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1 **RESIDUAL ADRENAL FUNCTION IN AUTOIMMUNE**
2 **ADDISON’S DISEASE – EFFECT OF DUAL THERAPY WITH**
3 **RITUXIMAB AND DEPOT TETRACOSACTIDE**

4
5 Catherine Napier¹, Earn H Gan¹, Anna L Mitchell¹, Lorna C Gilligan², D Aled
6 Rees³, Carla Moran⁴, Krishna Chatterjee⁴, Bijay Vaidya⁵, R Andrew James¹,
7 Yaasir Mamoojee¹, Simon Ashwell⁶, Wiebke Arlt^{2,7}, Simon HS Pearce¹
8
9

10
11 **Affiliations:**

12 1 Institute of Genetic Medicine, International Centre for Life, Newcastle University,
13 Newcastle upon Tyne, NE1 3BZ and Newcastle upon Tyne Hospitals, Queen Victoria Road,
14 NE1 4LP, UK.
15

16 2 Institute of Metabolism and Systems Research (IMSR), University of Birmingham,
17 Birmingham, B15 2TT, UK
18

19 3 Neuroscience and Mental Health Research Institute, Cardiff University, Cardiff, CF24 4HQ
20

21 4 University of Cambridge Metabolic Research Laboratories, Wellcome Trust-MRC, Institute
22 of Metabolic Science, Addenbrooke’s Hospital, Cambridge CB2 0QQ, UK.
23

24 5 Royal Devon & Exeter Hospital, University of Exeter Medical School, Exeter EX2 5DW,
25 UK.
26

27 6 The James Cook University Hospital, Marton Road, Middlesbrough, TS4 3BW, UK.
28

29 7 NIHR Birmingham Biomedical Research Centre, University of Birmingham and University
30 Hospitals Birmingham NHS Foundation Trust, Birmingham, B15 2GW, UK
31
32
33

34 **Address for Correspondence:**

35
36 Dr. Catherine Napier,
37 Endocrine Unit,
38 Leazes Wing,
39 Royal Victoria Infirmary,
40 Newcastle upon Tyne Hospitals,
41 Queen Victoria Road,
42 NE1 4LP, UK
43

44 Tel: (+44) 191 2820590

45 Email: Catherine.Napier@nuth.nhs.uk
46

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48 **ABSTRACT**

49 **CONTEXT** In autoimmune Addison's disease (AAD), exogenous glucocorticoid (GC) therapy is an
50 imperfect substitute for physiological GC secretion. Patients on long-term steroid replacement have
51 increased morbidity, reduced life expectancy and poorer quality of life.

52 **OBJECTIVE** To restore adrenocortical steroidogenic function in recent onset AAD.

53 **DESIGN** Open-label, multi-centre trial of immunotherapy and trophic stimulation in new-onset
54 AAD. Serial measurement of serum and urine corticosteroids at baseline and throughout 72-week
55 follow-up period.

56 **SETTING** Endocrine Departments and Clinical Research Facilities at 5 UK tertiary centres.

57 **PATIENTS** Thirteen subjects (9 female, 4 male; aged 19-64 years) with AAD confirmed by high
58 ACTH, low circulating cortisol (basal <100nmol/L or post-tetracosactide <300nmol/L) and positive
59 serum 21-hydroxylase antibodies.

60 **INTERVENTION** All subjects received dual therapy with B-lymphocyte depleting immunotherapy
61 (rituximab 1g given twice) and repeated depot tetracosactide (1mg alternate days for 12 weeks).

62 **MAIN OUTCOME MEASURE** Restoration of normal glucocorticoid secretion (stimulated
63 cortisol >550nmol/L) at Week 48.

64 **RESULTS** Ten of 13 (77%) had detectable stimulated serum cortisol (26-265nmol/L) at trial entry.
65 Following intervention, 7/13 (54%) had an increase in stimulated cortisol measurement, with a peak
66 response of 325nmol/L at Week 18 in one subject. Increased steroid metabolites, assayed by urine
67 GC-MS at Week 12 and Week 48, was detected in 8/13 (62%), reflecting an increase in endogenous
68 steroidogenesis. Four of 13 had Residual Adrenal Function at 72 weeks.

69 **CONCLUSION** Combined treatment with rituximab and depot tetracosactide did not restore normal
70 adrenal function. Nevertheless, adrenocortical plasticity is demonstrated in some patients and this has
71 the potential to be exploited to improve adrenal function.

72 **Clinical Trial registration (ISRCTN) 20220821**

73 **Introduction**

74 Autoimmune Addison's disease (AAD) is a rare disease in which immune-mediated
75 destruction of steroid-producing cells in the adrenal cortex culminates in a potentially fatal
76 state of steroid deficiency (1,2). Steroid 21-hydroxylase and other adrenal steroidogenic
77 enzymes are the target of immunological attack (3). Once levels of circulating
78 glucocorticoids (GC) and mineralocorticoids (MC) fall to a critical state, patients are
79 absolutely dependent on daily steroid replacement for survival.

80

81 The advent of cortisone acetate in the 1940s transformed the disease from certainly fatal to a
82 manageable chronic condition. Nonetheless, synthetic GCs cannot mimic the intrinsic diurnal
83 rhythm of cortisol production (4), thus current steroid replacement regimens are imperfect.
84 Side-effects from even a subtle excess of glucocorticoid pose a risk to bone health,
85 cardiovascular risk and glucose tolerance (5-10). Despite regular steroid replacement, the risk
86 of adrenal crisis remains an unpredictable and dangerous threat to health, and life expectancy
87 is reduced in patients with AAD (11-13).

88

89 Adrenocortical plasticity has long been established (14), with several examples in clinical
90 practice: patients receiving exogenous steroid therapy develop adrenal atrophy and functional
91 adrenal failure; conversely, hypertrophy of the adrenal glands is seen in the setting of ACTH
92 excess (e.g. Cushing's disease). Recent early-phase studies of novel therapies have
93 significantly advanced our understanding of the concept of Residual Adrenal Function (RAF)
94 in AAD, and have suggested that adrenocortical plasticity may be amenable to intervention
95 (15,16). The use of B cell-depleting immunotherapy in autoimmune disorders that share
96 pathophysiological features with AAD has now translated to routine clinical care for some
97 but not all conditions (17-19), and the first study in AAD treated 6 newly diagnosed patients
98 with rituximab with some success (15). This B-lymphocyte depleting anti-CD20

99 immunotherapy ameliorated the immunological destruction of steroid-producing cells in the
100 adrenal gland in one patient - progressively rising concentrations of endogenous GCs and
101 MCs were seen, allowing a temporary complete cessation of replacement steroids (15).
102 Thereafter, a second study of regenerative therapy in AAD was performed: 13 patients with
103 established AAD of greater than 1 year duration were treated with repeated doses of
104 tetracosactide (ACTH₁₋₂₄, depot Synacthen). This trophic stimulation harnessed and exploited
105 RAF in two patients (4 and 8 years from diagnosis) - levels of intrinsic GCs and MCs rose,
106 and both patients stopped exogenous steroids entirely (16). One patient remains off steroid
107 replacement seven years later.

108

109 These early-phase studies have greatly enhanced our understanding of the potential of RAF
110 and the impact regenerative medicine therapy could have in AAD. This paper reports the
111 RADS2 study, which combined therapy with B-lymphocyte depleting immunotherapy and
112 trophic ACTH stimulation in newly diagnosed patients for the first time, with the aim of
113 harnessing and exploiting endogenous adrenal steroidogenesis and ultimately delivering
114 better outcomes for patients with this chronic disease.

115

116 **Patients and Methods**

117 Thirteen subjects (9 female, 4 male; aged 19-64 years), with a diagnosis of new onset AAD
118 within the preceding four weeks were recruited from endocrine or acute medical services in
119 Newcastle, Exeter, Cambridge or Cardiff, UK. Patients underwent robust clinical and
120 biochemical screening at the point of trial entry to confirm unequivocally that adrenal failure
121 was primary and of autoimmune origin. Eligibility criteria included: age 10-65 years, clinical
122 features to confirm primary adrenal failure, high ACTH (>47 ng/L), low circulating cortisol
123 concentrations (basal <100nmol/L or stimulated 30 or 60 minutes post-tetracosactide
124 <300nmol/L) and positive serum 21-hydroxylase antibodies (≥ 1 U/mL). A computed

125 tomography scan and chest x-ray were also performed to exclude intercurrent illness or
126 malignancy and to assess adrenal gland appearances. Exclusion criteria were: significant
127 cardiovascular or respiratory disease (including asthma), renal or hepatic disease,
128 malignancy, pregnancy or breastfeeding, current infectious disease (including HIV, hepatitis
129 B/C, shingles/zoster, tuberculosis), unexplained abnormality on chest x-ray and previous use
130 of immunosuppressive or cytotoxic drugs (excluding GC).

131

132 33 potential recruits were identified and underwent preliminary screening across 4 sites. 17
133 patients were consented and formally recruited into the 72-week study (**Figure 1**). Of those
134 consented, 4/17 patients failed one or more eligibility criteria for treatment (**Figure 2**).

135 The study was registered at ISRCTN with ID 20220821. Ethical approval was granted by the
136 National Research Ethics Service North East-Sunderland, reference 12/NE/0339.

137

138 *Design and Intervention Regimen*

139 This open-label study of rituximab and depot tetracosactide followed newly diagnosed
140 patients for 72 weeks after intervention to assess for any improvement in adrenocortical
141 function. The schedule of visits for screening, intervention and follow-up is outlined in
142 **Figure 1**.

143

144 Rituximab (1 gram by intravenous infusion) was administered on Day 1 and Day 15 of the
145 study. Patients were taught to self-inject 1mg subcutaneous depot tetracosactide on Day 1 and
146 this was administered on alternate days for a minimum period of 12 weeks. In participants
147 who had any biochemical evidence of a rising stimulated-cortisol, tetracosactide was
148 continued for a maximum period of 20 weeks in total.

149

150 At recruitment, all participants were taking hydrocortisone as GC replacement (in doses
151 ranging between a total of 15-50mg daily). A subset of patients had not yet commenced
152 fludrocortisone, so this was started at the first clinical encounter with the trial team. GC
153 replacement doses were lowered to a total daily dose of 10-15mg hydrocortisone where
154 possible, to promote maximal endogenous ACTH secretion. Throughout the study, in
155 participants with a measurable improvement in endogenous steroidogenesis (any rise in basal
156 or stimulated-cortisol concentrations), GC replacement was judiciously weaned, with regular
157 monitoring of clinical symptoms, blood pressure and serum electrolytes. The lowest daily GC
158 replacement dose reached was 5mg hydrocortisone daily in one patient.

159

160 *Outcome Measures and Assessments*

161 The primary outcome measure was restoration of normal GC secretion at Week 48, defined
162 as a peak stimulated cortisol of >550nmol/L. Secondary outcome measures were: restoration
163 of normal GC secretion at Weeks 6, 12, 24 and 72, improvement of basal or peak cortisol
164 (>100nmol/L over baseline), changes of other biochemical parameters (DHEA-S, 17OHP)
165 and the safety and tolerability of the regimen.

166

167 Participants had regular follow-up during the first 24 weeks of the study, with clinical
168 assessment +/- biochemical assessment on Day 1, 7, 14, 28 and then at 6, 12, 18 and 24
169 weeks. Major outcome visits included a SST and detailed serum biochemistry sampling and
170 were performed at Week 6, 12, 24, 48 and 72, during a 36-hour 'steroid medication-free'
171 window to allow assessment of endogenous steroid production (**Figure 1**). Overnight urine
172 collections were performed during the steroid-free window and a comprehensive panel of
173 urine steroids were measured at Baseline, Week 12 and Week 48. Participants underwent
174 robust education and overnight hospital admission while steroid-free to ensure safety. In the
175 event of intercurrent illness, the visit was postponed for a short time; in one instance, a visit

176 was cancelled. Electrolytes, full blood count, lymphocyte subsets and short synacthen tests
177 (SST, off replacement steroids) were analysed in real-time. All other blood samples and urine
178 collections were stored at -80°C, and batch analysed on trial completion.

179

180 Flow cytometry analysis of B-lymphocyte subsets was performed on fresh material
181 throughout the study to assess the depth of B-lymphocyte depletion. CD19+ cells were
182 measured at baseline and following intervention; 10,000 lymphocyte events were counted
183 twice at each measurement. Complete depletion was judged as CD19+ <0.1% of
184 lymphocytes.

185

186 Short synacthen tests (SST with cortisol measured by competitive chemoluminescent assay,
187 lower limit of detection (LLD) 24nmol/L) were performed at Baseline, 6, 12, 24, 48 and 72
188 weeks and processed centrally with analysis performed in real time. 250µg soluble Synacthen
189 (ACTH₁₋₂₄) was administered intramuscularly following a baseline blood sample drawn for
190 cortisol measurement, with further samples drawn at 30 and 60 minutes. Prior to SST, a
191 series of serum and plasma samples were drawn and stored to allow batch analysis of ACTH
192 (solid-phase, chemoluminescent assay, LLD=5ng/L), dehydroepiandrosterone sulfate
193 (DHEA-S; solid-phase competitive chemoluminescent assay, LLD=0.1µmol/L),
194 androstenedione (solid-phase competitive chemoluminescent assay, LLD=1.05nmol/L),
195 aldosterone (solid-phase radioimmunoassay, LLD=70pmol/L), 17-hydroxyprogesterone
196 levels (17OHP; radioimmunoassay, LLD=1nmol/L) and 21OH Abs (ELISA kit from RSR
197 Ltd (Cardiff); positive result $\geq 1.0\text{U/mL}$) (20).

198

199 A comprehensive panel of urine steroids, collected in the steroid medication-free window,
200 were measured at Baseline, Week 12 and Week 48: GC precursors, GC metabolites, MC
201 precursors, MC metabolites and androgens were measured by gas chromatography-mass

202 spectrometry (GC-MS) in the laboratory at the Steroid Metabolome Analysis Core, Institute
203 of Metabolism and Systems Research, Birmingham. 32 individual urinary steroids was
204 quantified on an Agilent 5975 instrument after free and conjugated steroids were extracted
205 from 1ml of urine by solid-phase extraction (21). Urine metabolomic results were corrected
206 for collection duration. No female patients were taking the oral contraceptive pill or hormone
207 replacement therapy during the urine sample collections.

208

209 One major outcome visit was missed entirely due to illness (unsafe to stop steroid
210 replacement medication) and other safety visits were delayed by several days because of
211 unavoidable commitments that participants could not reschedule. This was an uncontrolled
212 exploratory study and descriptive statistics are used to present outcome measurements.
213 Where appropriate, continuous variables were analysed by paired t-tests.

214

215 **Results**

216 *Participant baseline characteristics*

217 Twelve of 13 participants (mean age 44; range 19-64 years) reported they had experienced
218 weight loss, nausea or vomiting and postural symptoms prior to diagnosis. Eleven of 13
219 reported salt craving, and all 13 described fatigue or lethargy. Eleven of 13 were pigmented
220 and 9/13 were in 'crisis' at the point of diagnosis (with adrenal crisis defined as requiring
221 hospital admission for parental steroids and intravenous fluids). 7/13 had concurrent
222 autoimmune diseases (hypothyroidism n=5, pernicious anaemia n=2, Graves' disease n=1
223 and premature ovarian failure n=1), with one participant having a triad of autoimmune
224 hypothyroidism, pernicious anaemia and premature ovarian failure (**Table 1**).

225

226 Ten of 13 participants had detectable but subnormal stimulated cortisol on SST at formal
227 screening at trial entry (26-265nmol/L). All had elevated ACTH levels (68-2630ng/L; NR 0-
228 47ng/L) and positive 21OH Abs (2.8-3648U/mL; NR<1U/mL)(**Table 1**).

229

230 *Adrenal steroidogenic function: Serum*

231 Seven of 13 participants demonstrated a rise in endogenous cortisol following intervention
232 (an increase in stimulated cortisol on SST detectable during sampling at least one major
233 outcome visit; $P=0.45$ at Week 48 vs Baseline visit; paired t-test)(**Figure 3**). No participants
234 met the primary study outcome with restoration of normal endogenous steroidogenesis
235 (stimulated cortisol >550nmol/L), but one participant (Participant 5) did achieve a secondary
236 outcome measure with an increase in stimulated cortisol from 55nmol/L to 155nmol/L
237 following intervention. This female patient (aged 24) had clear evidence of sustained
238 endogenous steroidogenesis following rituximab therapy and adrenocortical stimulation. Of
239 note, this patient had the highest titre of 21-hydroxylase antibodies (21OH Abs) at trial entry
240 (3648U/mL) and the longest duration of symptoms prior to diagnosis (fatigue and
241 hyperpigmentation of several years duration).

242

243 Participant 4 had the highest recorded serum cortisol during the study – 284nmol/L post-
244 synacthen at Week 12, (an early morning cortisol measurement taken as part of safety
245 surveillance at Week 18 was 325nmol/L). This 56 year old female participant retained clear
246 evidence of endogenous steroidogenesis for over 12 months after trial entry.

247

248 Participant 10 did not meet any biochemical endpoints, but did have noteworthy endogenous
249 function throughout the study. At trial entry, his peak cortisol was 145nmol/L, with a
250 significant rise to a peak cortisol of 234nmol/L at Week 6. Depot tetracosactide was
251 continued for 20 weeks - he retained detectable endogenous steroidogenesis at Week 72

252 (peak cortisol 114nmol/L on stimulation). A further male patient (Participant 13) maintained
253 endogenous steroidogenesis throughout the 72-week follow-up period with a stimulated
254 cortisol at trial entry of 81nmol/L, 127nmol/L at Week 48 and 116nmol/L at Week 72. At
255 week 72, 4 of the 13 (31%) participants had stimulated serum cortisol concentrations of
256 99nmol/l or above, suggesting residual adrenal function. These four participants had higher
257 mean serum cortisol at baseline than the rest of the cohort (129nmol/l vs 41nmol/l; p=0.03)
258 but were not different with regard other baseline characteristics.

259

260 Measurements of DHEA-S, androstenedione, aldosterone and 17OHP in serum are shown in
261 **Figure 4**. Aldosterone was undetectable throughout in all patients, except at Baseline and at
262 Week 12 in Participant 4, who had the highest recorded stimulated cortisol in study. 17OHP
263 was higher in female subjects, reflecting the contribution of an ovarian source. Similarly,
264 serum DHEA-S concentrations were higher in men.

265

266 *Adrenal steroidogenic function: Urine*

267 Eight of 13 participants demonstrated rising urinary steroid metabolite excretion post-
268 intervention, indicating an increase in endogenous adrenal steroidogenesis. In these eight
269 participants, we saw a pattern of increased GC precursor excretion, notably 17-
270 hydroxypregnanolone, pregnanetriol and tetrahydro-11-deoxycortisol, during the first 12
271 weeks of the study in several participants, with a subsequent decline by Week 48. Total GC
272 metabolite production followed a similar pattern in a smaller numbers of participants (4/13),
273 with an increase in urinary GC excretion between Baseline and Week 12 (**Figure 5**). Three
274 participants (4, 10 and 13) who maintained a peak serum cortisol >100nmol/L at Week 72
275 had excreted the highest amounts of GC metabolites amongst all participants (**Figure 5**). A
276 fourth individual (participant 01) had significant changes in urinary steroid output, with

277 increases in a range of GC precursors (pregnanediol, 17hydroxy-pregnanolone, pregnanetriol,
278 pregnanetriolone and tetrahydro-11-deoxycortisol). This increase in production of steroid
279 precursors indicates an authentic steroidogenic response to ACTH stimulation. Four of 13
280 participants had an increase in total MC metabolite production from Baseline to Week 12 and
281 5 of 13 participants had an increase androgen metabolite production during the same period
282 when comparing pre vs. post-intervention. Pooling results from all the participants, there was
283 no statistically significant increase in excretion of urine steroid metabolites between Baseline
284 vs. Week 48.

285 In terms of correlation between serum and urine steroid response, 7 of 13 participants had a
286 detectable serum response (any rise in stimulated cortisol from baseline), whereas 8 of 13
287 participants had a detectable rise in urine steroid excretion (overlapping with 6 of the serum
288 responders). Notably, the number and range of increasing urinary steroid metabolites
289 excreted post-treatment is not necessarily reflected in the serum steroid response. For
290 example, Participant 01 had only a small increase in peak stimulated cortisol following
291 intervention (increment of 30nmol/L in stimulated cortisol measurement at Week 12), but
292 demonstrated increased urinary steroid metabolite excretion across the spectrum including
293 GCs, GC precursors, MCs, androgens and androgen precursors.

294

295 *Immune parameters*

296 At baseline, 21OH Ab titres ranged from 2.8-3648U/mL (positive result ≥ 1.0 U/mL). Serum
297 immunoglobulin M levels fell over the course of the study, but remained within reference
298 range (supplementary figure; reference 22). Twelve of 13 participants achieved CD19+
299 counts measured as 0.0 or 0.1% of the lymphocyte population following immunotherapy,
300 with counts remaining low for several months (minimum of 12 – maximum of 48 depleted
301 weeks). Resurgence ($>0.5\%$ of lymphocyte population) was detectable in all participants by

302 the end of the study, and occurred after a median period of 48 weeks (range 12-72 weeks)
303 (supplementary figure)(22). Participant 08 did not achieve complete CD19+ depletion (lowest
304 count 0.2% at week 6), and did not have a rise in stimulated cortisol following intervention.

305

306 *Safety and tolerability*

307 Trial medications were well tolerated by all participants. All infusions of rituximab were
308 completed. All patients completed the active treatment phase; 1/13 did not complete the 72
309 week follow-up period (attended until Week 48, did not attend final Week 72 visit).
310 Localised reactions to tetracosactide were frequently reported – redness, swelling and
311 bruising around abdominal injection sites (these reactions have been previously reported with
312 repeated doses of tetracosactide (16,23).

313

314 Four serious adverse events (SAEs) were recorded during the study; none were found to be
315 causally-related to the study interventions (listed in supplementary table) (22). Multiple
316 adverse events (AEs) were recorded across all sites during the study; frequently reported
317 symptoms or minor illnesses were headaches, back pain, sore throat and sinusitis.

318

319 **Discussion**

320 Recent early-phase experimental studies of novel therapies have enabled small numbers
321 of patients with AAD to wean and stop steroid replacement following intervention,
322 transforming a chronic disease to a potentially curable condition (15,16, reviewed 24): this
323 heralds an opportunity for a transformation in AAD management. The aim of this study was
324 to combine immunotherapy and trophic stimulation to harness residual adrenal function
325 (RAF), aiming to regenerate steroidogenic function, with the hope that dependence on steroid
326 replacement and patient outcomes could improve further with dual therapy. It was anticipated

327 that rises in serum and urine glucocorticoids post-intervention would reflect
328 improving endogenous steroidogenesis, with these biochemical changes potentially mirrored
329 by enhanced QoL indicators and fewer adrenal crises – ultimately less morbidity
330 and a reduced disease burden for patients.

331

332 No participants met the primary study outcome of restoration of endogenous steroidogenesis
333 demonstrated by a stimulated cortisol >550nmol/L; nevertheless, 54% (7/13) did demonstrate
334 a rise in serum cortisol post-intervention. The highest serum cortisol mid-study was achieved
335 by Participant 04 (stimulated cortisol was 284nmol/L at Week 12): this allowed GC
336 replacement to be weaned to 5mg hydrocortisone/day for 5 months. Participant 05 achieved a
337 secondary outcome measure with a rise in serum cortisol of 100nmol/L, and 4 others (04, 06,
338 10 and 13) had sound biochemical evidence of maintained RAF, manifest as serum cortisol of
339 99nmol/l or more at 72-week follow-up. Although pathophysiologically interesting, this
340 residual steroidogenic function is not of the magnitude required to make a difference to the
341 clinical wellbeing or hormone replacement of these patients. Furthermore, as there was no
342 control group, we cannot exclude that the low-level persisting adrenal function was owing to
343 the spontaneous natural history of the condition and unrelated to the trial medication.

344

345 Urine GC/MS did herald meaningful results with 62% (8/13) demonstrating a rising excretion
346 of urinary steroid metabolites post-intervention. This pattern of urinary steroid excretion was
347 variable between participants, but one (participant 01), had a demonstrable
348 increase across panels of glucocorticoids metabolites, glucocorticoid precursors and
349 androgens. Importantly, the increase in steroid precursor metabolites unequivocally indicates
350 improving endogenous adrenocortical function. While the numbers of participants in this
351 study, and therefore those with a detectable positive response, are low, this is objective
352 evidence of improving RAF many months after a proven diagnosis of AAD. In the previous

353 study using ACTH₁₋₂₄ stimulation (16), analysis of urine steroid profiles in the 2 participants
354 who responded revealed that urinary excretion of GC precursors and active GC metabolites
355 gradually increased from below the 5th centile to above the median of healthy female
356 controls at 10 weeks (in one responder) and at 40 weeks (in second responder), with a parallel
357 increase in urine MC metabolite excretion. In both patients, androgen precursor and active
358 androgen metabolite excretion was slower to rise.

359 In the current study, when urine response is compared to levels of steroids detected in serum,
360 it is apparent that serum steroid measurements cannot provide a comprehensive illustration of
361 endogenous gland function. Therefore, assessment of urinary steroid excretion should be
362 considered the most reliable method of analysis of endogenous steroidogenesis in future
363 studies: urine steroid assays are our most valuable and robust tool for appraising adrenal
364 gland function.

365

366 Around 40% of patients with AAD have never been hospitalised with an adrenal crisis (25,
367 26); perhaps a reduced risk of crisis might be correlated to detectable RAF which will have a
368 protective effect. The two participants who responded to ACTH stimulation in the previous
369 early phase study had never been hospitalised with an adrenal crisis (16). In the current study,
370 Participant 04 presented with a crisis (defined by hospital admission with requirement for
371 parenteral steroids and intravenous fluids), but Participant 05 did not. Clearly there is
372 insufficient data in this study to predict how protective RAF may be, but it does warrant
373 further exploration because emerging data suggest it is not rare amongst patients with
374 established AAD (16,27,28). Furthermore, RAF has the potential to positively impact the risk
375 of life-threatening crisis, alongside other morbidity and mortality factors.

376

377 In healthy individuals, pulsatile ACTH secretion from the pituitary starts around 0300h and
378 increases to a zenith around 0700h, accompanied by a concordant but delayed rise in adrenal
379 cortisol secretion. As the day progresses, ACTH pulse amplitude decreases, leading to
380 reduced levels of serum cortisol by the late afternoon and evening: this circadian variation is
381 the key to optimising GC replacement in patients with Addison's disease (4,29). Exogenous
382 steroid replacement cannot replicate this entirely, which is one of the key drivers for
383 continuing to investigate methods of harnessing and exploiting endogenous steroidogenic
384 capability. The presence of RAF and the fluctuating nature of endogenous steroidogenesis
385 observed in this and previous studies reflects the heterogeneity of AAD. Previously, it was a
386 widespread assumption that the instigation of exogenous steroids caused adrenal glands to
387 entirely cease to function. It seems probable that ACTH drive diminishes rapidly following
388 the start of steroid replacement in most patients, compounding functional steroidogenic
389 failure. However, these studies have accumulated evidence that intrinsic gland function can
390 exist, even years after diagnosis, and has the potential to be exploited. Discovery of this
391 heterogeneity is comparable to gains in knowledge in recent years of a spectrum of disease
392 and a subset of patients with persisting C-peptide positivity indicative of a degree of
393 maintained β -cell function in type 1 diabetes. In both conditions, the disease trajectory within
394 and between individuals is variable, resulting in greater scope for intervention to harness
395 residual gland function and potentially improve patient outcomes – a further example of
396 opportunity for the development of personalised medicine.

397

398 One significant challenge in detecting and monitoring RAF is the requirement for
399 daily steroids in AAD. This limits the assessment of intrinsic gland function in a routine,
400 outpatient clinical setting. The identification of a biomarker which could act as a surrogate
401 for endogenous glucocorticoid production would be particularly advantageous. Further
402 attention should also be given to the choice of immunotherapy administered as rescue therapy

403 in the setting of immune-mediated adrenocortical destruction. Rituximab was chosen in this
404 early-phase study because of its mechanism of action, efficacy in similar
405 diseases, partially successful outcome in our initial study (15) and its durable safety
406 record over two decades. While some participants experienced mild side-effects during
407 administration of the drug, it was essentially well-tolerated – a key consideration in an
408 experimental study utilising novel therapeutic approach. Alternative immunotherapies, such
409 as those which can maintain or enhance the activity of regulatory T-cells, may prove more
410 robust for tackling diseases with immune-mediated gland destruction, such as type 1 diabetes
411 (30) and AAD, but are likely to be less well tolerated. Consequently, they may not be
412 acceptable to patients considering participation in early-phase studies or panels
413 considering ethical approval. Furthermore, it is evident from this study that once repeated
414 ACTH stimulation is withdrawn, its effect on adrenocortical steroid production rapidly wanes
415 in most cases.

416

417 *Conclusion*

418 While harnessing and exploiting RAF remains a significant challenge, this experimental
419 study has added further weight to the evidence that a sizeable proportion of patients with
420 AAD have maintained endogenous steroidogenic potential after diagnosis. Our understanding
421 of physiological steroid production means we know that standard steroid replacement is an
422 imperfect therapy for patients with AAD – but what does detectable RAF or an improvement
423 in endogenous function really mean for patients? There is a wealth of evidence that patients
424 with the condition exhibit increased morbidity and reduced quality of life: improved
425 intrinsic gland function can be expected to counteract these problems to a degree, although
426 the numbers in pilot clinical studies are too small to provide robust evidence of superior
427 outcomes in the context of improving RAF. Nevertheless, there have been no trials of novel
428 therapies other than alternative steroid replacement for AAD for over half a century:

429 persisting with innovative therapeutic approaches is the only meaningful
430 prospect for delivering a tangible improvement in the lives of those with the condition.

431

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433

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443

444 **REFERENCES:**

445

446 1. Pazderska A, Pearce SH. Adrenal insufficiency: recognition and management. *Clinical*
447 *Medicine (JRCPL)*. 2017; 17(3): 258–262.

448

449 2. Husebye ES, Løvås K, Allolio B, Arlt W, Badenhoop K, Bensing S, Betterle C, Falorni A,
450 Gan EH, Hulting A-L, Kasperlik-Zaluska A, Kämpe O, Mayer G, Pearce SH. Consensus
451 statement on the diagnosis, treatment and follow-up of patients with primary adrenal
452 insufficiency. *J Intern Med*. 2014; 275(2):104–15

453

454 3. Winqvist O, Karlsson FA, Kämpe O. 21-Hydroxylase, a major autoantigen in idiopathic
455 Addison's disease. *Lancet*. 1992; 339(8809):1559–62.

456

457 4. Spiga F, Lightman SL. Dynamics of adrenal glucocorticoid steroidogenesis in health and
458 disease. *Mol Cell Endocrinol*. 2015; 408(6): 227–34.

459

460 5. Devogelaer JP, Crabbé J, Nagant de Deuxchaisnes C. Bone mineral density in Addison's
461 disease: evidence for an effect of adrenal androgens on bone mass. *Br Med J (Clin Res Ed)*.
462 1987; 294(6575):798–800.

463

464 6. Florkowski CM, Holmes SJ, Elliot JR, Donald RA, Espiner EA. Bone mineral density is
465 reduced in female but not male subjects with Addison's disease. *New Zealand Medical*
466 *Journal*. 1994; 107(972): 52–53.

467

468 7. Valero MA, Leon M, Ruiz Valdepeñas MP, Larrodera L, Lopez MB, Papapietro K, Jara A,
469 Hawkins F. Bone density and turnover in Addison's disease: effect of glucocorticoid
470 treatment. *Bone and Mineral*. 1994; 26(1): 9–17.

471

472 8. Plat L, Leproult R, L'Hermite-Baleriaux M, Fery F, Mockel J, Polonsky KS, Van Cauter E.
473 *Journal of Clinical Endocrinology and Metabolism*. 1999; 84(9): 3082–3092.

474

475 9. Walker BR. Glucocorticoids and cardiovascular disease. *European Journal of*
476 *Endocrinology*. 2007;157(5): 545–559.

477

478 10. Björnsdóttir S, Sääf M, Bensing S, Kämpe O, Michaëlsson K, Ludvigsson JF. Risk of hip
479 fracture in Addison's disease: A population-based cohort study. *Journal of Internal Medicine*.
480 2011; 270(2): 187–195.

481

482 11. Bergthorsdóttir R, Leonsson-Zachrisson M, Odén A, Johannsson G. (2006). Premature
483 mortality in patients with Addison's disease: A population-based study. *Journal of Clinical*
484 *Endocrinology and Metabolism*. 2006; 91(12): 4849–4853.

485

486 12. Bensing S, Brandt L, Tabaroj F, Sjöberg O, Nilsson B, Ekbom A, Blomqvist P, Kämpe O.
487 Increased death risk and altered cancer incidence pattern in patients with isolated or
488 combined autoimmune primary adrenocortical insufficiency. *Clinical Endocrinology*. 2008;
489 69(5): 697–704.

490 13. Erichsen MM, Løvås K, Fougner KJ, Svartberg J, Hauge ER, Bollerslev J, Berg JP, Mella
491 B, Husebye ES. Normal overall mortality rate in Addison's disease, but young patients are at
492 risk of premature death. *Eur J Endocrinol*. 2009; 160(2):233–7.

- 493 14. Ingle DJ, Higgins GM. Autotransplantation and regeneration of the adrenal gland.
494 *Endocrinology*. 1938; 22(4): 458–464.
495
- 496 15. Pearce SH, Mitchell AL, Bennett S, King P, Chandran S, Nag S, Chen S, Smith BR,
497 Isaacs JD, Vaidya B. Adrenal steroidogenesis after B lymphocyte depletion therapy in new-
498 onset Addison's disease. *Journal of Clinical Endocrinology and Metabolism*. 2012; 97(10):
499 E1927–E1932.
500
- 501 16. Gan EH, MacArthur K, Mitchell AL, Hughes BA, Perros P, Ball SG, James RA, Quinton
502 R, Chen S, Furmaniak J, Arlt W, Pearce SH. Residual adrenal function in autoimmune
503 addison's disease: Improvement after tetracosactide (ACTH₁₋₂₄) treatment. *Journal of Clinical*
504 *Endocrinology and Metabolism*. 2014; 99(1): 111–118.
505
- 506 17. Edwards JC, Szczepanski L, Szechinski J, Filipowicz-Sosnowska A, Emery P, Close DR,
507 Stevens RM, Shaw T. Efficacy of B-cell-targeted therapy with rituximab in patients with
508 rheumatoid arthritis. *N Engl J Med*. 2004; 350(25): 2572–81.
509
- 510 18. Hauser SL, Waubant E, Arnold DL, Vollmer T, Antel J, Fox RJ, Bar-Or A, Panzara M,
511 Sarkar N, Agarwal S, Langer-Gould A, Smith CH; HERMES Trial Group. B-cell depletion
512 with rituximab in relapsing-remitting multiple sclerosis. *N Engl J Med*. 2008; 358(7): 676–
513 88.
514
- 515 19. Pescovitz MD, Greenbaum CJ, Bundy B, Becker DJ, Gitelman SE, Goland R, Gottlieb
516 PA, Marks JB, Moran A, Raskin P, Rodriguez H, Schatz DA, Wherrett DK, Wilson DM,
517 Krischer JP, Skyler JS; Type 1 Diabetes TrialNet Anti-CD20 Study Group. B-lymphocyte
518 depletion with rituximab and β -cell function: two-year results. [Diabetes Care](#). 2014;
519 37(2):453–9.
520
- 521 20. Tanaka H, Perez MS, Powell M, Sanders JF, Sawicka J, Chen S, Prentice L, Asawa T,
522 Betterle C, Volpato M, Smith BR, Furmaniak J. Steroid 21-hydroxylase autoantibodies:
523 measurements with a new immunoprecipitation assay. *J Clin Endocrinol Metab*. 1997;
524 82:1440–1446.
525
- 526 21. Arlt W, Biehl M, Taylor AE, Hahner S, Libé R, Hughes BA, Schneider P, Smith DJ,
527 Stiekema H, Krone N, Porfiri E, Opocher G, Bertherat J, Mantero F, Allolio B, Terzolo M,
528 Nightingale P, Shackleton CH, Bertagna X, Fassnacht M, Stewart PM. Urine steroid
529 metabolomics as a biomarker tool for detecting malignancy in adrenal tumors. *Journal of*
530 *Clinical Endocrinology and Metabolism*. 2011; 96(12): 3775–3784.
531
- 532 22. Supplementary material. <https://doi.org/10.25405/data.ncl.10011821>
533
- 534 23. Gan EH, MacArthur K, Mitchell AL, Joshi A, Crock P, Pearce SH. Spontaneous and
535 tetracosactide-induced anti-ACTH antibodies in man. *Clin Endocrinol (Oxf)*. 2016;
536 84(4):489–95
537
- 538 24. Gan EH, Pearce SH. Regenerative therapies in autoimmune Addison's disease. *Eur J*
539 *Endocrinol*. 2017 Mar;176(3):R123-R135.
540
- 541 25. Hahner S, Loeffler M, Bleicken B, Drechsler C, Milovanovic D, Fassnacht M, Ventz M,
542 Quinkler M, Allolio B. Epidemiology of adrenal crisis in chronic adrenal insufficiency: The

- 543 need for new prevention strategies. *European Journal of Endocrinology*. 2010; 162(3): 597–
544 602.
545
- 546 26. White K and Arlt W. Adrenal crisis in treated Addison's disease: A predictable but under-
547 managed event. *European Journal of Endocrinology*. 2010; 162(1): 115–120.
548
- 549 27. Smans LC, Zelissen PM. Does recovery of adrenal function occur in patients with
550 autoimmune Addison's disease? *Clin Endocrinol (Oxf)*. 2011; 74(4):434–7
551
- 552 28. Vulto A, Bergthorsdottir R, van Faassen M, Kema IP, Johannsson G, van Beek AP.
553 Residual endogenous corticosteroid production in patients with adrenal insufficiency. *Clin*
554 *Endocrinol (Oxf)*. 2019; doi: 10.1111/cen.14006. (in press)
555
- 556 29. Debono M, Ghobadi C, Rostami-Hodjegan A, Huatan H, Campbell MJ, Newell-Price J,
557 Darzy K, Merke DP, Arlt W, Ross RJ. Modified-release hydrocortisone to provide circadian
558 cortisol profiles. *Journal of Clinical Endocrinology and Metabolism*. 2009; 94(5): 1548–
559 1554.
560
- 561 30. Seelig E, Howlett J, Porter L, Truman L, Heywood J, Kennet J, Arbon EL, Anselmiova
562 K, Walker NM, Atkar R, Pekalski ML, Rytina E, Evans M, Wicker LS, Todd JA, Mander
563 AP, Bond S, Waldron-Lynch F. The DILfrequency study is an adaptive trial to identify
564 optimal IL-2 dosing in patients with type 1 diabetes. *JCI Insight*. 2018;3(19). pii: 99306.
565

566 **Table 1. Clinical and Biochemical Characteristics at Baseline**

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PARTICIPANT	AGE	SEX	BASELINE STIMULATED CORTISOL (nmol/L)*	ACTH (ng/L) AT STUDY ENTRY	21OH ANTIBODIES (U/mL) AT STUDY ENTRY	OTHER AUTOIMMUNE DISEASE
1	45	F	40	1085	6.0	
2	64	F	<24	68	22.3	GD
3	36	F	<24	1050	39.6	PA
4	56	F	265	316	5.5	AH
5	24	F	55	827	3648	
6	56	F	26	915	11.6	AH
7	43	F	<24	850	71.7	AH
8	27	M	30	1054	413.3	
9	19	M	40	1542	581.7	
10	48	M	145	535	10.8	
11	60	F	45	1160	63.4	AH, PA, POF
12	39	F	88	393	17.0	AH
13	52	M	81	2630	2.8	

568 13 treated participants. Mean age 44 years (range 19-64). Stimulated cortisol on Short Synacthen Test
 569 (SST) at study entry ranges from <24 to 265nmol/L; the peak value of 30 or 60 minutes post-
 570 tetracosactide is shown. Demographic features of those who had serum cortisol \geq 99nmol/l at 72
 571 weeks are highlighted in bold. *To convert serum cortisol values to μ g/dl divide by 27.6. ACTH at
 572 baseline ranged between 68-2630ng/L (NR 0-47ng/L) and all 12 participants had positive 21OH
 573 antibodies (2.8-3648U/mL; NR<1U/mL).

574 GD = Graves' disease. PA = pernicious anaemia. AH = autoimmune hypothyroidism.

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596 **Figure Legends**

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598 **Figure 1. Screening, Treatment and Monitoring Schedule in the RADS2 Study.** Initial
599 screening was performed in-person, or by telephone or email. If this identified a patient with primary
600 adrenal failure who was willing to participate in the study, then formal eligibility testing took place.
601 Robust clinical and biochemical assessment was carried out at the study entry (Baseline visit), prior to
602 any intervention. Major outcome visits (shown in middle column) were performed with patients ‘free’
603 of exogenous steroids, allowing assessment of endogenous steroid production. Interim safety visits
604 (shown in right hand column) allowed a shorter clinical assessment to take place, with the primary
605 aim being patient safety.

606 *depot tetracosactide therapy started. 1mg administered subcutaneously on alternate days

607 **depot tetracosactide discontinued at Week 12, or continued to a maximum of Week 20, depending
608 upon response (rising stimulated cortisol on Week 6 and Week 12 SST) and tolerability

609

610

611 **Figure 2. ‘CONSORT’ flow diagram of Patients Screened, Enrolled and Treated in the**

612 **RADS2 Study.** 33 patients with adrenal failure diagnosed within the past 4 weeks were contacted by a
613 member of the study team, for discussion of the study and initial assessment of clinical history. 17/33
614 patients with primary adrenal failure who agreed to participate proceeded to formal consent and
615 recruitment into the study. These patients were then formally screened to ensure they unquestionably
616 met eligibility criteria. 4 patients did not meet eligibility criteria on comprehensive assessment and
617 could not proceed. 13 participants received treatment with rituximab and depot tetracosactide. 12
618 participants completed 72 weeks of follow-up.

619

620 **Figure 3. Peak Stimulated Cortisol on Short Synacthen testing at Baseline and Major**
621 **Outcome Visits.** Peak stimulated cortisol (higher value of 30 or 60 minutes post-tetracosactide) at
622 Baseline (study entry) and at each major outcome visit: Week 6, 12, 24, 48 (primary outcome
623 assessment) and 72. Seven of 13 participants had an increase in stimulated cortisol recorded on ≥ 1
624 follow-up visit. 4 participants (04, 06, 10 and 13) completed the study with a stimulated cortisol
625 measurement of $\geq 99\text{nmol/L}$. Male participants are shown in blue. Lower limit
626 detection/LLD= 24nmol/L .

627

628 **Figure 4. Serum Steroid Biochemistry at Baseline and Major Outcome Visits.** DHEA-S
629 ($\mu\text{mol/L}$; solid-phase competitive chemoluminescent assay, lower limit detection/LLD= $0.1\mu\text{mol/L}$),
630 17aOHP (nmol/L ; radioimmunoassay, LLD= 1nmol/L), aldosterone (pmol/L ; solid-phase
631 radioimmunoassay, LLD= 70pmol) and androstenedione (nmol/L ; solid-phase competitive
632 chemoluminescent assay, LLD= 1.05nmol/L) were measured in all treated participants at each major
633 outcome assessment (Baseline, Week 6, Week 12, Week 24, Week 48 and Week 72). Samples were
634 collected prior to each major outcome visit SST and batch analysis was performed on trial
635 completion. Grey shading denotes lower limit detection/LLD for each steroid measured. Male
636 participants are shown in blue. Participants with the highest levels of DHEA-S are all male, likely
637 representing a testicular source of DHEA-S. No patients were taking DHEA supplementation.

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639

640 **Figure 5. Urine Steroid Metabolite Excretion ($\mu\text{g}/24$ hours) at Baseline, Week 12 and Week**
641 **48**

642 A comprehensive urine steroid profile was measured by gas chromatography-mass spectrometry at
643 Baseline, Week 12 and Week 48 (primary outcome assessment) of the study (see methods section;
644 21). The sum of the metabolites of the glucocorticoid precursors (17-hydroxyprogesterone, 17-
645 hydroxy-pregnanolone, pregnanetriol) and 11-deoxycortisol (tetrahydro-11-deoxycortisol) are plotted
646 (panel A). The sum of the active glucocorticoid metabolites (cortisol, tetrahydrocortisol, 5 α -
647 tetrahydrocortisol, α -cortol, β -cortol, cortisone, tetrahydrocortisone, α -cortolone, and β -cortolone) are
648 shown (panel B). The sum of the mineralocorticoid metabolites (3 α ,5 β -tetrahydroaldosterone,
649 tetrahydrocorticosterone, 5 α -tetrahydrocorticosterone, tetrahydrodeoxycorticosterone, 5 α -
650 tetrahydrodeoxycorticosterone, tetrahydro-11-dehydrocorticosterone, and 5 α -tetrahydro-11-
651 dehydrocorticosterone) are plotted (panel C). The sum of the androgen precursor metabolites
652 dehydroepiandrosterone, 16 α -dehydroepiandrosterone, 5-pregnanediol and 5-pregnanetriol are plotted
653 (panel D). The sum of the major active androgen metabolites androsterone and etiocholanolone are
654 plotted over time (panel E). Total urine GC metabolite production (shown here; estimated production
655 in $\mu\text{g}/24$ hours) increases between study entry and Week 12 in 4/13 participants (01, 05, 12, and 13).
656 Three of 13 participants (04, 10 and 13) with stimulated cortisol $>100\text{nmol/L}$ at Week 72 had the
657 highest levels of GC metabolite production in urine. Baseline urine samples from participant 8 and
658 participant 10 were not available.