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Original Research

Expansion of the immature B lymphocyte compartment in Graves' disease

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Abstract

Objective: The specific mechanisms driving autoimmunity in Graves' disease (GD) remain largely unknown. Kappa-deleting recombination excision circles (KRECs) are circular DNA molecules generated during B cell maturation in the bone marrow which provide a measure of B cell production and proliferation. We aimed to investigate the association between KRECs and B cell subpopulations, with thyroid status and clinical outcome in GD patients.

Methods: Kappa-deleting recombination excision circles were measured by quantitative real-time PCR using a triple-insert plasmid control in 132 GD patients and 140 healthy controls. In addition, KRECs in GD patients on withdrawal of antithyroid drug (ATD) and 6-10 weeks later were analysed according to a clinical outcome at 1 year. Flow cytometry was performed on isolated $CD19^+$ B cells to quantitate 7 B lymphocyte subpopulations in 65 GD patients.

Results: Circulating KRECs were higher in GD vs. controls ($P = 1.5 \times 10^{-9}$) and demonstrated a positive correlation to thyroid hormones and autoantibodies (free thyroxine: $P = 2.14 \times 10^{-5}$, rho = .30; free triiodothyronine: $P = 1.99 \times 10^{-7}$, rho = .37; thyroid stimulating hormone receptor autoantibodies: $P = 1.36 \times 10^{-5}$, rho = .23). Higher KRECs in GD patients 6-10 weeks after ATD withdrawal were associated with relapse of hyperthyroidism at 1 year ($P = .04$). The KRECs were positively correlated to the total $CD19^+$ B cell count ($P = 3.2 \times 10^{-7}$).

Conclusions: This study reports a robust association between KRECs and GD, highlighting the importance of B cells in the pathogenesis of GD and the influence of thyroid status on B cell activity. The findings indicate a potential role for KRECs as a marker of disease activity and outcome in GD.

Keywords: thyroid, autoimmune, Graves' disease, B lymphocytes, hyperthyroidism, humoral, KREC

Significance

The hyperthyroidism in Graves' disease is driven by circulating TSH receptor autoantibodies, which are produced by terminally differentiated B lymphocytes (plasma cells). Using analysis of Kappa-deleting recombination excision circles to study B lymphocyte dynamics, we find that patients with Graves' disease, both in the hyperthyroid and euthyroid state (after more than a year of treatment) had an expanded immature B lymphocyte compartment. This contributes a new mechanistic insight into the immunopathology driving Graves' disease.

Introduction

Graves' disease (GD) is an autoimmune disorder characterised by the presence of autoantibodies that bind to the thyroid stimulating hormone receptor (TRAbs), resulting in excessive production of thyroid hormones (hyperthyroidism) and goitre. GD is one of the commonest autoimmune conditions and the most frequent cause of hyperthyroidism with an annual incidence of 20-50 cases per 100 000; affecting up to 3% of women.[1,2] The usual approach to treating GD involves the administration of a 12-18-month course of antithyroid drugs (ATD) to block thyroid hormone synthesis, however, relapse occurs in around half of adults after ATD withdrawal.[3] The central role of TRAbs in mediating the clinical manifestations of GD means that they are useful in monitoring disease activity and for predicting outcome following ATD treatment.[1]

Although the specific immune mechanisms driving GD remain unknown, B lymphocytes have an indisputable role in GD, not only in their capacity as plasma cells producing the pathogenic autoantibody, TRAb, but also in their ability to modulate the immune response through antigen presentation

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and secretion of cytokines.[4] Kappa-deleting recombination excision circles (KRECs) are circular DNA molecules generated during the B-cell receptor Variable-Diversity-Joining (V(D)J) recombination process that occurs during B cell maturation in the bone marrow.[5] The KRECs provide a quantitative measure of B cell output from the bone marrow and allow insight into the peripheral B cell replicative activity. Although not previously studied in GD, KREC levels have been associated with disease status and activity in another B-cell mediated autoimmune disorder, immune thrombocytopenic purpura (ITP),[6] and therefore may provide a mechanistic insight into the immunopathology driving autoimmunity in GD, as well as associating with relevant clinical phenotypes or outcome.

Specific peripheral B cell subpopulations have been associated with hyperthyroid GD. In particular, the transitional and naïve B lymphocytes, both of which are recent emigrants from the bone marrow, have been observed to be elevated in individuals with both treated and untreated GD, with correlation to thyroid hormone and TRAb concentrations.[7–9]

Despite the relevance of KRECs as a marker of humoral immune activity, they remain relatively understudied in autoimmunity. This study aimed to investigate the association of KRECs with GD and B cell subpopulations, to improve our understanding of the immunopathology driving GD and provide mechanistic insight into the relapse process.

Materials and methods

Participants

A total of 132 GD patients from a UK cohort were included in this study, including 65 patients for whom the outcome of their GD 1 year after stopping ATD was known (16/65 relapsed [25%], 49/65 remitted [75%]), and a further 67 GD patient samples from a previously collected cohort (Figure S1). In the 65 patients where an outcome was known, there were 2 samples taken, with the first sample taken in patients with controlled hyperthyroidism at the time of stopping ATD following at least a 12-month course (65/65 samples available), and the second sample 6-10 weeks after withdrawal of ATD (58/65 samples available). In total, this cohort of 190 samples (from both timepoints and the additional 67 GD patient samples) included 49 hyperthyroid and 141 euthyroid samples from GD patients. All GD patients had both biochemical evidence of hyperthyroidism (raised free triiodothyronine [FT3] and/or free thyroxine [FT4]), and positive serum TRAb (>1.8 u/L) at diagnosis. None had previously received radioiodine therapy. Serum FT4, FT3, and thyroid peroxidase antibody (TPO Ab) levels were determined by using the commercial Roche Elecsys immunoassay (Roche Diagnostics), and serum TRAb levels were determined using the BRAHMS TRAK immunoassay (Thermoscientific). The median age of the GD cohort was 46 years and included 111 (84%) females.

In addition, 140 healthy control samples provided by the NIHR BioResource Centre, Newcastle were used in the analysis. These control samples had no known medical history of autoimmune disease, including thyroid disease. The median age of this cohort was 51 years and included 87 (62%) females. Therefore, in total, there were 330 separate samples from which KREC levels were quantified, including controls and euthyroid and hyperthyroid GD samples.

The study complied with the Declaration of Helsinki. Informed, written consent was obtained from all participants. This study was carried out with approval by the NHS HRA Research Ethics Committee in East Midlands—Leicester South (REC reference: 18/EM/0371). The NIHR BioResource Steering Committee approved the use of the healthy control patient DNA samples from the Newcastle NIHR BioResource Centre.

Quantitative polymerase chain reaction (qPCR)

Genomic DNA was extracted from peripheral whole blood taken from the participants. Kappa-deleting recombination excision circles were quantified by real-time quantitative polymerase chain reaction (qPCR) using a comparator triple-insert plasmid (containing T-cell receptor excision circle [TREC] and KREC signal joint [SJ] fragments and the T-cell receptor alpha constant [TCRAC] reference gene) supplied by Sottini et al.[9] The primers and probes used in this experiment are detailed in Table S1.

Serial 1:10 dilutions of the plasmid were performed to generate a 6-point standard curve. Both the standard curve and samples were run in triplicate, and 10% of samples were repeated to evaluate the inter- and intra-assay coefficients of variation. Negative controls and standard curves were run on each plate, and the PCR efficiency was maintained between 92% and 100% with a slope value between $-3.5 \geq -3.3$.

The assay was run on the QuantStudio™ 7 Flex Real-Time PCR System in standard mode thermal cycling; 50 °C for 2 minutes, 95 °C for 10 minutes; 95 °C for 15 seconds, 60 °C for 1 minute—45 cycles.

KREC calculations

The quantity of KRECs is reported as number of KRECs per 10^6 total cells (nucleated). As previously described by Sottini et al.,[10] to determine the number of KRECs per 10^6 cells, the following formula was used:

$$\frac{(\text{mean quantity of KRECs})}{(\text{mean quantity of TCRAC}/2) \times 10^6}.$$

The reference gene, TCRAC, used as a denominator in this calculation is present in every cell and therefore represents the overall cell number from which DNA has been extracted. Each cell contains 2 TCRAC gene copies and therefore, the mean quantity of TCRAC is divided by 2.

B cell replicative activity was analysed by using the coding joint (CJ) which remains stable in the genome of the B cell, and the SJ or KREC which does not replicate during B cell division and therefore is diluted 2-fold with each cell division. Thus, by using the cycle threshold (Ct) values to calculate the ΔCT (Ct KREC – Ct CJ)) along with the formula $^2\log\Delta CT$, van Zelm et al.[5] demonstrated that this is equal to the coding/signal joint ratio (CJ:KREC ratio) and can be used to estimate the replicative history of an individual's B cell population.

Flow cytometry (B cell subpopulations)

There were a total of 64 GD patient samples that were taken at the time of ATD withdrawal available for flow cytometry analysis, using a 10 antibody panel (Table S2). The B cell subpopulations and phenotypes studied are presented in Table 1. The gating strategy used to determine the B cell subpopulations is presented in Figure S2. All flow cytometry data was recorded using the BD LSRFortessa™ Cell Analyzer and BD FACSDiva™ Software. Analysis of the flow cytometry data was performed using FCS Express™ (Version 7).

Table 1. Spearman's correlation of each B cell subpopulation and KRECs per 10^6 cells, including the phenotype of each B cell studied.

B cell subpopulation	Phenotype	KRECs per 10^6 cells	
		Rho	*P*
Transitional	$CD19^+$ $CD38^{++}$ $CD24^{++}$ $CD5^+$ $CD27^-$	.52	$\mathbf{1.5 \times 10^{-5}}$[a]
Naïve mature	$CD19^+$ IgD^+ $CD27^-$	.60	$\mathbf{3.7 \times 10^{-7}}$[a]
Unswitched memory	$CD19^+$ $CD27^+$ IgD^+ $CD38^{low}$	.53	$\mathbf{9.9 \times 10^{-6}}$[a]
Switched memory	$CD19^+$ $CD27^+$ IgD^- $CD38^{low}$	.28	**.03**[a]
Plasmablast	$CD19^+$ $CD27^{++}$ $CD38^{++}$ CD20	.17	.17
Double negative	$CD19^+$ $CD27^-$ IgD^-	.34	**.006**[a]
B regulatory	$CD19^+$ $CD27^+$ $CD24^{++}$	.27	**.03**[a]

Spearman's correlation coefficients (rho) and *P* value (*P*) are presented for each of the correlations.
Abbreviation: KRECs, Kappa-deleting recombination excision circles.
[a] Significant value ($P < 0.05$)

Table 2. Clinical and demographic characteristics of the Graves' disease patients and healthy controls.

Demographic/clinical variable	Graves' disease $n = 132$	Healthy controls $n = 140$
Total number of hyperthyroid samples[a]	49	0[b]
Total number of euthyroid samples[a]	141	140[b]
Age (years): median (IQR) [range]	46 (35-54) [20-92]	51 (39-57) [20-70]
Female: ***n*** (%)	111 (84%)	87 (62%)
Smoking status (at time of ATD withdrawal)[c]		Unknown
Current smoker	15/65 (23%)	
Non-smoker	50/65 (77%)	
Graves' orbitopathy (at time of ATD withdrawal)[c]		Unknown
Present	9/65 (14%)	
Absent	56/65 (86%)	
Thyroid function at time sample taken (hyperthyroid patients): median (IQR) [range]	($n = 49$)	N/A
Free T4 (pmol/L)	36 (27-52) [13-100]	
Free T3 (pmol/L)	15.4 (9-26) [6.2-50]	
TRAb (IU/L)	8.9 (4-29) [0-100]	
TPO Ab (IU/mL)	34 (8-228) [0-600]	
Thyroid function at time sample taken (euthyroid patients): median (IQR) [range]	($n = 141$)	N/A
Free T4 (pmol/L)	16 (15-18) [10.8-21.7]	
Free T3 (pmol/L)	4.7 (4.2-5.3) [3.8-5.8]	
TRAb (IU/L)	0 (0-1.2) [0-85.9]	
TPO Ab (IU/mL)	24 (12-79) [0-301]	

See Figure S1 for further information about patient cohorts.
Abbreviations: N/A, not applicable; FT4, free thyroxine; FT3, free triiodothyronine; TRAb, thyroid stimulating hormone receptor autoantibody; TPO Ab, thyroid peroxidase antibody; ATD, antithyroid drug.
[a]Includes the GD samples 6-10 weeks after ATD withdrawal (total number of GD samples studied = 190).
[b]Presumed normal thyroid function as all healthy control samples had no known medical history of thyroid disease.
[c]Data available from 65/132 GD patients.

Statistical analysis

All statistical analysis was performed using R, version 4.2.2.[11] A Shapiro-Wilk test was used to test for normality which determined the choice of statistical test. Spearman's rank correlation was used to assess correlations between 2 non-normally distributed variables. A chi-square test was undertaken to assess the change in KRECs between the 2 outcome groups. A change of less than 50 KREC copies was used for the purposes of defining "stable" KREC levels. Kruskal-Wallis tests were performed for multiple group comparisons, and pairwise comparisons using the Wilcoxon rank sum test were subject to multiple test correction using the false discovery rate (Benjamini and Hochberg[12]). Multivariable binary logistic regression analysis was undertaken to investigate KRECs and GD outcome. The level of statistical significance accepted was $P < .05$ (2-tailed significance).

Results

KRECs and clinical characteristics

The clinical and demographic characteristics of the patient samples used in this study are presented in Table 2. To evaluate the association between age and gender with KRECs, the total sample population was included (330 samples). There was no significant difference in age between the GD and control cohorts (46 years vs. 51 years; $P = .38$), but there were more females represented in the GD cohort (84% vs. 62%; $P = .00004$). However, overall, there was no association found between KRECs and age ($P = .20$) or gender ($P = .50$) (Figure S3). There was no association observed between KRECs and smoking status ($P = .64$) or presence of Graves' orbitopathy ($P = .83$) in 65 of the GD patients where this data was available.

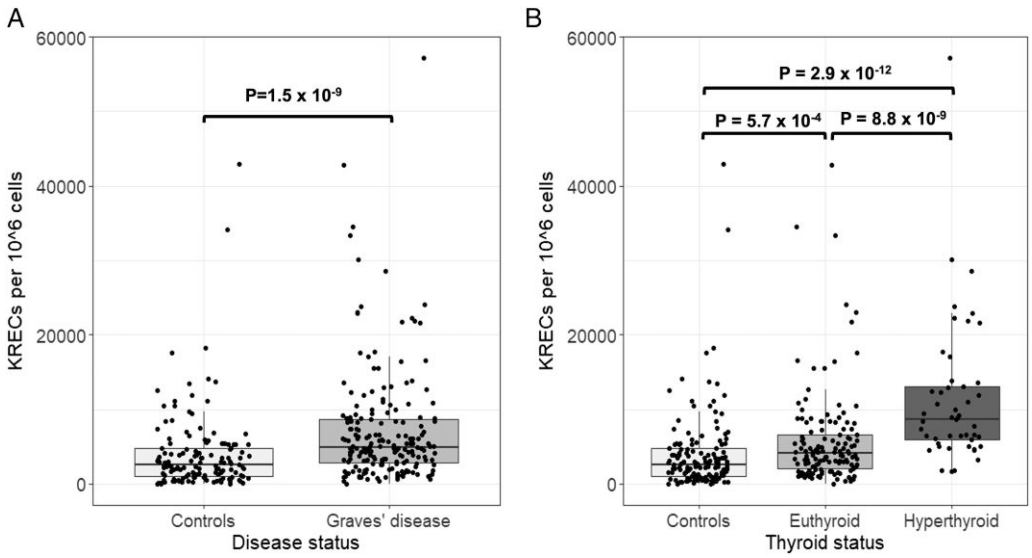

Figure 1. KRECs per 10^6 cells in controls vs. Graves' disease (A) and controls vs. euthyroid vs. hyperthyroid Graves' disease patients (B). Median, 25%-75% and 5%-95% quantiles are shown by the box and whiskers, respectively. KRECs, Kappa-deleting recombination excision circles.

KRECs and thyroid status in Graves' disease vs. healthy controls

To investigate the association of KREC levels with disease status, the GD patients were compared with healthy controls, followed by comparisons between both hyperthyroid and euthyroid GD patients with healthy controls (Figure 1).

There was a significantly higher quantity of KRECs per 10^6 cells in GD patients compared to controls (median 4952 vs. 2571; $P = 1.5 \times 10^{-9}$). Furthermore, there was a significant difference between the 3 groups (controls, euthyroid, and hyperthyroid; $P = 4.5 \times 10^{-14}$). Pairwise comparisons revealed a significant difference in KRECs per 10^6 cells between both controls vs. euthyroid (median 2571 vs. 4092; $P = 5.7 \times 10^{-4}$) and controls vs. hyperthyroid patients (median 2571 vs. 8614; $P = 2.9 \times 10^{-12}$). There was also a significant difference observed between hyperthyroid vs. euthyroid GD patients (median 8614 vs. 4092; $P = 8.8 \times 10^{-9}$), with higher thyroid hormone levels associated with higher KREC levels. Overall, these findings indicate that not only do individuals with Graves' hyperthyroidism have higher circulating KRECs, but that regardless of thyroid hormone status, those with GD are more likely to have elevated KREC levels.

To evaluate the association between thyroid status and KRECs in GD, all 190 GD samples where thyroid biochemistry was available (FT4 [all samples], FT3 [187/190; 98%], TPO Ab [134/190; 71%], and TRAb titre [168/190; 88%]) were entered into correlation analysis. There was a positive correlation observed between KRECs and FT4 ($P = 1.99 \times 10^{-5}$, rho = .30), FT3 ($P = 1.77 \times 10^{-7}$, rho = .37), TPO Ab ($P = .007$, rho = .23), and TRAb ($P = 1.34 \times 10^{-5}$, rho = .33) (Figure 2).

KRECs and Graves' disease outcome following ATD treatment

Differences between circulating KREC levels and outcome of GD at 12 months following ATD withdrawal were studied in the 65 GD patients where an outcome was available. There was no significant association between KRECs per 10^6 cells at the time of ATD withdrawal and outcome ($P = .87$), however, individuals with higher KREC levels 6-10 weeks after ATD cessation were more likely to relapse by 1 year ($P = .04$) (Figure 3). The FT3 was positively correlated to KRECs per 10^6 cells in the relapsing patients ($P = .018$, rho .58) (Figure S4), however, there was no significant association observed in these patients between KRECs and TRAb ($P = .13$) or FT4 concentrations ($P = .13$). Multivariable regression analysis including TRAb concentration at the time of ATD withdrawal and other risk factors associated with relapsing GD (age, gender, smoking status, and goitre size) demonstrated that KREC levels at 6-10 weeks were not an independent predictor of an outcome ($P = .08$).

Of the 16 patients that relapsed at 12 months and 49 patients that remitted, there were 15 and 42 paired samples available, respectively, from both the timepoint of ATD withdrawal and 6-10 weeks later. Of the relapsing patients, the majority (12/15; 80%) either had an increase (10/15; 67%) or stable (2/15; 13%) KREC levels from withdrawal of ATD to 6-10 weeks later (Figure 4A) compared to those that remitted where only 17/42 (40%) had an increase or stable KRECs levels (Figure 4B). This change in KRECs between the 2 outcome groups was nominally significant on chi-square analysis ($P = .046$). Interestingly, comparing patients that had an increasing or stable KREC trajectory following withdrawal of ATDs to those with falling KRECs, there was a trend towards a quicker relapse (mean 165 vs. 243 days) ($P = .24$). There was no correlation between relapse of GD and the different B cell populations defined by flow cytometry.

B cell replicative history

The decay of KREC signal joints with each cell division was used to investigate peripheral B cell replicative history in both the GD and control patients. When healthy controls were compared to GD patients, there was greater proliferative B cell activity observed in healthy controls (median 1.5 vs. 0.6; $P \leq 2 \times 10^{-16}$) (Figure 5).

Pairwise comparisons revealed a significant difference in the average number of B cell divisions between controls vs. euthyroid GD patients (median 1.45 vs. 0.74; $P \leq 2 \times 10^{-16}$) and controls vs. hyperthyroid patients (median 1.45 vs. 0.039; $<2 \times 10^{-16}$). There was also a significant difference observed

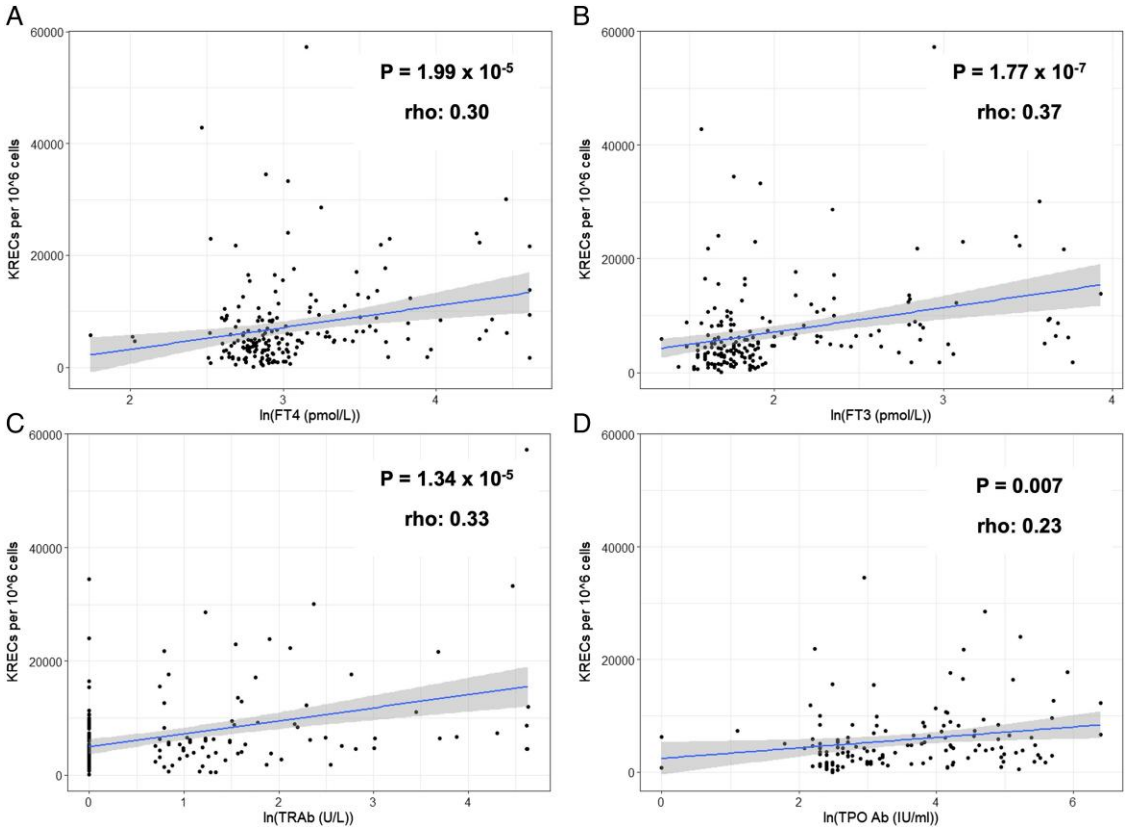

Figure 2. Association between log-transformed (ln) thyroid hormones and serum autoantibodies (A FT4, B FT3, C TRAb, and D TPO Ab) and KRECs per 10^6 cells. The grey shaded area represents the 95% confidence interval. KRECs, Kappa-deleting recombination excision circles; FT4, free thyroxine; FT3, free triiodothyronine; TRAb, thyroid stimulating hormone receptor autoantibody; TPO Ab, thyroid peroxidase antibody.

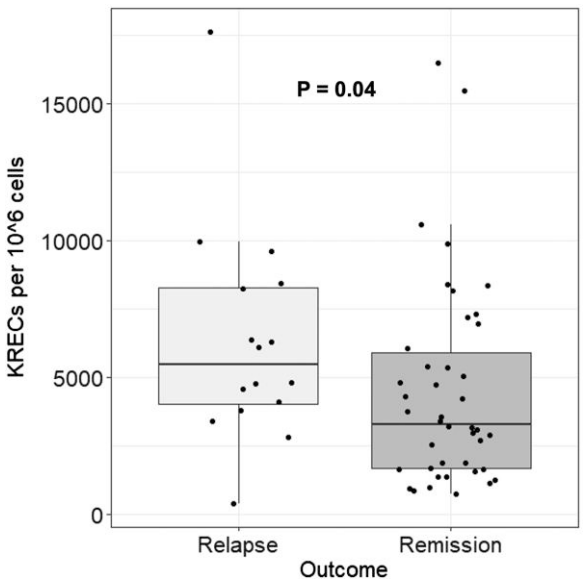

Figure 3. The association of KRECs per 10^6 cells 6-10 weeks after ATD withdrawal in Graves' disease with outcome (relapse/remission) at 1 year. KRECs, Kappa-deleting recombination excision circles.

between euthyroid vs. hyperthyroid GD patients (median 0.74 vs. 0.039; $P = 6.2 \times 10^{-7}$) (Figure 5). Furthermore, there was a significant overall difference observed between the 3 groups (controls, euthyroid, and hyperthyroid; $P \leq 2.2 \times 10^{-16}$). These findings suggest that healthy controls have a more mature peripheral B cell phenotype and fewer recently emergent B cells (which tend to have comparatively reduced proliferative activity), with the euthyroid and then hyperthyroid GD patients having a progressively less mature B cell population.

To determine whether there was any association between peripheral B cell proliferation and outcome in GD, the average number of B cell divisions at ATD withdrawal and 6-10 weeks later was compared between the GD outcome groups (relapse/remission), but this did not reveal any significant difference ($P = .16$, $P = .84$, respectively).

KRECs and B cell subpopulations

Analysis was undertaken to determine the relationships between the peripherally circulating B cell subpopulations and KRECs. The KRECs were positively correlated to the total $CD19^+$ B cell count ($P = 3.2 \times 10^{-7}$) and with all the B cell subpopulations studied, excluding the plasmablast population (Table 1). The strongest association was observed between KRECs and the early-stage B cell populations that are present shortly after emigration from the bone marrow (transitional and naïve B cell subsets) (Figure 6). Although there was no direct correlation observed between KRECs and plasmablasts, both the switched and unswitched memory B cell subsets were positively associated with plasmablasts (unswitched: $P = 1.3 \times 10^{-5}$, rho = .52, switched: $P = 1.7 \times 10^{-8}$, rho = .65). Additionally, the double negative (DN) B cells were positively associated with both KRECs ($P = .006$, rho = .34) and plasmablasts ($P = .018$, rho = .29) (Figure S5). This

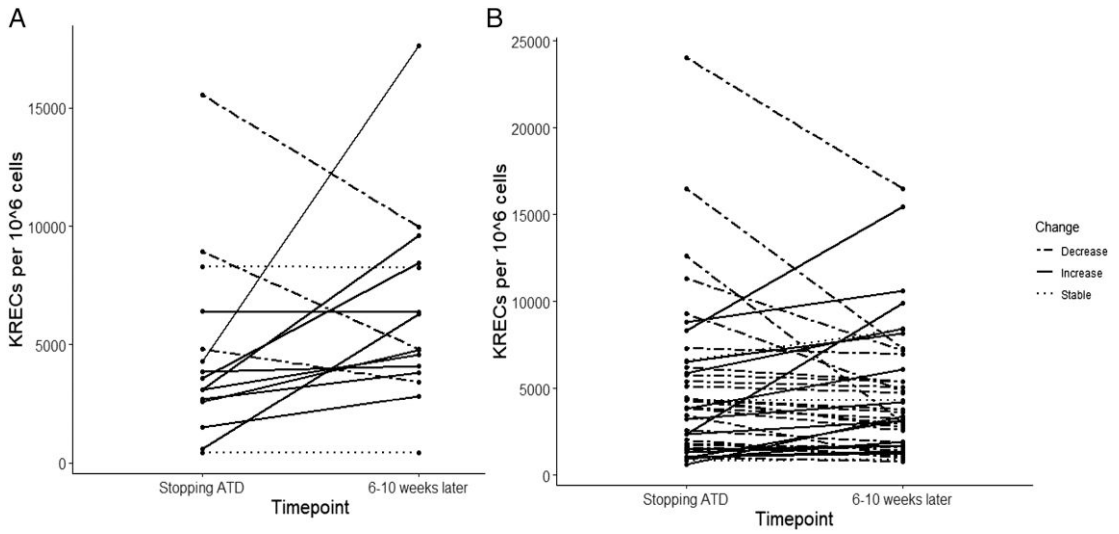

Figure 4. KRECs per 10^6 cells at the timepoint of stopping ATD and 6-10 weeks later in 15 of the Graves' disease patients that relapsed (A) and 42 of the patients that remitted (B) at 12 months. Lines are stratified by change in KRECs; decrease (2-dashed), increase (solid), and stable (dotted). ATD, antithyroid drug; KRECs, Kappa-deleting recombination excision circles.

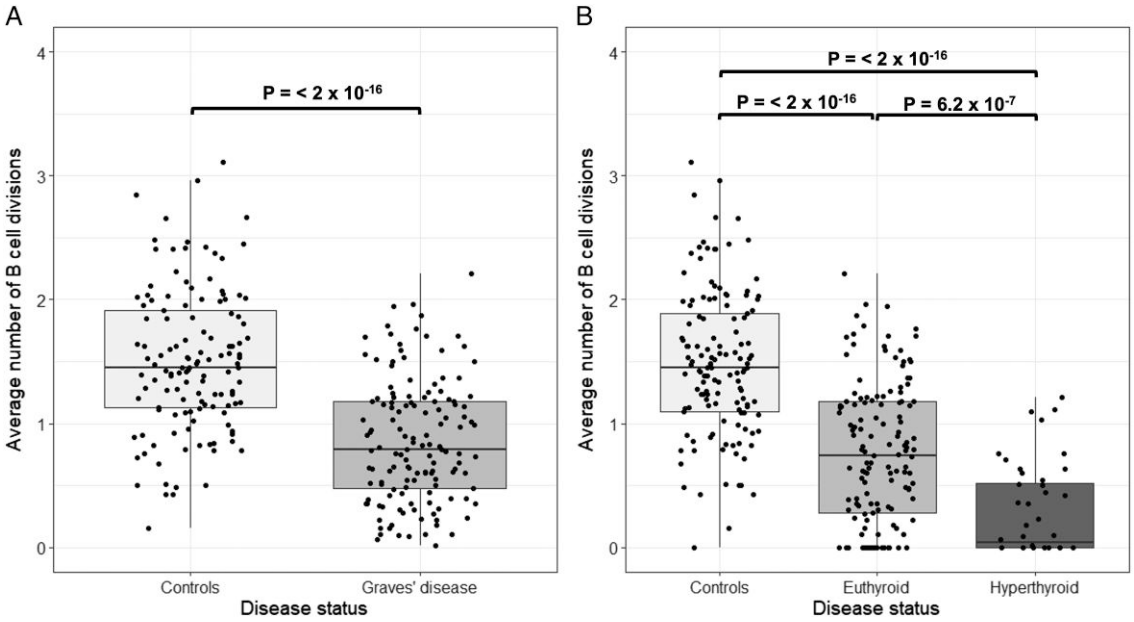

Figure 5. The average number of peripheral B cell divisions in controls vs. Graves' disease (A) and controls vs. euthyroid vs. hyperthyroid Graves' disease patients (B).

indicates that although the plasmablasts are not directly correlated to KRECs, the presence of memory and DN B cells, both of which are positively correlated to KRECs, may be consistent with their roles as precursors of antibody-secreting plasma cells.

Discussion

This study sought to understand more about the humoral immune system in GD, which might be regarded as a largely antibody-mediated condition.[1] We find a robust association between KRECs and GD and demonstrate a strong positive correlation between thyroid hormone and thyroid autoantibody concentrations with circulating KREC levels. Furthermore, the observed association with B cell subpopulations, particularly those recently emigrated from the bone marrow, suggests a less mature B cell compartment in both euthyroid and hyperthyroid GD patients.

Despite their obvious relevance in autoimmune disease, KRECs are most widely studied alongside TRECs in the field of primary immunodeficiencies to detect B and T cell developmental defects.[13] However, in addition to the association of KRECs with disease status and activity in ITP,[6] a recent proof-of-concept study demonstrated that KRECs, when used as a measure of oligoclonal peripheral B cell expansion, could predict response to the B-cell depleting agent rituximab in children with the autoimmune disease, juvenile dermatomyositis (JDM).[14] Although to the best of our knowledge KRECs have not previously been studied in GD, an increased

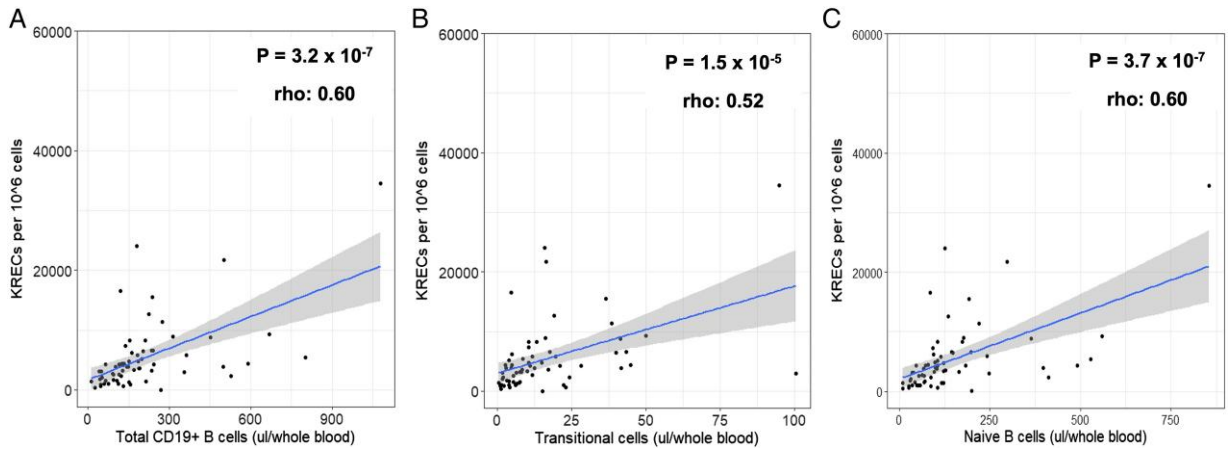

Figure 6. Association between the total CD19$^+$ B cell count (A) and early-stage B cell subpopulations; transitional (B) and naïve (C) with KRECs per 10^6 cells in 65 Graves' disease patients at the time of ATD withdrawal. The grey shaded area represents the 95% confidence interval. Spearman's correlation coefficients (rho) and *P* value (*P*) are presented for each of the correlations. KRECs, Kappa-deleting recombination excision circles.

concentration of TRECs has been observed in patients with GD and associated with thyroid status.[15] Unlike TRECs, KRECs are generally reported to remain stable with age and show no association with gender,[10] and our study confirms this finding.

This study demonstrated that higher thyroid hormone levels were positively correlated to KRECs, and it is well-established that higher thyroid hormone and TRAb concentrations are associated with a greater risk of relapse in GD.[3] Indeed, the higher circulating KRECs observed in the relapsing patients at 6-10 weeks were positively associated with serum FT3. There is growing evidence of "bidirectional crosstalk" between thyroid hormones and the immune system, with thyroid hormones proposed as direct regulators of the adaptive immune response and essential for primary B cell development and activation.[9,16–19] Specifically, FT4 concentrations have been demonstrated to be positively associated with circulating B lymphocytes and induce the proliferation of B cell subsets, including the naïve B cells.[19] Furthermore, immunophenotyping studies investigating the impact of thyroid hormones on immune homeostasis have demonstrated the enrichment of thyroid hormone sensitive gene expression in B-cell functional pathways, highlighting the role of thyroid hormones in regulating B lymphocyte function.[19] The increased B cell output, as quantified by KRECs, observed in this study of GD patients may reflect bone marrow hyperactivity that could in part be secondary to hyperthyroidism. Indeed, thyroid hormones are reported to be positively correlated to transitional and naïve B cells, and hyperthyroidism has been associated with the presence of hypercellular bone marrow, lymphocytosis, and splenomegaly, with a direct effect demonstrated on bone marrow activity.[8,20–22] However, the significantly higher KREC levels observed in euthyroid GD patients following at least a year of ATD treatment compared to healthy controls indicates that increased bone marrow B cell production may have an additional role in disease pathogenesis.

There is evidence that patients with GD, particularly in the untreated hyperthyroid phase, have an expanded immature B cell population, with increased numbers of CD5$^+$ transitional cells and naïve B cells.[7,9] Within this immature B cell population, even after passing the central tolerance checkpoint in the bone marrow, around 50% of transitional cells continue to demonstrate autoreactivity which only reduces once they differentiate into mature B cells.[23,24] This circulating pool of autoreactive B cells was studied in systemic lupus erythematous (SLE), where analysis of antinuclear antibodies (ANA) demonstrated that they were mostly of the naïve B cell subset with the potential to become activated due to a failure of anergy induction.[25] This was observed to have a direct effect on disease activity in SLE, as increased frequencies of anergic ANA$^+$ B cells were associated with decreased disease activity.[25] Additional studies have demonstrated the association of autoimmune conditions, such as JDM and systemic sclerosis, with an expanded immature transitional B cell population.[26–28] In this study, the increased KRECs observed in both hyperthyroid and euthyroid GD patients and their strong positive association with early-stage B cells may reflect this expanded autoreactive immature B cell population that ultimately gives rise to TRAb-producing B lymphocytes.

Unexpectedly, this study demonstrated that those with hyperthyroid GD had the lowest level of peripheral B cell maturation, followed by the euthyroid GD group, and then the healthy control group which had the highest B cell proliferative activity. The individual B cell subpopulations have differing levels of replicative activity, with immature B cells demonstrating substantially lower proliferative activity compared to their mature counterparts that have exited the highly replicative germinal centres.[5,29] Specifically, the CD5$^+$ naïve B lymphocytes have been demonstrated not to undergo proliferation in the peripheral B cell compartment.[30] This could therefore explain why individuals with active hyperthyroid or inactive euthyroid GD, whom may have an expanded immature B cell population relative to healthy controls, might demonstrate lower levels of peripheral B cell replicative activity. Additionally, it may be that those with greater B cell output and potentially more circulating autoreactive B cells have lower levels of peripheral proliferation in order to maintain B cell homeostasis.[31]

The differentiation of B lymphocytes into IgG$^+$ plasma and memory cells in autoimmunity is classically described to occur through the germinal centre pathway.[32] However, the activation and differentiation of naïve B lymphocytes into plasma cells has also been demonstrated to occur directly through the extrafollicular pathway in SLE.[32] The germinal centre

response results in memory cell formation and is also reported to produce higher affinity plasma cells,[33] however, it is slower to develop and therefore may be less important during an acute relapse of GD. Indeed, a large proportion of the circulating plasma cells during an active flare of SLE have been observed to be clonally related to naïve B cells rather than IgG^+ memory B cells, suggesting that they originate from an extrafollicular, rather than germinal centre, response.[33,34] Therefore, the extrafollicular pathway may activate the expanded immature naïve B lymphocytes in GD into TRAb-producing plasma cells and play a critical role in the humoral immune response during active and acutely relapsing GD. Thus, the association of KRECs with early-stage B lymphocytes suggests that they could represent a biomarker for disease activity in GD, with increased levels associated with active or relapsing GD.

As this study was performed in a relatively small cohort of GD patients, the findings should be validated on a larger scale. Wider analysis of B cell subpopulations at different stages of disease activity would help to establish the role of immature B cell subsets in relation to GD activity. To further explore the underlying mechanism of KRECs and peripheral B cell replicative activity in GD, analysis of KRECs on sorted B cell subpopulations may provide greater insight into the functional roles of specific B cell subsets in the pathogenesis of GD.

In conclusion, our study is the first to report an association between KRECs and GD, with the findings highlighting the importance of B cells in the pathogenesis of GD and the influence of thyroid status on B cell activity. The findings demonstrate the relationship between KRECs and B cell subsets, and provide mechanistic insight into the possible immunopathology driving GD. The association between KRECs and thyroid status indicates a potential role for KRECs as a marker of disease activity and outcome in GD.

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Supplementary material

Supplementary material is available at *European Journal of Endocrinology* online.

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