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1 **Longitudinal characterization of autoantibodies to the thyrotropin receptor (TRAb)**
2 **during alemtuzumab therapy; evidence that TRAb may precede thyroid dysfunction**
3 **by many years.**

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31

32 **Running title:** Longitudinal study of alemtuzumab-related TRAb

33

34 **Key words:** Thyroid Autoimmunity, Graves' Disease, Immune Reconstitution Syndrome,

35 Autoantibodies to the thyrotropin receptor, Alemtuzumab, Thyroid bioassays

36

37 **ABSTRACT**

38

39 **BACKGROUND**

40 Thyroid autoimmunity, especially Graves' disease or hypothyroidism with positive
41 autoantibodies (TRAb) to the thyrotropin receptor (TSHR), occurs in 30-40% of patients
42 with relapsing multiple sclerosis (MS) following treatment with alemtuzumab (ALTZ).
43 ALTZ therapy therefore provides a unique opportunity to study the evolution of TRAb
44 prior to clinical presentation. TRAb can stimulate (TSAb), block (TBAb) or not affect
45 ("neutral": TNAb) the TSHR function, causing hyperthyroidism, hypothyroidism or
46 euthyroidism, respectively.

47 **METHODS**

48 We conducted a longitudinal retrospective analysis of TRAb bioactivity over a period of 9
49 years in 45 MS patients receiving ALTZ using available stored serum; 31 developed
50 thyroid dysfunction (TD) and 14 remained euthyroid despite being followed for a
51 minimum of 5 years (NO-TD). The presence of TRAb was evaluated at standardized time
52 points: A) pre-ALTZ, B) latest time available post-ALTZ and before TD onset, C) post-
53 ALTZ during/after TD onset. Serum TRAb were detected by published in-house assays
54 (ihTRAb): flow cytometry (FC) detecting any TSHR-binding TRAb and luciferase
55 bioassays (LB) detecting TSAb/TBAb bioactivity. Purified IgGs were used to verify
56 TSAb/TBAb in selected hypothyroid cases. Standard clinical automated measurements of
57 TRAb (autTRAb), anti-thyroid peroxidase autoantibodies (TPOAb), thyroid stimulating
58 hormone, free-thyroxine and free-triiodothyronine were also collected.

59 **RESULTS**

60 Pre-ALTZ, combined ihTRAb (positive with FC and/or LB), but not autTRAb, were
61 present in 5/16 (31.2%) TD versus 0/14 (0%) NO-TD ($p=0.017$). Detectable ihTRAb

62 preceded TD development in 9/28 (32.1%) and by a median of 1.2 years (range 28 days –
63 7.3 years). Combination testing of ihTRAb and TPOAb at baseline predicted 20% of
64 subsequent cases of hyperthyroidism and 83% of hypothyroidism.

65 **CONCLUSIONS**

66 We present evidence that TRAb measured with custom-made assays can be detected prior
67 to any change in thyroid function in up to a third of cases of ALTZ-related TD.
68 Furthermore, The presence of ihTRAb prior to ALTZ treatment was strongly predictive of
69 subsequent TD. Our findings suggest that a period of affinity maturation of TRAb may
70 precede clinical disease onset in some cases. Combined testing of TPOAb and ihTRAb
71 may increase our ability to predict those who will develop thyroid dysfunction post
72 ALTZ.

73 **INTRODUCTION**

74 Alemtuzumab (ALTZ; Campath-1H) is an anti-CD52 humanized monoclonal
75 which has proven efficacy in relapsing multiple sclerosis (MS) (1). It is administered as a
76 standard treatment regime (two cycles one year apart), with subsequent courses
77 determined by evidence of returning central nervous system inflammatory activity. It
78 causes rapid complement mediated lysis of circulating lymphocytes and profound
79 lymphopenia. Since bone marrow derived lymphoid precursors are unaffected,
80 lymphocyte reconstitution subsequently occurs, which appears to have a beneficial effect.
81 Patterns of lymphocyte re-population vary between patients but circulating B cells return
82 most rapidly and can rise to higher levels than baseline (2), whilst CD4/CD8 T cells
83 numbers recover more slowly, and may never attain pre-treatment levels (3,4). Despite
84 prolonged T cell lymphopenia post-alemtuzumab immune competence is largely
85 preserved and significant infections occur only rarely (5). However 30-48% of patients
86 develop secondary autoimmunity, mainly humoral, 0.5–11 years after treatment (peak
87 incidence 2-3 years) (6-10). The commonest disease (41%) is thyroid autoimmunity (TA)
88 (11), followed by idiopathic thrombocytopenic purpura (1-3%). However, a range of other
89 rare autoimmune disorders including haemolytic anaemia, neutropenia and Goodpasture
90 syndrome have also been reported (1,12-14). The exact pathogenic mechanism for post-
91 ALTZ TA remains unclear, however it is considered to be an “immune reconstitution
92 syndrome”, i.e. an autoimmune phenomenon occurring during or after a phase of immune
93 restoration following lymphopenia. This has also been reported in HIV patients following
94 antiretroviral therapy and after bone marrow transplantation (7,8).

95 Among TA, ALTZ predominantly induces Graves’ disease (GD: 63%), followed
96 by hypothyroidism (34%), and rarely transient thyroiditis (11). GD is caused by anti-
97 thyrotropin (TSH) receptor (TSHR) autoantibodies (TRAb) persistently activating the

98 TSHR (TSHR-stimulating antibodies: TSAb), leading to hyperthyroidism (15). TRAb can
99 also block the TSHR (TSHR-blocking antibodies, TBAb), causing hypothyroidism
100 (16,17), and “neutral” TRAb (which bind the TSHR without affecting thyroid function:
101 TNAb) have been reported in around 12% of subjects with normal thyroid function, 59-
102 84% GD patients (depending on the assay type used), and patients with autoimmune
103 thyroiditis at lower rates (18-20). TNAb seem to bind TSHR but do not activate the
104 cAMP signaling cascade, which is the principal pathway leading to thyroid hormones
105 synthesis; however they may be able to trigger alternative and multiple signaling cascades
106 having complex downstream effects, including oxidative stress (20).

107 In spontaneous TA, TBAb account for a minority of cases of hypothyroidism
108 (around 9-10%) (16,21), the remainder being due to lymphocyte-mediated damaging of
109 the thyroid, as in classical Hashimoto’s thyroiditis (22). Autoantibodies to thyroid
110 peroxidase (TPOAb) are the hallmark of such autoimmune thyroiditis, however they are
111 very often positive in GD also, indicating that in TA the self-tolerance breakdown
112 involves multiple thyroid antigens (23). Surprisingly, TRAb are positive in 50.0%-76.7%
113 of patients with post-ALTZ hypothyroidism (9,10), with TBAb representing a common
114 mechanism of post-ALTZ hypothyroidism in a recent analysis (10).

115 TPOAb are very common in the general population (up to 20%) (24,25), and have
116 been identified as a predictive marker of TD subsequent to ALTZ (9). In particular, 69%
117 of MS subjects TPOAb positive before ALTZ developed subsequent TD, compared to
118 31% of TPOAb negative subjects. However, 85% patients who later developed TD were
119 TPOAb negative at baseline, indicating that TPOAb status alone has limited value in risk
120 stratification in the majority of patients (9).

121 The longitudinal study of ALTZ-treated patients provides a rare opportunity to
122 study TRAb prevalence and biological function prior to disease “triggering” in patients

123 who develop GD. The automated TRAb assays (autTRAb) used in clinical diagnostics are
124 unable to distinguish TSAb/TBAb (26); as a result several groups including ours have
125 developed in-house bioassays able to detect TSAb (27,28) and TBAb (21,29), as well as
126 TNAb (19). We postulated that TRAb, in particular TNAb, pre-existing before ALTZ
127 may be the precursors of the TSAb and TBAb that subsequently develop by somatic
128 hypermutation and affinity maturation in B cells (30,31). Detection of low titre or low
129 affinity TSAb/TBAb or the presence of TNAb prior to ALTZ therapy, in combination
130 with TPOAb testing, may also increase our ability to predict thyroid dysfunction after
131 ALTZ.

132 In addition we used the in-house TRAb bioassays (ihTRAb) to analyze TRAb
133 bioactivity arising after ALTZ therapy, which has so far only been described in
134 spontaneous TA (21,28,32). In a recent UK study conducted in collaboration between
135 Cambridge and Cardiff we have introduced TSAb/TBAb analysis in post-ALTZ TA,
136 however this was limited to only a few patients affected with hypothyroidism or
137 “fluctuating” GD, defined as multiple alternate phases of hyperthyroidism and
138 hypothyroidism (10). In the present study we extended this analysis to all available cases,
139 including a third different in-house assay to detect TSHR-binding TRAb independently
140 from their bioactivity (19).

141 **MATERIALS AND METHODS**

142 **Patients and sera**

143 Blood samples from Welsh MS patients consenting to research have been
144 consecutively collected for research purposes from 2006 (REC# 05/WSE03/111), and
145 stored within the Welsh Neuroscience Research Tissue Bank (WNRTB: Cardiff, UK,
146 REC# 14/WA/0073). Blood samples were processed within 3 hours of collection

147 following a standardized protocol including spin at 4500 rpm for 10 minutes at +4°C.

148 Serum and plasma were subsequently aliquoted and stored at -80°C.

149 Sera of 45 patients affected with relapsing MS treated with ALTZ with
150 longitudinal samples between August 2006 to October 2015 were identified including
151 samples from 31 consecutive subjects with post-ALTZ thyroid dysfunction (TD).
152 Samples from 14 patients who had not developed TD (NO-TD) were also selected based
153 on the availability of serum before ALTZ, and clinical follow-up of ≥ 5 years, in order to
154 exclude cases of late TD onset (11). Sera from pre-specified time-points were requested
155 for TD and NO-TD groups (Figure 1): A) first available pre-ALTZ time; B) the latest time
156 available post-ALTZ and before the TD onset; C) post-ALTZ at the TD onset, or
157 alternatively the earliest subsequent time available (TD only).

158 All patients were treated with ALTZ at the University Hospital of Wales (UHW)
159 in Cardiff, UK, and followed up both at UHW and local Welsh hospitals. ALTZ was
160 administered intravenously 5 consecutive days for the first cycle, with the majority of
161 subjects receiving a second cycle (3 consecutive days) 12 months later; in some patients
162 further doses were given at least one year apart, depending on clinical and radiological
163 outcomes. The date of the first ALTZ administration within our patient cohort ranged
164 from April 2002 to November 2012; the initiation dose was 24-30 mg/day prior to 2006,
165 then reduced to 12 mg/day. Since blood collection for research purposes commenced only
166 in 2006, this explains why time-point A is missing in several patients.

167 Information about patients' age, TSH, free-thyroxine (FT4), free-triiodothyronine
168 (FT3), TPOAb, TRAb determined by automated assays, thyroid treatment, and number of
169 ALTZ treatments were collected, when available. Demographic information and detailed
170 longitudinal clinical information was available for all patients, with last update in
171 February 2018.

172 **Luciferase bioassays (TSAb/TBAb)**

173 In-house luciferase bioassays (LB) to detect TSAb and TBAb were performed
174 using a Chinese Hamster Ovary (CHO) cell line stably transfected with the human TSHR
175 and a cAMP responsive luciferase reporter (pA3Luc), as previously described (Lulu*)
176 (27,29). Briefly, cells were seeded at 2×10^4 cells/well in 96-well plates in Ham's F12
177 containing 10% fetal calf serum, and switched to Ham's F12 containing 10% charcoal
178 stripped calf serum the day before the assay. In the assays cells were incubated for 4 hours
179 at 37°C in 5% CO₂ in air with whole human serum (1:10 dilution) in serum-free medium
180 (SFM: Ham's F-12 supplemented with 2.5% sodium bicarbonate) for the TSAb assay, and
181 SFM containing 1 mU/ml bovine TSH (bTSH; Sigma-Aldrich Company Ltd., Poole, UK)
182 in the TBAb assay. Cells were also incubated with SFM alone as negative control, and 5
183 mU/ml bTSH and 0.2 ng/μl M22 human monoclonal Ab to TSHR (RSR, Cardiff, UK) as
184 positive controls. Cells were finally lysed, and the luciferase activity measured using
185 commercially available kits (Promega, Madison, USA) and a luminometer machine
186 (Glomax®-Multi Detection System, Promega).

187 Randomly selected sera from 9 euthyroid participants from the Controlled
188 Antenatal Thyroid Screening II (CATS II) study (33,34) were used as euthyroid pool in
189 both TSAb/TBAb assays; they were all adult women (mean age ± standard deviation =
190 40.8 ± 5.3 years) with normal thyroid function and negative for TPOAb.

191 In the TSAb assay, CHO cells transfected with pA3Luc only (Zulu) were used in
192 parallel to Lulu*. The considered positivity cut-off was a stimulation index (SI) >1.5
193 calculated with the following formula:

$$\text{SI} = \frac{\text{light patient sample Lulu*} / \text{light patient sample Zulu}}{\text{light euthyroid pool Lulu*} / \text{light euthyroid pool Zulu}}$$

194
195
196
197 The TBAb assay positivity cut-off was an inhibition index (InI) >20% as

198 previously determined (formula A) (29) using Lulu* cultured with 1 mU/ml bTSH:

$$199 \quad \text{InI} = 100 \times \frac{(1 - \text{light patient sample})}{\text{light euthyroid pool}}$$

200

201 In order to exclude interference of high serum TSH levels with our in-house
202 TSAb/TBAb serum assay, especially among hypothyroid patients, experiments were
203 repeated using IgG (amount equivalent to 1:10 serum dilution) in place of serum; if
204 results were discordant we counted those using IgGs. IgGs were purified from selected
205 serum samples with the Melon Gel IgG Purification Kit (Pierce, Rockford, IL) according
206 to the manufacturer's protocol. Briefly, serum samples were diluted 1:10 and the diluted
207 serum was added to a spin column containing the Melon Gel resin. After 30 minutes
208 incubation, the purified IgGs were collected in the flow through by centrifugation of the
209 spin column, and the IgG concentration measured by ultraviolet optical absorption at 280
210 nm with a NanoDrop™ Lite spectrophotometer (Thermo Scientific). All IgG purified
211 samples were promptly used for downstream analysis, or aliquoted and stored at -20 °C.

212 **Flow Cytometry (TSHR-binding TRAb)**

213 In order to reduce the high non-specific background staining due to human
214 antibodies recognizing and/or cross-binding to surface CHO proteins, a serum pre-
215 adsorption step using Zulu cells was performed as previously described (35).

216 Flow cytometry (FC) detection of TSHR-binding TRAb (FC-TRAb) in pre-
217 adsorbed sera was then performed using CHO cells expressing the
218 glycosylphosphatidylinositol (GPI)-anchored TSHR extracellular domain (CHO-TSHR),
219 as previously described (19). As minor protocol modifications, 1:100 goat polyclonal anti-
220 human IgG (H+L) Alexa Fluor 488 (Life Technologies) and 1:1000 LIVE/DEAD®
221 Fixable Near-IR Dead Cell Stain Kit (Invitrogen) were used as second conjugated-
222 antibody and viability dye, respectively (35). Zulu cells were used as CHO control cell

223 line not expressing TSHR. The fluorescence of 10,000 cells/tube was assayed by BD
224 FACSCanto II flow cytometer, FACSDiva Software (BD Biosciences, San Jose,
225 USA); no FITC (TRAb) and Apc-Cy7 (LIVE/DEAD®) channels compensation was
226 needed (500-520 nm and 633-750 nm excitation-emission peaks wavelengths
227 respectively)(35).

228 Flow Cytometric data were analyzed using FlowJo 8.8.6 Software (TreeStar Inc.,
229 Ashland, USA), and damaged or dead cells (Apc-Cy7 positive) gated and excluded from
230 analysis (35). The geometric mean FITC fluorescence intensity values of CHO-TSHR and
231 Zulu cells were compared for all sera and the Kolmogorov–Smirnov univariate two-
232 sample test was used to obtain the greatest difference between the two histograms, quoted
233 as D value (D) (36). Cut-off values were defined based on the mean D +2 SD of
234 individual pre-adsorbed sera from 9 healthy women from the CATS II study (33,34) used
235 as controls; all values higher than this were considered positive (FC-TRAb+) (35).

236 **Automated Laboratory Measurements**

237 Automated TRAb (autTRAb) were measured with the Brahms Diagnostika
238 Lumitest TRAK assay (Germany; Reference Ranges IU/L = Negative <1, Borderline 1–
239 1.5, Positive >1.5) until January 2014, then using the Roche Cobas® e411 assay
240 (Switzerland; Reference Ranges IU/L = Negative <0.9, Borderline 0.9–1.6, Positive
241 >1.6). According to Thermoscientific, human TSH does not interfere with TRAb
242 measurement in the Lumitest TRAK assay, up to TSH values of at least 500mU/L. UHW
243 Biochemistry Department also run specific cross-reactivity tests using patient serum with
244 a TSH concentration of 179 mU/L, confirming no interference with neither Brahms nor
245 Roche TRAb assays.

246 TPOAb, TSH, FT4 and FT3 analyses were performed using an ADVIA Centaur
247 automated immunoassay analyser (Bayer plc, UK) until 31/05/2010, followed by

248 Chemiluminescent Microparticle Immunoassay methods by the ARCHITECT® System
249 (ABBOTT Laboratories, USA) until the end of the observation period. Supplemental
250 Table 1 summarizes the changes of reference ranges occurred during this time period.

251 **Definitions of Thyroid Function**

252 All 45 patients included in the study were euthyroid when receiving the first
253 ALTZ treatment, and had no clinical history of thyroid disease. The time of TD onset was
254 defined as the first alteration of the thyroid function defined as persistent (i.e. detectable
255 in consecutive blood tests at least 3 months apart) and/or significant (i.e. requiring
256 immediate thyroid treatment). Hyperthyroidism was defined as low TSH with or without
257 raised FT4/FT3 levels; hypothyroidism was defined as raised TSH with or without low
258 FT4/FT3 levels.

259 Thyroid diagnosis was defined as:

260 I) GD: TRAb+ hyperthyroidism

261 II) Fluctuating GD: TRAb+ cases with multiple alternate phases of hyperthyroidism and
262 hypothyroidism, not explained by overtreatment or poor treatment compliance

263 III) TRAb+ hypothyroidism

264 IV) Chronic autoimmune thyroiditis (37): persistent hypothyroidism (≥ 6 months) with
265 positive TPOAb and negative TRAb

266 V) Subacute thyroiditis: transient hyperthyroidism, hypothyroidism or both with TD
267 lasting in total <6 months, TRAb negative, with or without TPOAb

268 VI) TPOAb-/TRAb- hypothyroidism: persistent hypothyroidism (≥ 6 months) with
269 negative TPOAb and TRAb

270 **Statistical Analysis**

271 According to the TRAb prevalence in the general population of 12% (19), our *a*
272 *priori* power calculation indicated 12 versus 12 subjects required to provide 80% power to

273 detect a 5-fold TRAb prevalence (60%) in patients that will later develop ALTZ-induced
274 thyroid dysfunction, with a 0.05 significance level (two-tailed).

275 Presence of TRAb at different time-points was compared between TD and NO-TD
276 groups using the Fisher Exact Test, considering $p < 0.05$ as significance level. As
277 explorative analysis, positivity of TPOAb and TRAb measured with automated assays
278 was also considered.

279 Fisher exact test and t-test were used also to compare the characteristics of TD and
280 NO-TD groups, considering $p < 0.05$ as significance level.

281 **RESULTS**

282 **Patients**

283 The date of first ALTZ treatment ranged from 2002 to 2012 (median 2008), and
284 the mean \pm SD follow-up was 9.0 ± 2.5 years post-ALTZ (range: 4.3 – 14.0 years). The
285 TD group comprised patients showing post-ALTZ hyperthyroidism (n=19) or
286 hypothyroidism (n=12) as first clinical manifestation (TD onset).

287 Table 1 summarizes the characteristics of TD and NO-TD groups; no significant
288 differences were detected between the different groups. Before TD onset (time-points A
289 and B) all patients were euthyroid and free of persistent thyroid function abnormalities.
290 Note that at time-point C (TD group) many patients who developed thyroid dysfunction
291 were already on thyroid medication: a detailed description of their treatments and
292 outcomes has been reported elsewhere (10).

293 **Combined in-house TRAb (ihTRAb) results at all time-points**

294 We compared the overall results obtained with the three different ihTRAb assays
295 (FC-TRAb, LB-TSAb, LB-TBAb) at all time-points in TD and NO-TD groups. Due to the
296 retrospective nature of this study, sera from some time-points were unavailable for the TD

297 group (Table 1). As shown in Figure 2, at time-point A (before ALTZ) 5/16 (31.2%) TD
298 patients were found to be ihTRAb positive (ihTRAb+), compared with 0/14 (0%) NO-TD
299 patients (p=0.017). Following ALTZ, 6/25 (24.0%) TD patients were ihTRAb+ at time-
300 point B (before TD onset); as expected, at time-point C (during or after TD onset)
301 ihTRAb+ cases markedly increased to 18/29 (62.1%). This prevalence is likely to be
302 underestimated, considering the late average collection time of time-point C compared
303 with disease onset (Table 1). Among NO-TD patients, 4/14 (28.6%) were ihTRAb+ at
304 time-point B. When splitting the overall ihTRAb+ results according to the TD subtype at
305 onset (hyperthyroidism or hypothyroidism), time-point A ihTRAb were predominantly
306 positive in those who subsequently developed hypothyroidism (4/6: 66.7%) rather than
307 hyperthyroidism (1/10: 10%), p=0.036. It is worth noting that two initially hypothyroid
308 ihTRAb+ patients subsequently showed a fluctuating thyroid function and were classified
309 as fluctuating GD.

310 **Time-point A: predictors of post-ALTZ TD**

311 To validate TRAb as an independent predictor of ALTZ-induced TD, we
312 compared ihTRAb results with autTRAb and TPOAb data at time-point A (Table 2). Pre-
313 ALTZ ihTRAb and TPOAb had a very similar predictive value for future TD
314 development. When ihTRAb and TPOAb testing were combined together, 7/16 (43.8%)
315 TD patients were positive, versus 0/14 of NO-TD group (p=0.007); in particular 83.3%
316 hypothyroid and 20% hyperthyroid cases were predicted, versus 50% and 20%
317 respectively when considering TPOAb alone (Table 2, last two columns). Detailed TRAb
318 and/or TPOAb predictive values, sensitivity and specificity have been reported in
319 supplemental Table 2.

320 Considering this from a different perspective, TD developed in 7/7 (100%)
321 baseline ihTRAb and/or TPOAb positive patients, versus 9/23 (39.1%) baseline ihTRAb
322 and/or TPOAb negative patients ($p=0.007$).

323 AutTRAb were positive before ALTZ in only 1 patient of the TD hyperthyroid
324 subgroup (14.3%) and none in the hypothyroid group, suggesting that autTRAb do not
325 appear to be a useful predictive marker of subsequent TD development.

326 **In depth analysis of ihTRAb+ cases**

327 Table 3 reports in more detail the ihTRAb+ cases only, describing the different
328 ihTRAb subtypes in comparison with autTRAb, TPOAb, and TSH results, when
329 available. Here the hyperthyroid group was further subdivided into classic hyperthyroid
330 GD and fluctuating GD. At time-point A ihTRAb+ cases as expected were predominantly
331 TNAb (3/5: 60%), defined as FC-TRAb+ but both LB-TSAb/TBAb negative (Table 3).

332 At time-point B, ihTRAb+ cases were represented by a similar proportion of
333 TNAb, TSAb and TBAb (Table 3). In combination, TNAb or TSAb/TBAb preceded TD
334 onset in 9 cases (32.1%, considering a total of 28 TD patients with time-point A and/or B
335 available) with an interval before TD onset of a median of 1.2 years (range 28 days – 7.3
336 years).

337 At time-point C (Table 3), as expected all ihTRAb+ hyperthyroid GD and
338 fluctuating GD patients were also autTRAb+, confirming a GD diagnosis. Among
339 ihTRAb+ hyperthyroid and fluctuating GD cases, FC-TRAb was the most sensitive assay
340 with 13/14 (92.9%) positive, versus 9/14 (64.3%) of TSAb. TBAb were positive in 3/10
341 (30%) purely hyperthyroid ihTRAb+ GD patients. As expected fluctuating GD cases had
342 a documented TSAb/TBAb coexistence in 2/4 (50%) cases (IDs 35, 42); the other two
343 cases were positive for TSAb only (IDs 1, 7).

344 Among the whole hypothyroid group (n=10), 4 (40%) were ihTRAb+ at time-point C, in
345 particular 2/4 (50%) FC-TRAb+ and 2/4 (50%) both FC-TRAb+ and TBAb+; autTRAb
346 results were concordant (Table 3). Surprisingly, both ihTRAb+ hypothyroid patients at
347 time-point A (IDs 15, 37) resulted ihTRAb negative at time-point C.

348 **Final thyroid diagnosis**

349 TPOAb titres measured anytime post-ALTZ were available in 17/19 (89.5%) of
350 hyperthyroid group, and were positive in 15/17 (88.2%) cases; in fact two TPOAb
351 negative GD patients at time-point C (Table 3) later became TPOAb positive, for example
352 ID 27. Anytime post-ALTZ, TPOAb were positive in 11/12 (91.7%) of hypothyroid
353 patients. None of the 14 NO-TD patients developed post-ALTZ TPOAb.

354 According to their clinical course, TD patients were classified as pure
355 hyperthyroid GD (n=17), fluctuating GD (n=4), and hypothyroid patients (n=10).
356 AutTRAb were positive in 17/17 (100%) GD and 4/4 (100%) fluctuating GD. Taking our
357 ihTRAb results into account to better define the final thyroid diagnosis among the 31 TD
358 patients according to the criteria given in the methods identified 17 (54.8%) GD, 4
359 (12.9%) fluctuating GD (2 started with hypothyroidism, 2 with hyperthyroidism), 4
360 (12.9%) TRAb+ hypothyroidism, 3 (9.7%) chronic autoimmune thyroiditis, 2 (6.5%)
361 TPOAb+ subacute thyroiditis, 1 (3.2%) TPOAb-/TRAb- hypothyroidism.

362 **DISCUSSION**

363 We have described for the first time the biological function of TRAb in a
364 longitudinal cohort of patients developing ALTZ-induced thyroid dysfunction (TD) using
365 three different in-house TRAb assays (ihTRAb). Importantly, as a result of a structured
366 monitoring and sampling process for patients with MS in south Wales and suitable for
367 ALTZ treatment, serum was available before the onset of TD enabling us to describe how

368 and when TRAb become positive in patients with ALTZ-induced TD. This setting is
369 unique, as serum is not generally available before disease onset in sporadic GD.
370 Interestingly, serum ihTRAb, but not TRAb detected with standard automated assays
371 (autTRAb), were detected before ALTZ in one third of patients who later developed TD,
372 and in none of those who remained free of TD (NO-TD) over a minimum follow-up
373 period of 5 years. The appearance of ihTRAb was detected a mean of 1.2 years (range 28
374 days – 7.3 years) prior to the development of thyroid dysfunction. We believe this is the
375 first report of the detection of TRAb prior to the onset of ALTZ-induced TD. Similar
376 findings have been previously described for spontaneous TD in a retrospective study
377 showing progressively increasing TRAb positivity, as well as TPOAb and anti-
378 thyroglobulin antibodies, in patients who will later develop GD. In particular TRAb
379 positivity increased from 2% at 7 years before diagnosis to 55% at diagnosis, with
380 intermediate percentages of 7% and 20% at -5 and -2 years, respectively (38).

381 Furthermore, in our study for the first time we provided details about TRAb
382 biological function over time. We have previously reported the presence neutral TRAb
383 (TNAAb), detected using flow cytometry, in healthy euthyroid subjects, but without any
384 follow-up clinical data to indicate whether they later did develop TD (19). Information
385 similar to our data in ALTZ-induced disease are difficult to collect in the setting of
386 spontaneous autoimmune TD, requiring very large and long-term cohort studies. The fact
387 that the rates of TD post ALTZ are much higher than generally seen in MS, suggests that
388 the two settings are not necessarily comparable, however the principle that autoimmunity
389 to the TSHR may precede TD by many months or years applies to both ALTZ-induced
390 (this study) and spontaneous forms as reported by others (38). Note that the wide range of
391 pre-TD intervals (28 days – 7.3 years) is partly a consequence of the retrospective nature
392 of this study, not providing systematic and identical time-points for all patients. Future

393 prospective studies are needed to precisely define how long ihTRAb may precede the
394 onset of TD in some cases.

395 In cases of TRAb positivity pre-dating TD, we hypothesize that TSHR-reactive B
396 cell clones may undergo progressive antigen-driven affinity maturation by somatic
397 hypermutation within germinal centres, and finally generate high affinity stimulating
398 (TSAb) or blocking (TBAb) TRAb. The phenomenon of multiple different pathogenic
399 TRAb arising from single B cell clones by somatic hypermutation has already been
400 described in mouse models of GD (30,31). In this context, our finding that ihTRAb more
401 commonly preceded hypo- than hyperthyroidism is interesting, but may reflect that once a
402 stimulatory TSAb-secreting clone develops, TD follows rapidly whereas it may take
403 longer for TBAb to achieve clinically relevant inhibition of thyroid function such that
404 TSH levels rise. Our observations are in accordance with previous evidence that TSAb are
405 potent at low concentrations, therefore inducing hyperthyroidism rapidly after their
406 appearance (23), while TBAb levels needed to trigger hypothyroidism are usually much
407 higher than TSAb levels inducing hyperthyroidism (26). Further prospective studies with
408 large numbers of subjects should clarify this. It also has to be mentioned that we did not
409 sub-classify TD patients into subclinical and overt disease since the vast majority of
410 patients diagnosed with subclinical disease went on to develop overt thyroid dysfunction,
411 or were treated immediately after diagnosis, preventing the possible evolution to overt
412 disease.

413 Although it is understandable that TNAb can exist without altering thyroid
414 function, it is less clear how this is possible with TSAb and TBAb. Possible explanations
415 for TSAb/TBAb positive cases in euthyroid patients are: i) they are low-affinity, therefore
416 not able to exert a significant function on TSHR activity with clinical consequence; ii) the
417 *in vitro* assays in some cases do not reflect the different and more complex human thyroid

418 environment, providing slightly different results from *in vivo*. For example luciferase
419 bioassays use bovine and not human TSH, CHO cells instead of human thyrocytes, and
420 only the cAMP pathway is investigated. The same observations about TNAb or low-
421 affinity TSAb/TBAb apply to 28.6% euthyroid NO-TD patients developing post-ALTZ
422 TRAb; in the future they might remain positive with no long-term clinical consequences,
423 or might develop late onset TD.

424 TD post ALTZ is often delayed by several years and the ability to reliably predict
425 those at risk would allow targeted monitoring and possibly early intervention or
426 prevention. TPOAb are already known to identify subjects at risk, with 69% of
427 individuals TPOAb+ at baseline developing TD. However, TPOAb testing only detects
428 around 15% of all future cases of TD post-ALTZ (9). Our data suggests that custom-made
429 TRAb testing in combination with TPOAb testing at baseline might increase this to
430 predicting around 20% of hyperthyroid cases and 80% of hypothyroid cases. Interestingly,
431 in 2 hypothyroid patients pre-ALTZ ihTRAb positivity was no longer detectable at the
432 time of disease onset, suggesting that TRAb titres fluctuate over time and are not always
433 detectable. Furthermore, in these cases they also might have become spontaneously
434 negative, and destructive thyroiditis might represent the sole mechanism of
435 hypothyroidism.

436 The analysis of ihTRAb proved less valuable for predicting the disease course
437 after the onset of TD than expected. For example, not all subjects who developed
438 hypothyroidism or GD with fluctuating course had detectable TBAb. Several explanations
439 are possible: i) non-optimal timing of time-point C, often several months after the disease
440 onset and the commencing of anti-thyroid treatments, usually associated with TRAb titres
441 decrease and negativization; ii) TSAb/TBAb levels might fluctuate over time, not being
442 always positive at the same time; iii) TBAb might interact with the TSHR with a lower

443 affinity compared with TSAb, and therefore might be masked by TSAb coexistence in our
444 biological assays. Similarly, TBAb false positive cases have been described due to the
445 concomitant presence of TSAb; if TSAb act as weak agonists, they interfere with the
446 bTSH in the TBAb assay resulting in a signal reduction. In general, TSAb/TBAb
447 coexistence can be challenging to demonstrate due to their mutual interference, depending
448 on relative concentrations, affinities and potencies, varying over time. Sometimes serum
449 serial dilutions are needed to properly distinguish between the two TRAb populations
450 (26).

451 However our findings show that around 40% of hypothyroidism post ALTZ is
452 TBAb mediated, as suggested in previous studies (9,10); this is nonetheless substantially
453 higher than reports in spontaneous disease (around 10%) (16,21). By contrast, 91.7%
454 (11/12) of hypothyroid subjects were TPOAb positive, consistent with TBAb negative
455 hypothyroidism post-ALTZ still being autoimmune in the majority of cases, but perhaps
456 cell-mediated. However, it was notable that autTRAb were detectable in many subjects
457 who developed hypothyroidism or a switching course as well as all those with
458 hyperthyroidism. Currently, autTRAb measurement is recommended only in patients
459 developing hyperthyroidism; if our observations are confirmed in larger prospective
460 studies, autTRAb testing should probably be extended to all cases of post-ALTZ TD,
461 including hypothyroidism, since they appear to predict a more complex clinical course
462 (i.e. possibility of thyroid function switching) requiring close observation.

463 The strength of our study is the long follow-up to define outcome (≥ 5 years where no
464 TD is reported) and the wide range of thyroid autoantibody assays used. However, ALTZ
465 has only recently been licensed for use in relapsing/remitting MS (since 2014) and hence
466 ALTZ-induced TD is currently not very common, especially cases with the long follow-
467 up required to define outcome. As a result, our cohort is relatively small (n=45) and this is

468 a limitation. Furthermore, due to the retrospective nature of the study, serum was not
469 available at all time-points in the whole cohort, and in particular, samples at the time of
470 TD onset were not always available. However we believe our finding that TRAb can
471 precede disease onset and are associated with subsequent TD is robust as our numbers
472 were consistent with our *a priori* power calculations.

473

474 In conclusion we have observed that TRAb can precede TD by many years and, if
475 present before ALTZ, can increase the risk of subsequent development of TD. Future
476 prospective studies are needed to determine the exact value of baseline and follow-up
477 TRAb testing in subjects treated with ALTZ and the most valuable assay to use. Such
478 studies, as well as large cohort studies in spontaneous thyroid autoimmunity may also be
479 used to investigate and define the process of affinity maturation in TRAb further. Now
480 that ALTZ is licensed for the treatment of relapsing/remitting MS in more than 60
481 countries, the available case load for prospective studies is likely to substantially increase
482 and make at least the studies in ALTZ induced disease feasible.

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492

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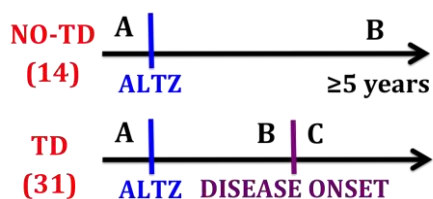
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677

678 FIGURES

679

680 **Figure 1: Serum time-points**



681

682 ALTZ: first alemtuzumab treatment. TD: patients developing thyroid dysfunction. NO-

683 TD: patients not developing thyroid dysfunction.

684 A: first available time before ALTZ (both TD and NO-TD). B: post ALTZ, first available

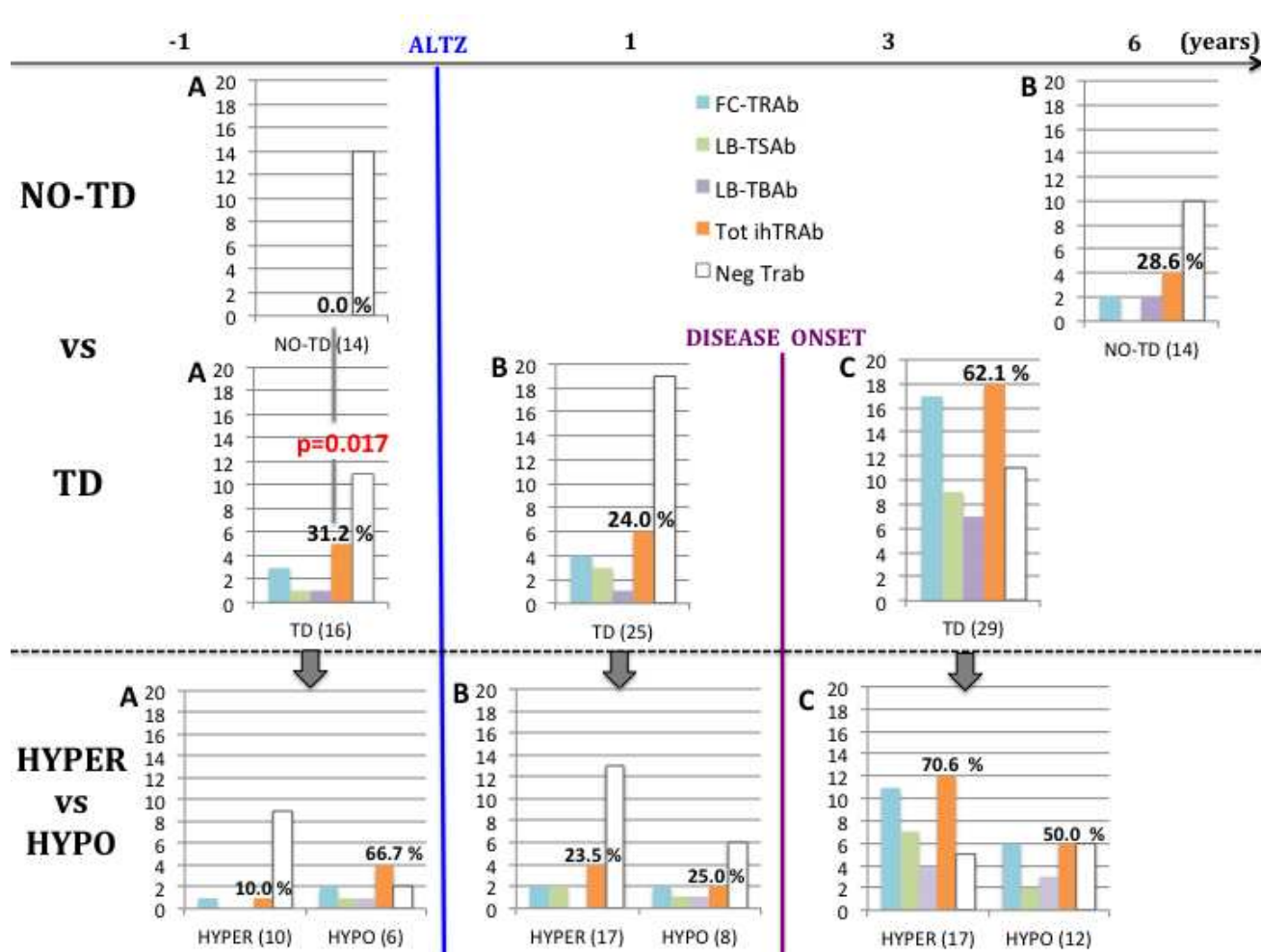
685 time before the onset of thyroid dysfunction (TD) or the latest available time post-ALTZ

686 (NO-TD). C: post ALTZ during the onset of thyroid function abnormalities, or in

687 alternative the earliest available time after it (TD only).

688

689 **Figure 2: ihTRAb positive results at all time-points**



690

691

692 Cross-sectional results of all available sera at pre-specified time-points (see Figure 1)
693 analysed with in-house assays to detect autoantibodies to the thyrotropin receptor
694 (ihTRAb), obtained in patients developing thyroid dysfunction (TD) and patients not
695 developing any thyroid dysfunction (NO-TD). Below the dashed line TD patients were
696 further sub-grouped into hyperthyroidism (HYPER) or hypothyroidism (HYPO) as first
697 clinical manifestation. Numbers in brackets indicate the total number of available sera for
698 each time-point and patient subgroup.

699 A: time-point A = before the first treatment with alemtuzumab (ALTZ). B: time-point B =
700 latest available time post-ALTZ and before TD onset, when applicable. C: time-point C
701 (TD only) = post ALTZ during the onset of thyroid function abnormalities, or in
702 alternative the earliest available time after it.

703 FC-TRAb (azure) = TRAb detected by flow cytometry. LB-TSAb (green) = Stimulating
704 TRAb detected by luciferase bioassays. LB-TBAb (purple) = Blocking TRAb detected by
705 luciferase bioassays. Tot ihTRAb (orange) = positive FC-TRAb and/or LB-TSAb and/or
706 LB-TBAb; percentages refer to this column. Neg TRAb (white) = ihTRAb negative
707 results with all the three FC, LB-TSAb and LB-TBAb techniques.
708

709 **TABLES**

710

711 **Table 1: Patients' characteristics**

		TD			NO-TD (n=14)
		1 st manifestation: Hyperthyroidism (n=19)	1 st manifestation: Hypothyroidism (n=12)	Overall (n=31)	
Female: n (%)		15 (78.9%)	8 (66.7%)	23 (74.2%)	10 (71.4%)
Age (years) at 1 st ALTZ: mean (SD)		32.0 (7.2)	37.0 (10.6)	33.8 (8.9)	35.0 (9.5)
Tot n ALTZ treatments received: mean (SD)		1.8 (0.7) ¹	2.2 (1.0) ¹	2.0 (0.8) ¹	2.8 (0.8) ²
years from 1 st ALTZ to TD onset: mean (SD), median (range)		3.1 (2.2) 2.0 (1.0-8.7)	3.2 (2.1) 3.0 (0.8-7.3)	3.0 (1.9) 2.7 (0.8-8.7)	NA
Time-point A ³ : median (range)	days before 1 st ALTZ	171.5 (26-700)	141 (0-418)	162.0 (0-700)	121.5 (0-375)
	years after 1 st ALTZ	1.4 (0.3-7.8)	2.5 (0.6-6.6)	1.63 (0.3-7.8)	6.2 (5.1-8.1)
Time-point B ⁴ : median (range)	days before TD onset	192.0 (56-680)	179 (28-583)	192.0 (28-680)	NA
	years after 1 st ALTZ	3.1 (1.3-8.8)	3.4 (0.8-9.0)	3.1 (0.8-9.0)	NA
Time-point C ⁵ : median (range)	days after TD onset	89.0 (0-794)	82.5 (0-829)	89.0 (0-829)	NA

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713 ALTZ = alemtuzumab treatment. n = number. NA = Not Applicable. TD = thyroid dysfunction (abnormal thyroid hormones). TD onset = time of the
714 first TD defined as persistent (i.e. detectable in consecutive blood tests at least 3 months apart) and/or significant (i.e. requiring to immediate start a
715 thyroid treatment).

716 Fisher exact test (gender distribution) and t-test (other variables) excluded significant differences between the groups, when comparable (p= ns).

717 ¹= until TD onset

718 ²= until the end of observational period (time-point B)

719 ³= Time-point A serum was available in 10 hyperthyroid, 6 hypothyroid (16 overall TD) and 14 NO-TD patients.

720 ⁴= Time-point B serum was available in 17 hyperthyroid, 8 hypothyroid (25 overall TD) and 14 NO-TD patients.

721 ⁵= Time-point C serum was available in 17 hyperthyroid, 12 hypothyroid (29 overall TD) patients.

Table 2: Time-point A: Predictive value of baseline TRAb versus TPOAb

	No of Patients	FC TRAb	LB TSAb	LB TBAb	Aut TRAb	TPOAb	TPOAb and/or TRAb
HYPERTHYROID	1						
	1				NA		
	2				NA		
	6						
	Tot: 10	1/10 (10%)	0/10 (0%)	0/10 (0%)	1/7 (14.3%)	2/10 (20%)	2/10 (20%)
HYPOTHYROID	1				NA		
	1						
	1*				NA		
	1*						
	1						
	1						
	Tot: 6	2/6 (33.3%)	1/6 (16.7%)	1/6 (16.7%)	0/4 (0%)	3/6 (50%)	5/6 (83.3%)
NO-TD	4				NA		
	10						
	Tot: 14	0/14 (0%)	0/14 (0%)	0/14 (0%)	0/10 (0%)	0/14 (0%)	0/14 (0%)

Cross-sectional results of all available sera at time-point A (before alemtuzumab treatment; see Figure 1) for autoantibodies to the thyrotropin receptor (TRAb) and autoantibodies to thyroid peroxidase (TPOAb), obtained in patients developing subsequent hyperthyroidism or hypothyroidism as first clinical manifestation, and patients not developing any thyroid dysfunction (NO-TD) following alemtuzumab treatment.

White cells = negative TRAb/TPOAb results. Colored squares represent positive results: azure = TRAb detected by flow cytometry (FC-TRAb); green = stimulating TRAb detected by luciferase bioassays (LB-TSAb); purple = blocking TRAb detected by luciferase bioassays (LB-TBAb); grey = TRAb detected by automated systems (Aut-TRAb); yellow = TPOAb (automated assay); red = TRAb (any test) and/or TPOAb. NA = Not Available.

* Fluctuating Graves' disease (GD) presenting hypothyroidism as first clinical manifestation.

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Table 3: TRAb, TPOAb and TSH status in patients with positive in-house TRAb assays (ihTRAb+)

Hyperthyroid patients (GD)

ID	Time-point A: Pre-ALTZ						Time-point B: Last euthyroid time						Time-point C: Post thyroid dysfunction							
	TSH (mU/L)	FC TRAb	LB TSAb	LB TBAb	Aut TRAb	TPO Ab	Time α (d)	TSH (mU/L)	FC TRAb	LB TSAb	LB TBAb	Aut TRAb	TPO Ab	Time β (d)	TSH (mU/L)	FC TRAb	LB TSAb	LB TBAb	Aut TRAb	TPO Ab
8	1.05	0.33 ²	0.99	-8.58	2.2	80 ^A	159	1.34	0.45 ²	0.98	-12.98	1.9	225 ^A	238	< 0.01	0.30 ²	1.38	8.56	>40	66 ^B
4	1.86	0.15 ¹	1.12	-7.95	NA	<2 ^B	105	0.27	0.24 ³	1.54	-4.72	<1	58 ^A	35	< 0.01	0.57 ¹	4.24	3.52	>40	<2 ^B
5	0.96	0.28 ²	0.92	-28.85	<1	<2 ^A	418	1.01	0.49 ²	1.12	-8.40	<1	NA	0	< 0.01	0.44 ²	0.76	21.03	12.0	403 ^B
2	0.4	0.06 ¹	1.34	-22.75	NA	<2 ^B	387	^	NA	1.00	-11.74	0.3	NA	166	#	0.49 ¹	0.92	16.26	38.0	352 ^B
6	1.02	0.18 ²	0.87	-0.76	<1	18 ^A	302	1.27	0.13 ²	0.75	4.71	0.6	<2 ^B	22	< 0.01	0.75 ²	2.78	0.19	17.6	14 ^B
27	2.37	0.21 ³	1.10	4.57	0.3	<2 ^B	NA	NA	NA	NA	NA	NA	NA	182	3.87	0.31 ³	1.12	26.84	2.4	9 ^B
40	NA	NA	NA	NA	NA	NA	334	0.86	0.28 ⁵	1.65	-13.88	1.7	13 ^A	37	< 0.01	0.53 ⁵	3.96	-21.73	33.6	2 ^B
36	NA	NA	NA	NA	NA	NA	680	1.45	0.06 ⁵	1.46	-6.20	0.3	56 ^A	0	< 0.01	0.65 ⁵	3.04	-22.92	>40	22 ^B
38	NA	NA	NA	NA	NA	NA	142	4.10	0.11 ⁴	0.78	-48.12	0.5	36 ^A	0	< 0.01	0.34 ⁴	1.32	-77.57	6.9	124 ^A
41	NA	NA	NA	NA	NA	NA	294	1.00	0.02 ⁴	1.15	-43.97	<1	5 ^B	177	< 0.01	0.87 ⁴	2.97	48.78	>40	728 ^A

739 ID 2: missing TSH values for both time-points B (^) and C (#), so the closest TSH results are provided:

740 ^ previous TSH= 0.36 mU/L (233 days before); next TSH <0.02 mU/L (202 days after, during a transient subclinical hyperthyroidism phase lasted <3 months).

741 # previous TSH <0.02 mU/L (166 days before, same day of thyroid dysfunction onset); next TSH= 17.81 mU/L (55 days after, during carbimazole treatment).

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Fluctuating GD

ID	Time-point A: Pre-ALTZ						Time-point B: Last euthyroid time						Time-point C: Post thyroid dysfunction							
	TSH (mU/L)	FC TRAb	LB TSAb	LB TBAb	Aut TRAb	TPO Ab	Time α (d)	TSH (mU/L)	FC TRAb	LB TSAb	LB TBAb	Aut TRAb	TPO Ab	Time β (d)	TSH (mU/L)	FC TRAb	LB TSAb	LB TBAb	Aut TRAb	TPO Ab
1	1.40	0.16 ¹	0.6	32.58	<1	26 ^A	118	0.53	0.18 ²	0.95	-20.00	<1	31 ^A	829	1.76	0.38 ¹	2.46	-16.31	>40	491 ^A
35	2.14	0.23 ⁴	0.88	8.98	<1	3 ^B	583	□	0.23 ⁴	1.19	-13.41	0.6	NA	0	159.68	0.76 ⁴	3.34	38.02	>40	442 ^B
7	0.89	0.05 ²	1.21	-5.97	<1	44 ^A	441	0.34	0.09 ²	1.02	4.57	NA	<2 ^B	196	69.25	0.68 ²	3.46	11.93	>40	82 ^B
42	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	197	52.15	0.78 ⁴	2.16	40.90	>40	>1300 ^A

744 □ TSH not available. Previous TSH = 1.96 mU/L (182 days before); next TSH = 159.68 mU/L (583 days after, corresponding to time-point C, same day of thyroid dysfunction onset).

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748 **Hypothyroid patients**

ID	Time-point A: Pre-ALTZ						Time-point B: Last euthyroid time						Time-point C: Post thyroid dysfunction							
	TSH (mU/L)	FC TRAb	LB TSAb	LB TBAb	Aut TRAb	TPO Ab	Time α (d)	TSH (mU/L)	FC TRAb	LB TSAb	LB TBAb	Aut TRAb	TPO Ab	Time β (d)	TSH (mU/L)	FC TRAb	LB TSAb	LB TBAb	Aut TRAb	TPO Ab
15	2.34	0.26 ¹	0.72	-1.44	0.6	688 ^A	NA	NA	NA	NA	NA	NA	NA	0	6.58	0.10 ¹	1.10	15.79	NA	377 ^A
37	2.26	0.09 ⁴	1.54	-31.97	NA	68 ^A	337	NA	0.07 ⁴	1.63	-9.95	NA	NA	6	35.46	0.10 ⁴	0.63	-12.25	<1	>1300 ^A
44	1.34	0.07 ⁴	1.09	7.16	<1	21 ^B	NA	NA	NA	NA	NA	NA	NA	0	9.18	0.25 ⁴	0.86	-6.08	NA	377 ^B
33	NA	NA	NA	NA	NA	NA	28	0.18	0.87 ⁵	2.33	66.27	NA	>1300 ^A	159	5.39	0.68 ⁵	1.21	21.56	>40	>1300 ^A
31	NA	NA	NA	NA	NA	NA	203	1.49	0.10 ⁵	1.26	-7.90	0.8	672 ^A	672	*	0.08 ⁵	0.94	2.44	NA	>1000 ^B
29	NA	NA	NA	NA	NA	NA	210	1.34	0.24 ⁵	1.17	-27.18	0.5	37 ^A	224	5.26	0.90 ⁵	1.21	53.82	>40	496 ^A
32	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	0	80.42	0.78 ⁵	0.99	-11.59	20.7	>1300 ^A

749 * TSH not available, but likely within the normal range, considering the evidence of stable euthyroidism under levothyroxine treatment for nearly 4 years post-ALTZ.
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751 **NO-TD patients**

ID	Time-point A: Pre-ALTZ						Time-point B: Latest time post-ALTZ					
	TSH (mU/L)	FC TRAb	LB TSAb	LB TBAb	Aut TRAb	TPO Ab	TSH (mU/L)	FC TRAb	LB TSAb	LB TBAb	Aut TRAb	TPO Ab
13	1.63	0.15 ¹	1.13	13.88	0.85	<10 ^A	1.84	0.05 ¹	1.03	30.92	0.3	<2 ^B
25	1.30	0.23 ³	0.82	8.99	<1	<2 ^B	0.90	0.19 ³	1.15	26.62	<0.9	<2 ^B
17	0.82	0.21 ²	1.04	0.58	<1	<2 ^B	1.54	0.39 ²	1.08	-2.39	<0.9	<2 ^B
19	1.22	0.25 ⁵	0.75	-2.13	NA	<2 ^B	★	0.33 ⁵	0.88	-1.84	<0.9	<2 ^B

752 ★ TSH not available, but likely within the normal range, considering the evidence of stable euthyroidism for 5.9 years post-ALTZ.
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754 Summary of autoantibodies to the thyrotropin receptor (TRAb), autoantibodies to thyroid peroxidase (TPOAb) and thyroid
755 stimulating hormone (TSH) status among patients positive for in-house TRAb assays (ihTRAb+). Only patients resulted ihTRAb+ in
756 at least one time-point are represented.

757 White cells = negative TRAb/TPOAb results. Colored squares represent positive results: azure = TRAb detected by flow cytometry
758 (FC-TRAb); green = stimulating TRAb detected by luciferase bioassays (LB-TSAb); purple = blocking TRAb detected by luciferase
759 bioassays (LB-TBAb); grey = TRAb detected by automated systems (AutTRAb); yellow = TPOAb (automated assay).

760 ALTZ = Alemtuzumab. GD = Graves' disease. ID = patient's identification number. NA = Not Applicable or Not Available.

761 TSH normal reference range varies between 0.30 – 4.4 mU/L and 0.35 – 5.5 mU/L, depending on the assay used and the date of test
762 (see supplemental table 1).
763 α = Time (days) before onset of thyroid dysfunction
764 β = Time (days) after onset of thyroid dysfunction
765 Reference Ranges and Positivity Cut-offs
766 AutTRAb (IU/L) reference ranges: negative <1, borderline 1–1.5, positive >1.5 (until January 2014); negative <0.9, borderline
767 0.9–1.6, positive (from February 2014 onwards)
768 A,B = TPOAb (U/ml) reference ranges: A = negative <60, positive ≥ 60 (until May 2010); B = negative <6, positive ≥ 6 (from
769 June 2010 onwards)
770 FC-TRAb positivity cut-offs. Samples have been tested in 5 different sets of experiments, each producing slightly different
771 mean of greatest differences in fluorescence intensity between the two histograms (D) and relative standard deviation (SD) among
772 pooled controls. Samples were considered positive if $D_{\text{sample}} > D_{\text{controls}} + 2 \text{SD}$.
773 $^1 = \text{Set 1 } D_{\text{controls}} + 2 \text{SD} = 0.26$
774 $^2 = \text{Set 2 } D_{\text{controls}} + 2 \text{SD} = 0.30$
775 $^3 = \text{Set 3 } D_{\text{controls}} + 2 \text{SD} = 0.42$
776 $^4 = \text{Set 4 } D_{\text{controls}} + 2 \text{SD} = 0.21$
777 $^5 = \text{Set 5 } D_{\text{controls}} + 2 \text{SD} = 0.32$
778 LB-TSAb positive if stimulation index (SI) >1.5.
779 LB-TBAb (%) positive if inhibition index (InI) >20%.
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SUPPLEMENTAL MATERIAL

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Supplemental Table 1: Automated Laboratory Assays and Reference Ranges for TPOAb, FT4, FT3 and TSH

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TIME PERIOD	ASSAY	REFERENCE RANGES			
		TPOAb (U/mL)	TSH (mU/L)	FT4 (pmol/L)	FT3 (pmol/L)
From 1/1/2006 To 31/5/2010	Siemens ADVIA Centaur	<60	0.35 – 5.5	10.0 – 25.0 (from 1/1/2006) 9.8 – 23.1 (from 21/5/2009)	3.5 – 6.5
From 01/06/2010 To current	Abbott Architect	<6	0.30 – 4.40 0.35 – 5.0 (from 29/6/10) 0.30 – 4.4 (from 31/1/2014)	9.0 – 19.1 9.2 – 21.0 (from 31/1/2014) 9.0 – 19.1 (from 5/11/2014)	2.6 – 5.7

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TSH = thyroid stimulating hormone. FT4 = free-thyroxine. FT3 = free-triiodothyronine.

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Supplemental Table 2: TRAb and TPOAb predictive values, sensitivity and specificity at time-point A

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		Sensitivity	PPV	Specificity	NPV
TD versus NO-TD	ihTRAb	5/16 (31.2%)	5/5 (100%)	14/14 (100%)	14/25 (56.0%)
	TPOAb	5/16 (31.2%)	5/5 (100%)	14/14 (100%)	14/25 (56.0%)
	ihTRAb and/or TPOAb	7/16 (43.8%)	7/7 (100%)	14/14 (100%)	14/23 (60.9%)
HYPER versus NO-TD	ihTRAb	1/10 (10.0%)	1/1 (100%)	14/14 (100%)	14/23 (60.9%)
	TPOAb	2/10 (20.0%)	2/2 (100%)	14/14 (100%)	14/22 (63.6%)
	ihTRAb and/or TPOAb	2/10 (20.0%)	2/2 (100%)	14/14 (100%)	14/22 (63.6%)
HYPO versus NO-TD	ihTRAb	4/6 (66.7%)	4/4 (100%)	14/14 (100%)	14/16 (87.5%)
	TPOAb	2/10 (50.0%)	3/3 (100%)	14/14 (100%)	14/17 (82.3%)
	ihTRAb and/or TPOAb	5/6 (83.3%)	5/5 (100%)	14/14 (100%)	14/15 (93.3%)

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TD = thyroid dysfunction; NO-TD = absence of thyroid dysfunction; HYPER = hyperthyroidism; HYPO = hypothyroidism; ihTRAb = TRAb measured with in-house assays; PPV = positive predictive value; NPV = negative predictive value.

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