on IgE mediated respiratory allergy is the first study conducted on this subject.

Normal healthy volunteers show very little change in their TREC values over time, supporting the concept that their

Figure 3 - Number of TRECs per 100.000 CD4 + CD31 + cells in patients submitted to specific immunotherapy (SCIT and SLIT). The results shown are for each patient, both baseline and data obtained 60 and 240 minutes after the first blood sample.

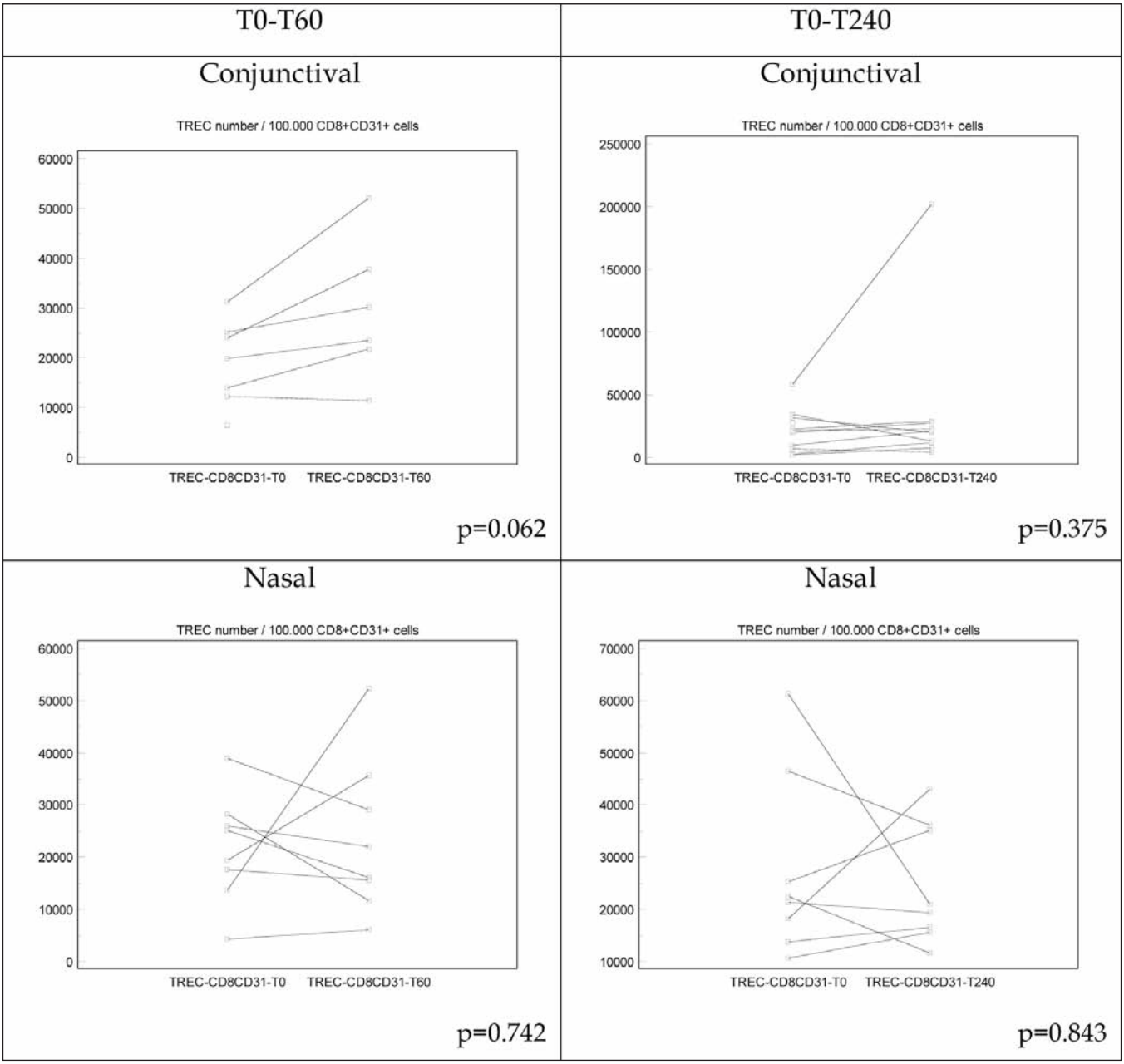


thymus works at a constant level, despite the function decline with increasing age (20). Our control group had, also, widely spread ranges that are stable at least on the 4 hours of evaluation. Several studies demonstrated a reduction of total TRECs in chronic inflammatory diseases compared to healthy controls, namely in atopic dermatitis, rheumatoid arthritis or lupus erythematosus (20, 29, 30).

In previous works, our group was able to demonstrate *in vivo*, that the central immune system reacts earlier to an allergic induction (12-14), than previously thought using bone marrow biopsies (26-28). The immunological integrity of T-cells throughout life is assured by a functional thymic activity, despite the anatomical involution of the thymus. The functional activity can be observed by radioisotopes label leukocyte scintigraphy, which can also be observed very early in an IgE mediated reaction.

The determination of TRECs depends not only from the thymic function but also from the periphery T-cell metab-

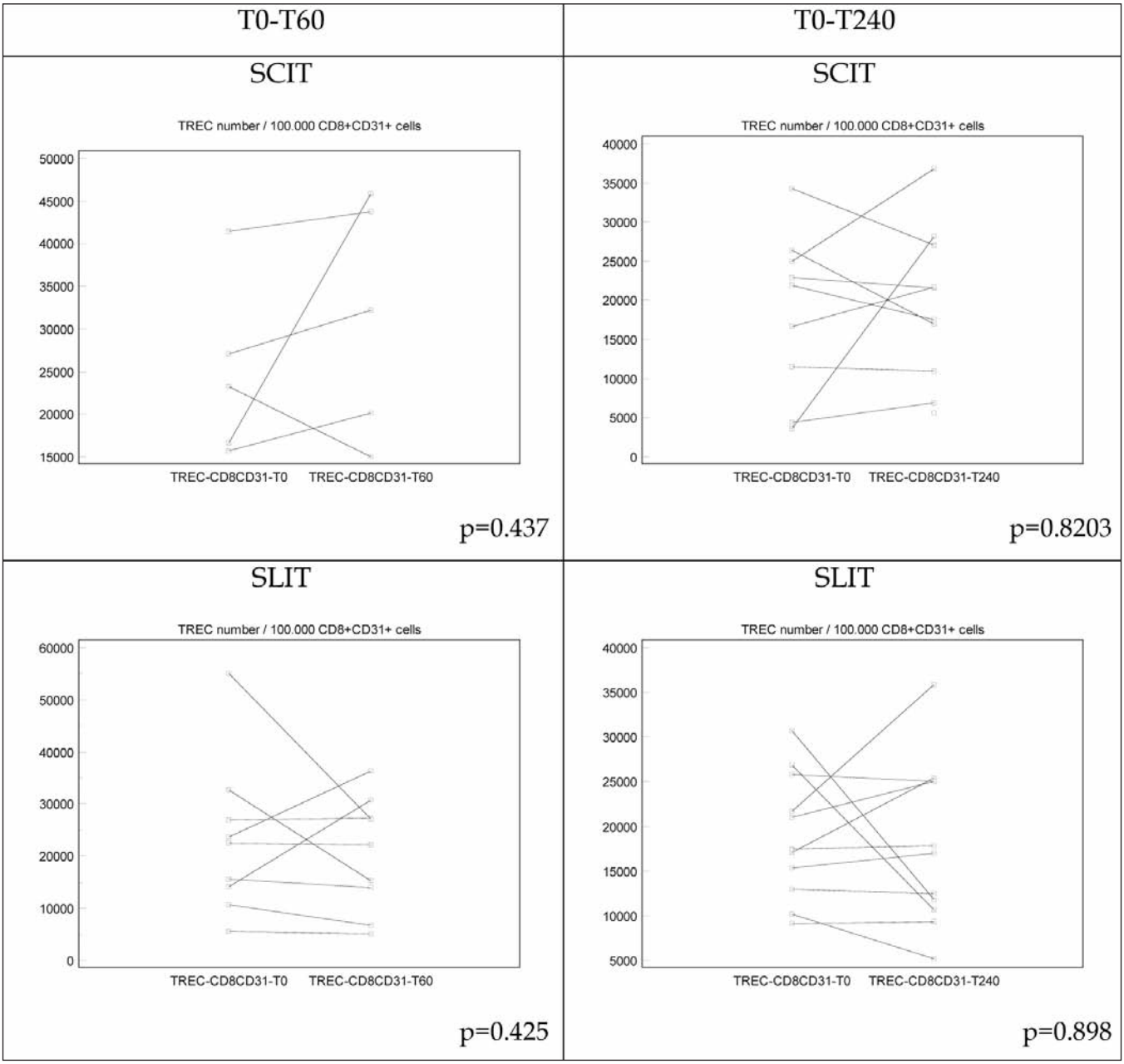
Figure 4 - Number of TRECs per 100.000 CD8 + CD31 + cells in patients submitted to allergen challenge test (nasal and conjunctival) (Group I). The results shown are for each patient, both baseline and data obtained 60 and 240 minutes after the first blood sample.



olism. The evaluation of the number of TRECs present in 100.000 $CD31^+$ cells of two different T-cells populations gives a better perception of the real thymic activity, with the indication of the production of young and immature RTEs. RTEs are naive peripheral T cells, which have only recently exited the thymus and have not undergone further peripheral proliferation and antigen selection (18). Despite the lack of an exclusive marker for these cells, the methodology that we designed (determination of TREC in $CD31^+$ cells) is currently the most accurate to establish the thymic activity in humans.

In this context, it is very important to analyse the individual results in the different subgroups, since the high individual variation does not allow an unequivocal evaluation of the av-

Figure 5 - Number of TRECs per 100.000 CD8 + CD31 + cells in patients submitted to specific immunotherapy (SCIT and SLIT) (Group II). The results shown are for each patient, both baseline and data obtained 60 and 240 minutes after the first blood sample.



erage values of the sample. For this work we did not considered relevant the existence of a separate control group of allergic patients, since the time point T0 would be a better internal control.

In the different groups and subgroups, the usage of the average values has a reduced relevance, since it would be more relevant to evaluate the dynamics of the individual response at the time points established in the protocol. In fact, despite each patient evidenced changes in the levels of RTEs, there was no significant alteration in the average values of $CD4^+CD31^+$ or $CD8^+CD31^+$ regarding the total number of $CD4^+$ or $CD8^+$ cells, respectively.

In our patients with respiratory allergy, although the study occurred during a clinical stabilization stage, the base value

Figure 6 - Basal average number of TRECs per 100.000 cells in all patients of the three groups.

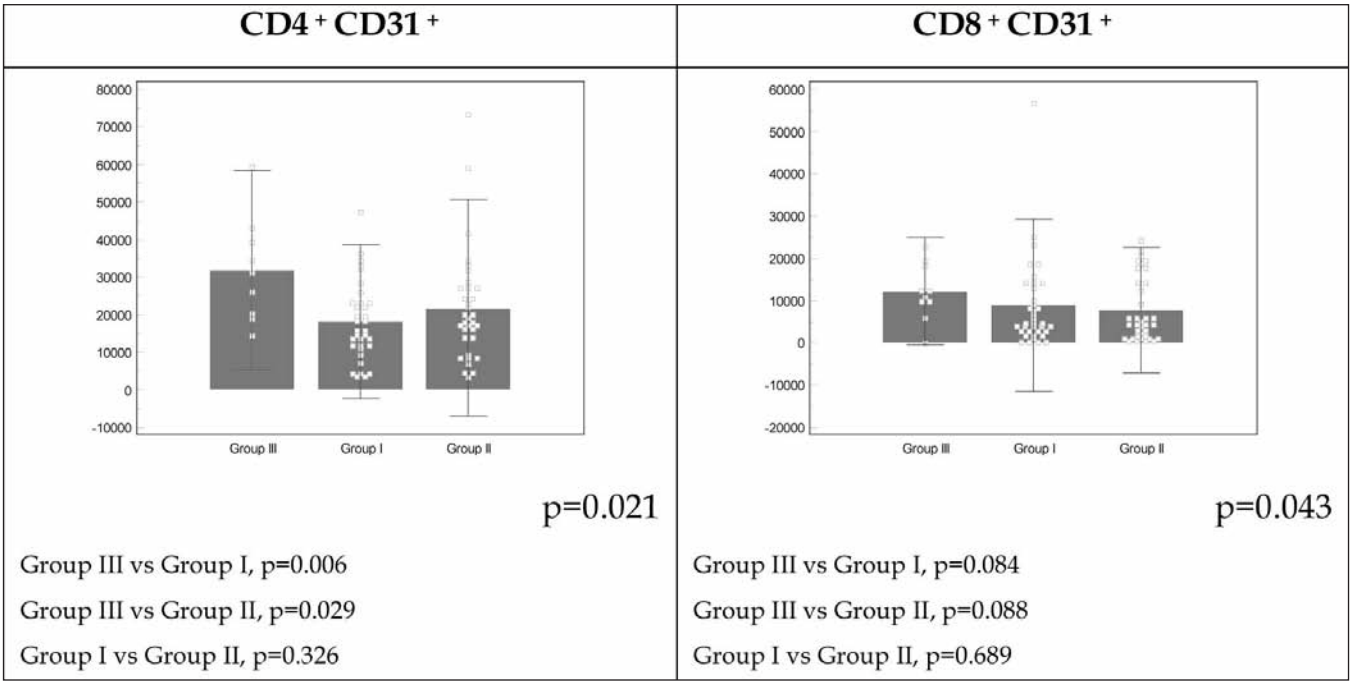
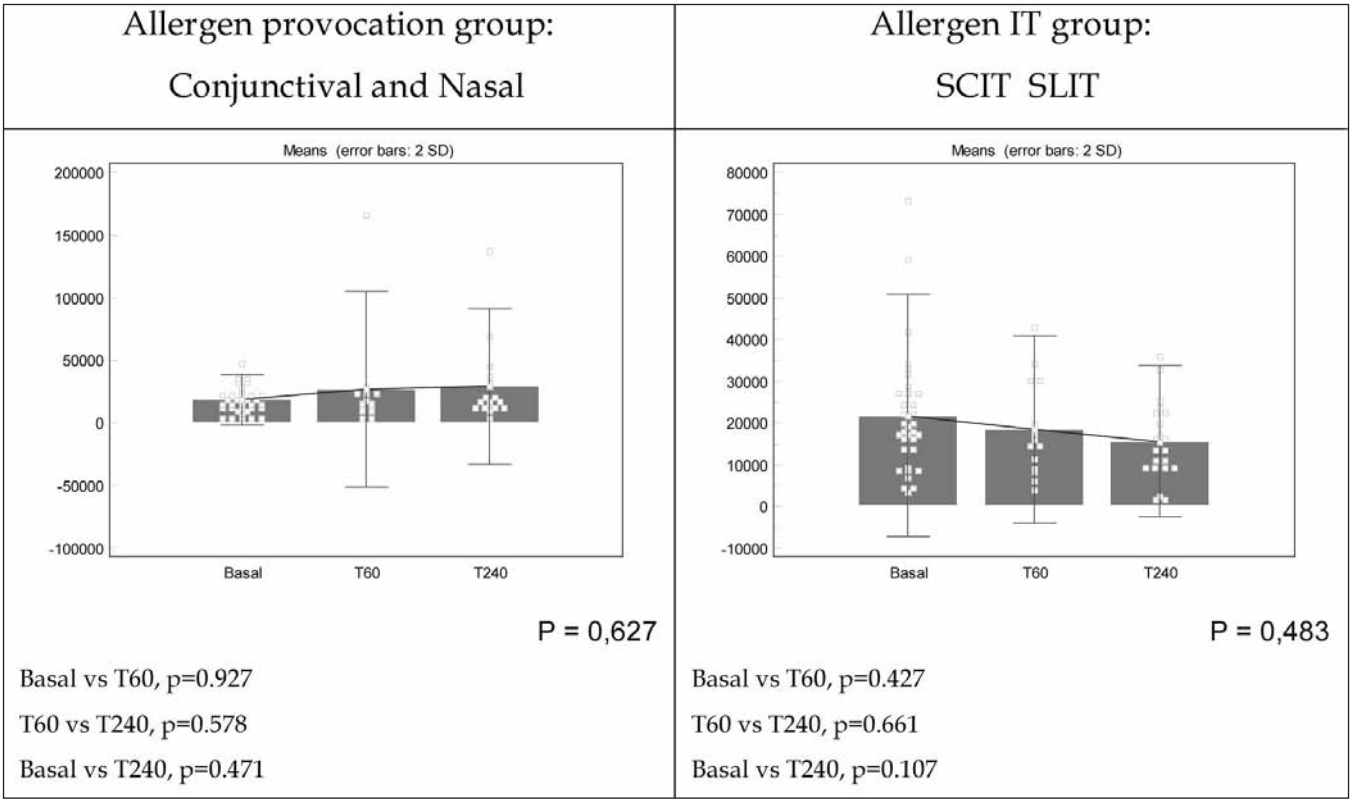


Figure 7 - Average number of TRECs/100.000 CD4+CD31+ cells obtained for all the patients in the three groups.



of TRECs in $CD4^+CD31^+$ cells was lower than the control group. This low basal level was higher in the group treated with SIT, probably evidencing the central mechanism of this treatment.

The variability in the determination of this value was quite high in all groups, further supporting the functional activity of the central immune system. It was also evident that the individual response of each patient is highly variable, most likely depending on the genetic expression of each patient, although the clinical pattern and the allergic sensitization (Dpt) was the same.

The nasal and conjunctival allergic challenge test produced similar RTEs profiles in peripheral blood after 1 hour. Even though no second value was obtained for each patient, the group of patients analyzed 4 hours after the provocation seemed to present a time-dependent increase (statistical significant, Figure 2).

Although the total population of circulating $CD4^+$ e $CD8^+$ T-cells is conserved in allergic patients, when compared to the healthy controls, the reduction of RTEs can be due to a chronic inflammatory status (20,29).

The local release of mast cell mediators increases the expression of adhesion molecules on postcapillary venules. This can lead to homing of circulating leukocytes, which may infiltrate distant tissues. There is significant evidence to suggest that during the early or initiation phase, there is selective recruitment of $CD4^+$ T lymphocytes into the extravascular space at the challenge site (31). This cell recirculation and focalization makes the IgE mediated allergic disease a dynamic and systemic process. Previous results showed that this cell response starts at an early stage, in parallel with the immediate allergic response (12). The IgE mediated response induces immunolymphatic involvement of the adjacent structures which amplifies the allergic response to locoregional lymphoid organs, while circulating leukocytes recirculation compromise the primary lymphoid organs (thymus and bone marrow). These central organs are responsible for the systemic immune response induced by a focused allergen challenge.

In contrast to the atopic dermatitis, the most implicated cell population in the allergic reaction seems to be $TREC/CD4^+CD31^+$ (20). Most patients analyzed evidenced an increase of circulating naïve T-cells, which can indicate the ability to increase the maturation of thymic cells in order to control the magnitude of the allergic reaction.

In SIT, the induction of T-reg cells is one of the most relevant events in the regulatory mechanism (6). The therapeutical administration via injection in the lymph nodes assumes the direct intervention of the immune system, although similar results are obtained when the treatment is performed either subcutaneously or sublingual (13,14). In our patients undergoing a maintenance treatment, we observe a reduction of circulating RTEs when compared to healthy control population, although their average is higher than the average measured in patients not exposed to this type of treatment. The early involvement of bone marrow and the functional thymic tissue is also observed, but obviously their mechanisms and targets are surely distinct from the allergic reaction.

The induction of T-reg cells is determinant in the immunotherapy mechanism, even though they consist on a heterogeneous population that includes: naturally occurring $CD4^+CD25^+$ T cells and cells induced in the periphery following antigen exposure (Tr1 cells, Th3 cells, and $CD8^+$ regulatory T cells) (32). From the result analysis, we admit that the need from this production requires a maturation time within the thymic tissue, which can explain the reduction in circulating RTEs ($TRECs/CD31^+$) observed after the treatment with the therapeutical extract.

Our results support the idea that the immune system is actually a target from the IgE mediated allergic reaction and SIT. The functional thymic tissue plays an early role, since the production of RTEs is highly variable, contrasting with the results observed in the healthy population where these levels are constant throughout the experiment. We suggest that in allergic reaction the prompt release of naive thymic cells results from the pathogenic mechanism itself and/or the regulation of its own reaction. In SIT mechanism, however, the apparent reduction of these cells can result from a cellular influx to the functional thymic tissue and for further maturation of the T-reg lymphocytes.

We suggest that a later evaluation of the allergic reaction or the SIT effect could lead to more significant differences, both in terms of individual and group analysis. We believe that further studies to characterize RTEs specific cell markers are required. These studies should also focus on RTEs abilities to repopulate the T compartment and in the evaluation of new therapeutical approaches for IgE mediated allergic disease.

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Declaration of Interest

All the authors disclose any financial, consulting, and personal relationships with other people or organizations that could influence (bias) the author's work.
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