Pharmacodynamic studies of nasal tetracosactide with salivary glucocorticoids for a noninvasive Short Synacthen Test

Charlotte J. Elder,[1,2] Ruben Vilela,[1] Trevor N. Johnson,[3] Rosie N Taylor,[4] E. Helen Kemp,[1] Brian G. Keevil,[5] Alexandra S. Cross,[1] Richard J. Ross,[1] and Neil P. Wright[2]

[1]Department of Oncology and Metabolism, The University of Sheffield, Sheffield S10 2RX, United Kingdom; [2]Department of Endocrinology, Sheffield Children's NHS Foundation Trust, Sheffield S10 2TH, United Kingdom; [3]Certara UK Limited, Sheffield S1 2BJ, United Kingdom; [4]Statistical Services Unit, The University of Sheffield, Sheffield S3 7RH, United Kingdom; [5]Department of Clinical Biology, Manchester University NHS Trust, Manchester M23 9LT, United Kingdom

ORCiD numbers: 0000-0003-2390-5593 (C. J. Elder); 0000-0002-0313-8916 (E. H. Kemp); 0000-0001-9222-9678 (R. J. Ross).

Context: The Short Synacthen Test (SST) is the gold standard for diagnosing adrenal insufficiency. It requires invasive administration of Synacthen, venous sampling, and is resource-intensive.

Objective: To develop a nasally administered SST, with salivary glucocorticoids measurement, to assess the adrenal response.

Design: We conducted 5 studies: 4 open-label, sequence-randomized, crossover, pharmacodynamic studies testing 6 doses/formulations and a repeatability study. Additionally, pharmacokinetic analysis was undertaken using our chosen formulation, 500 µg tetracosactide with mucoadhesive chitosan, Nasacthin003, in our pediatric study.

Setting: Adult and children's clinical research facilities.

Participants: A total of 36 healthy adult males and 24 healthy children.

Intervention: We administered all 6 nasal formulations using an European regulator endorsed atomization device. The IV comparators were 250 µg or 1 µg SST.

Main Outcome Measures: We analyzed paired blood and saliva samples for plasma cortisol and salivary cortisol and cortisone.

Results: The addition of chitosan to tetracosactide and dose escalation increased peak cortisol response ($P = 0.01$ and 0.001, respectively). The bioavailability of Nasacthin003 was 14.3%. There was no significant difference in plasma cortisol at 60 minutes between 500 µg Nasacthin003 and 250 µg IV Synacthen ($P = 0.17$). The repeatability coefficient at 60 minutes was 105 nmol/L for IV Synacthen and salivary cortisol and cortisone was 10.3 and 21.1 nmol/L, respectively. The glucocorticoid response in children was indistinguishable from that of adults.

ISSN Print 0021-972X ISSN Online 1945-7197
Printed in USA

Received 27 March 2020. Accepted 27 May 2020.
First Published Online 2 June 2020.
Corrected and Typeset 27 June 2020.

Abbreviations: AI, adrenal insufficiency; AUC, area under the curve; BMI, body mass index; CI, confidence interval; Cmax, maximum plasma concentration; CV, coefficient of variation; IQR, interquartile range; MS, mass spectrometry; PK, pharmacokinetic, SST, Short Synacthen test; Tmax, time to maximum plasma concentration.

Conclusions: Nasal administration of Nasacthin003 generates equivalent plasma cortisol values to the 250-µg IV SST and, with measurement at 60 minutes of salivary cortisol or cortisone, provides a noninvasive test for adrenal insufficiency. (***J Clin Endocrinol Metab*** **105: 2692–2703, 2020)**

Key Words: Adrenal insufficiency, short Synacthen test, salivary cortisol, salivary cortisone, chitosan

The Short Synacthen Test (SST), or ACTH (cosyntropin) stimulation test, is the most commonly used diagnostic test for adrenal insufficiency (AI) and the recommended, gold standard test for primary AI (1-3). The test involves either IV or IM injection of synthetic ACTH(1-24) (tetracosactide) and blood sampling at 30 to 60 minutes to quantify the plasma cortisol response. Use of the test is increasing (3, 4). For most health care providers the test is time-, labor-, and resource-intensive. Cannulation (or administration of intramuscular Synacthen) and venous sampling may be distressing and painful, especially for children. These barriers may lead to delayed or missed diagnoses, with a risk of death through an adrenal crisis (5). There is a need for a less invasive, less resource-intensive, and therefore more cost-effective, test for AI.

The label for Synacthen recommends a 250-µg IV dose; however, in clinical practice, both a high- and low-dose test are used as 250 µg provides a supraphysiological dose of Synacthen (3). Meta-analyses demonstrate similar outcomes for both the high- and low-dose test (6, 7). There is a threshold tetracosactide level that elicits a maximal cortisol response at 30 minutes; this is achieved with 1 µg IV Synacthen, with higher doses generating more prolonged stimulation (8-10). Salivary cortisol is a validated and well-established alternative to invasive glucocorticoid sampling (11). It has been investigated as an alternative to plasma cortisol following the administration of Synacthen in both healthy volunteers and patient populations (12-14). Salivary cortisone is emerging as the preferable salivary biomarker because it is more abundant in saliva than cortisol, more sensitive at low plasma cortisol levels, and better reflects plasma total and free cortisol than salivary cortisol (12-16).

We have investigated alternative routes for Synacthen administration. The intranasal route has advantages: minimal training to administer drugs in a rapid and tolerable way; good absorption because of the richly vascular nasal mucosa; avoidance of first-pass metabolism; and a rapid onset of action (17). The side effects of intranasal drugs are few and are generally attributable to the drug itself rather than the method of delivery. Previous studies have examined the potential of nasally administered ACTH analogues but as a replacement for depot ACTH, historically used as an alternative to corticosteroid treatment in inflammatory conditions. Despite a demonstrable adrenal response, the short duration of Synacthen activity limited their therapeutic advancement (18, 19). We have developed an intranasal formulation of Synacthen and tested it in both adults and children, with the aim of generating a noninvasive test.

Materials and Methods

Studies design and participants

We conducted 5 studies (S1-S5) at the Clinical Research Facilities of Sheffield Children's NHS Foundation Trust and Sheffield Teaching Hospitals NHS Trust, UK, between 2010 and 2017. S1-S3 and S5 were open-label, multiarm, sequence-randomized, crossover, pharmacodynamic studies. S4 was a repeatability study. S1-S4 were conducted in healthy adult males and study S5 in healthy children. As a "first in man" study, ethical restrictions precluded administration of the novel drug product to women of childbearing age. The numbers assessed for eligibility, recruited, completing study visits, and included in data analyses for each study are displayed in the CONSORT table (Table 1). We recruited 12 different adults to each of the pharmacodynamic studies, with 6 volunteers from the dose-response study (S3) re-enrolled for the repeatability study (S4), and 24 children participating in the pediatric study (S5). We excluded volunteers if they smoked, had been diagnosed with an endocrinopathy, intracranial or adrenal pathology, asthma, allergic rhinitis, anemia, peptic ulcer disease, gastrointestinal bleed or dyspepsia, experienced a severe allergic reaction or any hypersensitivity to Synacthen, were on any regular or prescribed medication, received any formulation of corticosteroid in the previous 3 months or had ever had a course of oral corticosteroids lasting more than 1 month. The first study was approved by Leeds (West) Research Ethics Committee, UK, and all subsequent studies by London-Hampstead Research Ethics Committee, UK. Written informed consent was given by all participants or their parents/carers.

Nasal tetracosactide formulations

Our initial study (S1) used 25 µg and 100 µg of the licensed IV Synacthen formulation (250 µg/mL, Alliance Pharmaceuticals Wiltshire, UK), which we administered intranasally. Doses were based on the results of a murine study but were poorly absorbed (20). For subsequent studies,

Table 1. CONSORT Table: Overview of the 5 Studies

Study Identifier, Purpose	Study Design and Population[a]	Enrollment: Recruitment and Eligibility Screening	Test Product[b]; Dosage; Route of Administration	N Completing Study	N Included in Final Analysis[c]
Study 1: Formulation development	Open-label, multiarm, crossover, PK Healthy adult males	Recruitment target = 12 Screened for eligibility = 13[d]	Synacthen: Single nasal dose, 25 µg and 100 µg	11[e]	10
			Single IV dose, 1 µg	12	12
Study 2: Formulation optimization	Open-label, multiarm, crossover, PK Healthy adult males	Recruitment target = 12 Screened for eligibility = 12	Nasacthin001, single nasal dose, 100 µg	12	10
			Nasacthin002, single nasal dose, 500 µg	12	12
			Nasacthin003, single nasal dose, 500 µg	10[e]	8
			Synacthen, single IV dose, 1 µg	11[f]	10
Study 3: Dose response	Open-label, multiarm, crossover, PK Healthy adult males	Recruitment target = 12 Screened for eligibility = 12	Nasacthin003, single nasal dose, 500 µg	12	11
			Nasacthin003, single nasal dose, 1 mg	12	12
			Synacthen, single IV dose, 250 µg	12	12
Study 4: Repeatability	PK repeatability Healthy adult males from S3	Recruitment target = 6	Participants received 2 further doses of: Nasacthin003, single nasal dose, 500 µg	6	6
Study 5: Pediatric study	Open-label, multiarm, crossover, PK Healthy children	Recruitment target = 24 (12 females) Screened for eligibility = 36	Nasacthin003, single nasal dose, 500 µg	24	23
			Synacthen, single IV dose, 1 µg	12	9
			Synacthen, single IV dose, 250 µg	12	11

PK, pharmacokinetics.
[a]Order of visits was randomized for studies S2, S3, and S5; visits separated by a minimum of 1 week.
[b]Nasacthin001, 100 µg tetracosactide and chitosan; Nasacthin002, 500 µg tetracosactide; Nasacthin003, 500 µg tetracosactide and chitosan.
[c]Number of participants completing the study and included in final data analyses. Dexamethasone suppression confirmed by baseline undetectable/low cortisol (< 50 nmol/L), and subjects excluded from analysis if not adequately suppressed (13 of the total 175 participant visits).
[d]Participant screened but excluded because of mild asthma.
[e]Participant failed to attend subsequent visits.
[f]Participants removed from study because of mild gastrointestinal side effects to dexamethasone.

the tetracosactide was specifically manufactured (Archimedes Pharma, Nottingham, UK), presenting us with the opportunity to formulate with concentrations of tetracosactide (Bachem AG, Bubendorf, Switzerland) in volumes suitable for nasal administration (0.1-0.2 mL per nostril) and with chitosan (FMC BioPolymer AS, Sandvika, Norway), a drug enhancer to optimize nasal absorption. Chitosan, a polysaccharide comprising copolymers of glucosamine and N-acetyl-glucosamine, is derived by partial deacetylation of chitin from crustacea. It is a cationic biopolymer, acting as a bioadhesive film-forming agent to increase drug residence time in the nose, slow mucociliary clearance, and may facilitate paracellular transport of large polar molecules. It is not systemically absorbed and has an excellent safety profile, with a multitude of applications including clarification agent, fungicide, nutritional supplement, and cosmetics constituent (17).

The nasal formulations we chose for the optimization study (S2) examined the effects of chitosan addition, dose escalation, and the additive effect of both. The nasal formulations were: Nasacthin001 containing 100 µg tetracosactide with chitosan (0.2 mL of 0.5 mg/mL), Nasacthin002 containing 500 µg tetracosactide (0.2 mL of 2.5 mg/mL), and Nasacthin003 containing 500 µg tetracosactide with chitosan (0.2 mL of 2.5 mg/mL). Nasacthin003 was subsequently given in double dose (0.4 mL of 2.5 mg/mL) to yield 1000 µg tetracosactide with chitosan.

Procedures

All studies were carried out using a similar methodology. Participants were given 1 mg (0.5 mg for those younger than 8 years old) dexamethasone the night before and the morning of each visit to establish a uniform glucocorticoid baseline and to allow for plasma tetracosactide quantification. We verified adherence by low or undetectable plasma cortisol (< 50 nmol/L) on a baseline sample. Additionally, in S1, we tested -1 minute (baseline) samples for plasma ACTH on an immunochemiluminometric assay (Immulite 2000, Siemens Healthineers, Munich, Germany), verifying adherence as an ACTH level of < 5 IU/L.

All visits commenced before 9:30 AM. Volunteers rested for 30 minutes following IV cannulation and remained supine throughout. Participants attended for nasal tetracosactide visits or the ID comparator visit (high-dose Synacthen [250 µg, 12 adults; 145 µg/m^2, 12 children] or low dose [1 µg, 23 adults, 12 children]) in a randomized order, with no fewer than 7 days between visits. Our dilution method for low-dose

Synacthen has been described previously (14). We administered nasal preparations via a readily available, European regulator endorsed (Conformité Européenne-CE marked) Mucosal Atomisation Device (Teleflex, Wayne, PA, USA), 0.1 mL to each nostril (0.2 mL for 1000-µg dose). Paired blood, taken from the indwelling cannula, and saliva samples, a minimum of 1 mL collected by passive drool into a Salicap tube (IBL, Hamburg, Germany), were taken at the following times (administration of tetracosactide at 0 minutes): -15, -1, 2, 5, 10, 15, 20, 30, 40, 50, 60, 75, 90, and 120 minutes. We requested participants rinse their mouths thoroughly with water 10 minutes before the first salivary sample and not to eat or drink anything, other than water, until after the final sample collection. Participants initiated the salivary drool at the same time the syringe was connected to the cannula to withdraw the discard, before blood sampling.

The 6 participants we re-recruited from the dose response study (S3) to participate in the repeatability study (S4) underwent 2 further SST using 500 µg Nasacthin003 with sampling times at -1, 5, 10, 30, 40, 60, and 90 minutes. In the pediatric study (S5), we recruited 24 children, all of whom received the 500-µg Nasacthin003 formulation for their intranasal visit, but were randomized to receive either high-dose (145 µg/m^2) or low-dose (1 µg) Synacthen as their IV comparator. After completion of their visits, participants were invited to complete a poststudy questionnaire to gauge their personal experience of the intravenous and nasal tests.

We froze and stored samples, batch analyzing at the end of each of the 5 studies. There were no changes to the assay platforms between studies. We analyzed plasma cortisol samples using the Abbott Architect i1000 chemiluminescent microparticle immunoassay (Abbott Diagnostics Ltd, Berkshire, UK). Our salivary cortisol and cortisone analyses were performed by a modified liquid chromatography-tandem mass spectrometry (MS) assay using a Waters Xevo TQ-MS mass spectrometer and a Waters Acquity LC system with an electrospray source operated in positive-ionization mode. We have reported assay characteristics previously (14). In S5, we measured plasma tetracosactide levels by an ACTH(1-24) EIA Kit (Peninsula Laboratories International, Inc., San Carlos, CA, USA). Assay sensitivity, 72 pg/mL; intra-assay precision, 2.73% (at 165 pg/mL) and 1.76% (at 400 pg/mL); and inter-assay precision 9.32% (at 160 pg/mL) and 6.50% (at 412 pg/mL). The EIA ACTH(1-24) data sheet reports 100% cross-reaction with ACTH(1-39) in the assay.

Randomization

We randomized formulation to participant visits in S2, S3 and S5, using an online randomization program. The randomization used a block permutation method that created a balanced randomization, which was produced for 20 participants to allow for further volunteers to be randomized should any of the original recruits drop out.

Outcomes

Our primary outcome was to develop a noninvasive alternative to the IV SST. Our secondary outcomes were: comparison of plasma cortisol at different time points between the nasal and IV formulations; comparison of the response both within and between participants to assess repeatability; comparison of the response between children and adults; and (in the pediatric study only) comparison of the pharmacokinetic parameters (time to maximum plasma concentration [Tmax], maximum plasma concentration [Cmax], area under the concentration time curve [AUC], and bioavailability) achieved.

Statistical analysis

We selected the sample size for each study in accordance with European Medicines Agency bioavailability study guidance (21). Only participants who were adequately dexamethasone-suppressed and completed at least 1 nasal and the IV comparator visit were included in the final analyses (Table 1). Our safety analysis includes all participants who received nasal formulations. We examined the plasma cortisol response to each formulation over time for each participant. To mirror the most popular sampling times following IV Synacthen, we truncated cortisol response over time graphs at 60 minutes (3, 4). We used descriptive statistics, mean, and SD to describe the plasma cortisol, salivary cortisol, and salivary cortisone response to each formulation of tetracosactide. Where participants had both formulations, we used paired *t*-tests and where the formulations had been given to different participant groups, we used independent samples *t*-tests to compare the difference of the peak plasma cortisol and 30-minute and 60-minute cortisol between the IV comparator and the nasal formulation. A P value < 0.05 indicated a statistically significant difference. We used coefficient of variation (CV) to quantify the variability between formulations, between-participants, and within-participants and SDs to further examine between-participant variability. Additionally, we assessed the within-participant repeatability using data from S4 to calculate a 30- and 60-minute repeatability coefficient. The repeatability coefficient is calculated from the within participant SD (s_w) as $1.96\sqrt{2}\,s_w$ and describes the range within which 2 observations on the same individual would be expected to fall 95% of the time. We used SAS v9.3 for statistical analyses.

Pharmacokinetic analysis of tetracosactide data in pediatric study (S5)

We calculated the pharmacokinetic (PK) parameters: Tmax, Cmax, AUC from time 0 until the last quantifiable time point ($AUC_{0\text{-}t}$), and AUC from time 0 until infinity ($AUC_{0\text{-}\infty}$) for each individual using noncompartmental analysis in Phoenix WinNonLin 6.4. For determination of AUC values, we used the linear up and log down trapezoidal method. For $AUC_{0\text{-}\infty}$, the terminal slope (Lambda Z) of the concentration-time profile was determined using the "Best fit" method in WinNonLin. We obtained descriptive statistics for the PK parameters for the IV and nasal tetracosactide formulations. The absolute bioavailability of the Nasacthin003 was calculated based on $AUC_{0\text{-}\infty}$ compared with the IV Synacthen 250-µg dose.

Results

Participants

We enrolled the first participant to study S1 on July 7, 2010, and the last to study S5 on August 17, 2017. The numbers screened for eligibility, participating in each study, and included in the final analyses are displayed in

Table 1. The adult studies (S1-S4) recruited 36 healthy males, aged 19 to 46 years (median, 22; interquartile range [IQR], 21.5, 23.0), with body mass index (BMI) ranges of 19.1 to 29.4 kg/m^2 (median, 23.2; IQR, 21.7, 24.4). There was no indication that BMI influenced the efficacy of the nasal formulations. In the pediatric study (S5), we recruited 36 children but 12 did not complete their initial visit because of unsuccessful cannulation or difficulties obtaining samples and did not continue in the study. Thus, 24 healthy children (12 females) participated, aged 5 to 14 years (median, 10.5; IQR, 9.0, 12.5), with BMIs between the 4th and 93rd centiles (median, 51.5; IQR, 31.5, 74.0).

Formulation development and optimization

These were based on the pharmacodynamic cortisol response to Synacthen (Tables 1 and 2). Our initial study (S1) used a commercially available IV formulation of Synacthen administered intranasally at 25 and 100 µg and the peak cortisol response was significantly lower when compared with the 1-µg IV comparator (mean difference and 95% confidence interval [CI], -320 nmol/L (-370 to -271) and -222 nmol/L (-297 to -146); $P < 0.0001$ and $P = 0.0001$, respectively). We then tested 3 novel intranasal formulations (study S2) to examine the effect of dose escalation and the addition of the mucoadhesive chitosan: Nasacthin001 (100 µg tetracosactide with chitosan), Nasacthin002 (500 µg tetracosactide), and Nasacthin003 (500 µg tetracosactide with chitosan). The peak cortisol responses showed an increase with the addition of chitosan with mean difference (95% CI): 177 nmol/L (54-299) $P = 0.007$ for the 100-µg formulation and 222 nmol/L (73-370) $P = 0.01$ for the 500-µg formulation. The increase in dose from 100 to 500 µg also significantly increased the cortisol response: 242 nmol/L (112-372) $P = 0.0011$ for formulations without chitosan and 339 nmol/L (137-541) $P = 0.0063$ for formulations with chitosan. We therefore selected to progress with formulation Nasacthin003 (500 µg tetracosactide and chitosan) for S3, S4, and S5 because it gave the maximal cortisol response.

Nasacthin003 compared with IV Synacthen

We compared 250 µg IV Synacthen with Nasacthin003 at 500 µg and 1000 µg in S3 (Table 3). As can be seen in Fig. 1A, the mean plasma cortisol concentrations of all 3 formulations are similar up to 60 minutes. There was no significant difference in the 60-minute plasma cortisol between either the 500 µg or 1000 µg Nasacthin003 nasal formulation compared with 250 µg IV Synacthen, with mean difference (95% CI): -28 nmol/L (-70 to 14) $P = 0.17$ and -16 nmol/L (-48 to 16) $P = 0.30$, respectively. For salivary cortisol and cortisone, the shape of the curve was similar following administration with both 250-µg IV Synacthen and Nasacthin003 (Fig. 1B and 1C). However, the salivary cortisol and cortisone

Table 2. Maximum Glucocorticoid Responses to Different Doses and Formulations of Tetracosactide Administered in Studies 1 and 2

Maximum concentration nmol/L			Plasma Cortisol	Salivary Cortisol	Salivary Cortisone
Study	**Dose**				
1	**Intranasal Synacthen, 100 µg**	**N**	9	10	10
		Mean	159.6	1.1	8.1
		SD	95.0	0.6	6.5
	Intranasal Synacthen, 25 µg	**N**	10	10	10
		Mean	63.9	1.0	3.5
		SD	57.7	0.5	2.8
	IV Synacthen, 1 µg	**N**	11	11	11
		Mean	388.2	7.2	29.1
		SD	48.6	4.0	7.7
2	**Nasacthin001, 100 µg**	**N**	11	11	11
		Mean	336.1	8.6	34.7
		SD	160.8	7.2	22.8
	Nasacthin002, 500 µg	**N**	12	12	12
		Mean	401.6	16.2	55.2
		SD	183.7	14.3	38.0
	Nasacthin003, 500 µg	**N**	8	8	8
		Mean	648.7	28.7	82.1
		SD	141.0	12.9	23.9
	IV Synacthen, 1 µg	**N**	10	10	10
		Mean	391.4	10.2	39.5
		SD	90.5	4.6	13.9

Data displayed as mean and standard deviation (SD).

doi:10.1210/clinem/dgaa323

Table 3. Summary of Adult and Pediatric Glucocorticoid Response Following Tetracosactide Challenge by Single-Dose Administration of 250 µg or 1 µg IV Synacthen or 500 µg Nasacthin003

Parameter (All nmol/L)		Nasacthin003, 500 µg Intranasal			Synacthen, 250 µg IV			Synacthen, 1 µg IV		
		Plasma Cortisol	Salivary Cortisol	Salivary Cortisone	Plasma Cortisol	Salivary Cortisol	Salivary Cortisone	Plasma Cortisol	Salivary Cortisol	Salivary Cortisone
Peak glucocorticoid concentration (nmol/L)	***Adult*** Mean (± SD)	547 (± 106)	25.4 (± 9.2)	64.7 (± 19.4)	615 (± 51)	35.8 (± 10.7)	78.1 (± 11.5)	388 (± 67)	8.5 (± 4.5)	33.7 (± 11.6)
	Children Mean (± SD)	568 (± 81)	30.7 (± 9.0)	65.9 (± 14.9)	626 (± 54)	37.2 (± 9.0)	80.0 (± 15.9)	401 (± 80)	12.8 (± 4.9)	40.3 (± 10.9)
	All Mean (± SD)	556 (± 97)	27.7 (± 9.5)	65.2 (± 17.7)	620 (± 53)	36.5 (± 9.9)	79.0 (± 13.8)	392 (± 72)	9.8 (± 5.0)	35.6 (± 11.8)
Time at peak glucocorticoid concentration (min)	***Adult*** Median (Range)	75 (40-120)	90 (40-120)	90 (40-180)	120 (120-120)	120 (120-120)	120 (120-120)	30 (15-40)	40 (20-150)	40 (30-60)
	Children Median (Range)	75 (40-120)	75 (50-120)	90 (50-120)	105 (90-120)	120 (90-120)	120 (90-120)	40 (30-50)	40 (30-50)	40 (30-50)
	All Median (Range)	75 (40-120)	82.5 (40-120)	90 (40-180)	120 (90-120)	120 (90-120)	120 (90-120)	30 (15-50)	40 (20-150)	40 (30-60)
Mean concentration at 30 minutes Mean (±SD)		398 (± 61)	10.6 (± 4.2)	27.0 (± 10.1)	428 (± 53)	12.1 (± 4.4)	31.2 (± 9.4)	379 (± 61)	8.2 (± 4.0)	27.6 (± 10.8)
Mean concentration at 60 minutes Mean (± SD)		511 (± 88)	21.6 (± 7.1)	54.4 (± 13.4)	531 (± 59)	24.9 (± 7.5)	59.0 (± 12.1)	270 (± 75)	5.5 (± 4.1)	28.5 (± 10.4)
Number of subjects		Adults: N=19 (31 doses); Children: N=23			Adults: N=12; Children: N=11			Adults: N=21; Children: N=9		

SD, standard deviation.

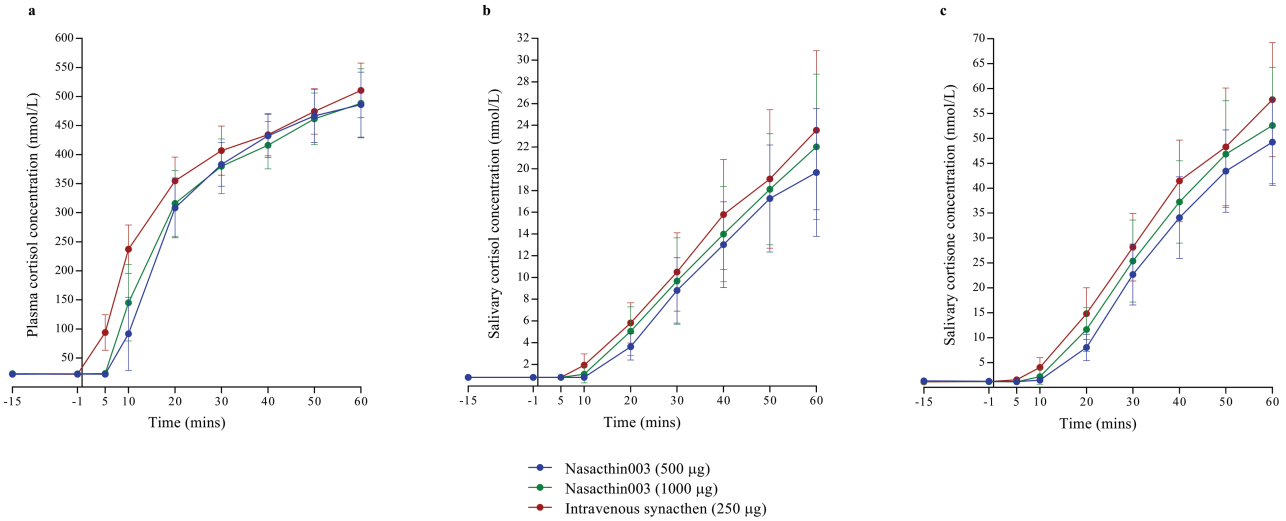

Figure 1. Mean (A) plasma cortisol, (B) salivary cortisol, and (C) salivary cortisone concentration over time following single dose administration of 250 µg IV Synacthen (red line), 500 µg Nasacthin003 (blue line), and 1000 µg Nasacthin003 (green line) in dexamethasone-suppressed healthy adult male participants. Standard deviation shown as error bars.

levels at 60 minutes were lower following intranasal administration. The mean difference (95% CI) for salivary cortisol and cortisone at 1000 µg was -1.5 nmol/L (-3.8 to 0.7) $P = 0.16$ and -5.2 nmol/L (-7.5 to -2.8) $P = 0.0005$, respectively, and at 500 µg was -4.5 nmol/L (-8.3 to -0.6) $P = 0.028$ and -8.4 nmol/L (-15.2 to -1.6) $P = 0.02$, respectively.

Reproducibility

The between-participant variability of cortisol response at 60 minutes, represented by the SD, calculated using all participants who received 500 µg Nasacthin003 (n = 46, excluding repeated observations), was 95 nmol/L for plasma cortisol, 7 nmol/L for salivary cortisol, and 14 nmol/L for salivary cortisone. The CVs were 18%, 32%, and 24%, respectively, and were significantly different (all pairwise comparisons $P < 0.0001$). For participants receiving 250 µg IV Synacthen (n = 23) the SD was 60 nmol/L for plasma cortisol, 7 nmol/L for salivary cortisol, and 12 nmol/L for salivary cortisone. The CVs were 11%, 30%, and 21%, respectively, and were significantly different (all pairwise comparisons $P < 0.0001$). The within-subject variability (CV) for the cortisol response at 60 minutes in 6 participants who received 500 µg Nasacthin003 on 3 separate occasions (S3 and S4) ranged from 1.0% to 11.2% for plasma cortisol, 1.1% to 31.4% for salivary cortisol, and 3.1% to 23.4% for salivary cortisone (Fig. 2). Additionally, we assessed the within-participant variability of plasma cortisol by calculation of a repeatability coefficient, which was 70.6 nmol/L at 30 minutes, such that 2 observations on the same individual would be expected to fall within -70.6 and + 70.6 nmol/L plasma cortisol for 95% of the time. At 60 minutes, the repeatability coefficient was 104.6 nmol/L. The repeatability coefficients calculated for the 60-minute concentration of salivary cortisol and cortisone were 10.3 and 21.1 nmol/L, respectively.

Nasacthin003 in children (S5)

All 24 participating children received 500 µg Nasacthin003 (Fig. 3). Half received 1 µg (low-dose) IV Synacthen as their comparator and the other half 145 µg/m^2 (high-dose) IV Synacthen and the results were compared with those in adults from S2 and S3 (Table 3). We found no significant difference in the peak and 30- and 60-minute plasma cortisol between adults and children for 500 µg Nasacthin003 or either of the 2 IV Synacthen doses. There was no significant difference in the 60-minute plasma cortisol between the 500 µg Nasacthin003 formulation compared with 250 µg IV Synacthen with mean difference (95% CI): -15.9 nmol/L (-46.1 to 14.4) $P = 0.26$. Similarly, we found no difference at 60 minutes when measuring the glucocorticoid response in saliva: mean difference (95% CI): -3.8 nmol/L (-7.7 to 0.2) $P = 0.06$ for salivary cortisol and -5.8 nmol/L (-14.7 to 3.1) $P = 0.17$ for salivary cortisone.

Absolute bioavailability of Nasacthin003 (S5)

A summary of the pharmacokinetic results from the pediatric cohort are shown in Table 4. Our calculated absolute bioavailability of 500 µg Nascthin003 against IV 145 µg/m^2 Synacthen was 0.143 (14.3%). The mean $AUC_{0-\infty}$ for Nasacthin003 was more than 3-fold higher than the 1 µg IV SST (Fig. 4) but approximately one-third that of the 145 µg/m^2 IV dose. Similarly, the mean Cmax for Nasacthin003 was higher than for 1 µg IV

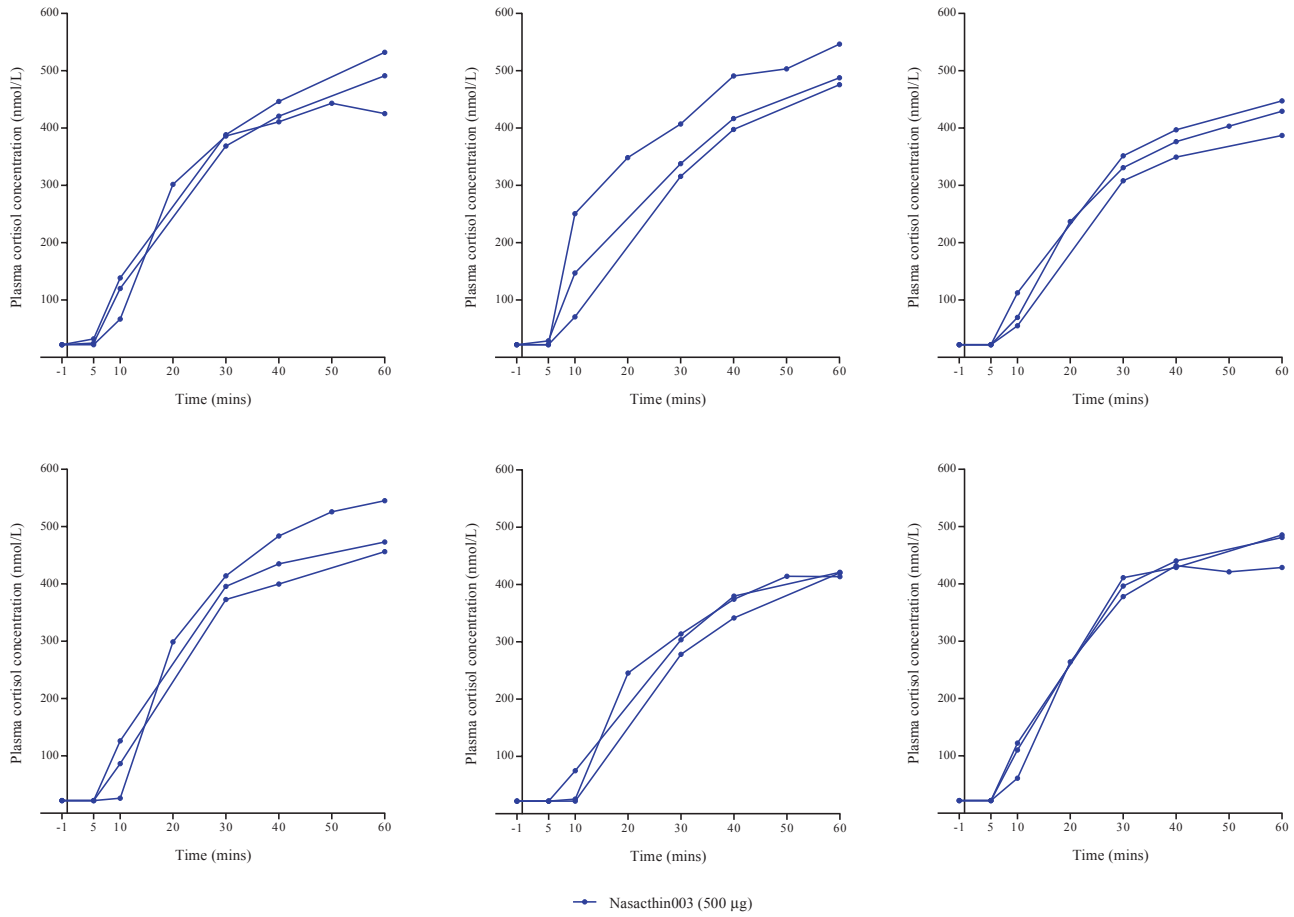

Figure 2. Individual plasma cortisol concentration over time graphs following 3 separate single-dose administrations of 500 µg Nasacthin003 in dexamethasone-suppressed healthy adult male participants.

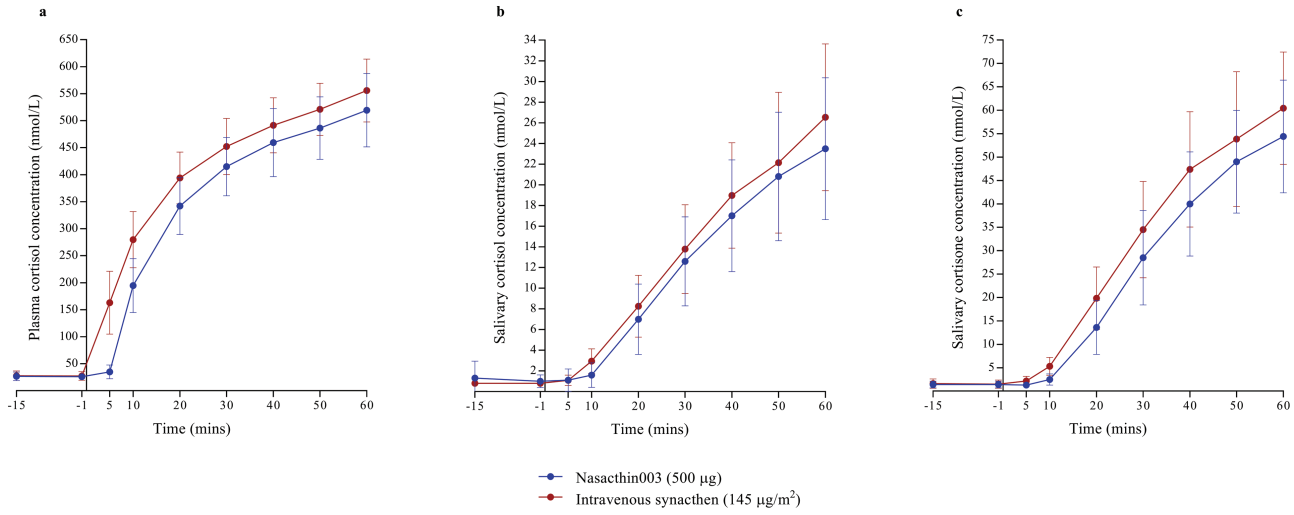

Figure 3. Mean (A) plasma cortisol, (B) salivary cortisol, and (C) salivary cortisone concentration over time following single-dose administration of 250 µg IV Synacthen (red line) and 500 µg Nasacthin003 (blue line) in dexamethasone-suppressed healthy children. Standard deviation shown as error bars.

Synacthen (433 vs 245 pg/mL) but lower than the 145 µg/m^2 IV SST (433 vs 6702 pg/mL).

Safety

We found nasal tetracosactide formulations to be well tolerated by adults and children. We administered a total of 70 doses of Nasacthin003 to 46 subjects (22 adults, 24 children) with no serious adverse events.

Table 4. Summary of Pediatric Pharmacokinetic Parameters for Plasma Tetracosactide Following Administration of IV Synacthen and Nasacthin003 in Study 5

	Nasacthin003	Synacthen	
PK parameter GM (95% CI)	**500 µg, Intranasal**	**250 µg, IV**	**1 µg, IV**
Tetracosactide C_{max}(pg/mL)	433 (324-602)	6702 (4346-10,335)	245 (199-302)
$AUC_{0\text{-}Last}$ (pg/mL × min)	16672 (12,845-20,192)	75329 (45,537-124,611)	3409 (2691-3904)
$AUC_{0\text{-}\infty}$ (pg/mL × min)	20934 (16,274-24,156)	77058 (46,899-126,611)	6212 (4789-8058)
$F_{0\text{-}\infty}$	0.143 (0.082-0.249)	-	-
T_{max} (min) Median (range)	10 (5-20)	-	-

$AUC_{0\text{-}\infty}$, area under the concentration-time curve extrapolated to infinity; $AUC_{0\text{-}Last}$, area under the concentration-time curve to last measurable concentration; CI, confidence interval; C_{max} maximum plasma concentration; F, bioavailability; GM, geometric mean; T_{max}, time of maximum concentration.

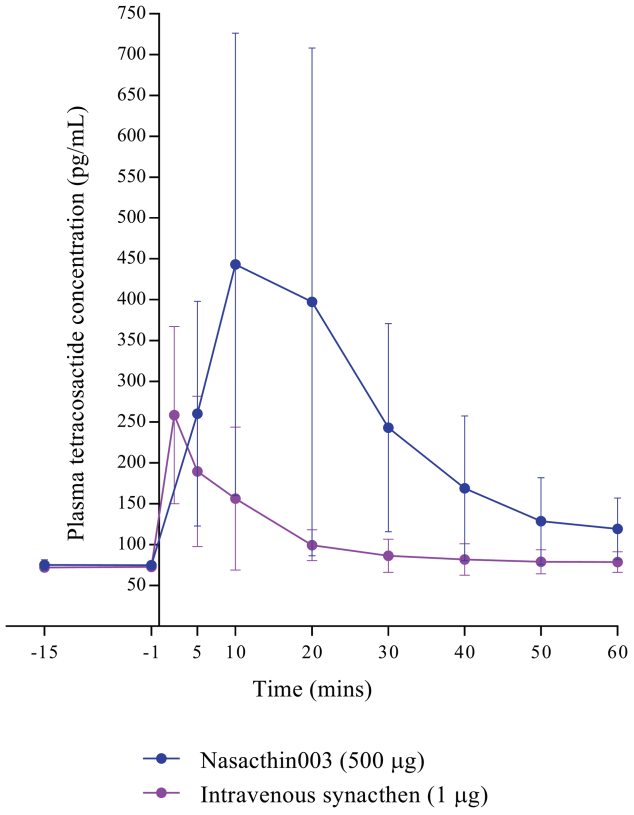

Figure 4. Mean plasma tetracosactide concentration over time following single-dose administration of 1 µg IV Synacthen (purple line) and 500 µg Nasacthin003 (blue line) in dexamethasone-suppressed healthy pediatric subjects. Standard deviation shown as error bars.

Although 32% of participants did not report adverse events, the 68% who did experienced events that were anticipated following nasal drug administration; specifically, watery eyes, coughing, sneezing, and a vinegary taste (acetic acid is a constituent of the drug product). These were all mild, with full and rapid resolution, and considered to be treatment-related. In addition, 12 doses of Nasacthin001, 12 doses of Nasacthin002, and 11 doses of 25-µg and 100-µg Synacthen were all administered intranasally with no serious adverse events reported. On poststudy questionnaires, the majority of participants (77%) reported nasal administration to be "easy" or "very easy," receiving the nasal drug as "no problem" or "slightly unpleasant" (86%), and "much the same," "better," or "much better" compared with the IV administration (71%).

Discussion

Over our 4 open-label multiarm, sequence-randomized, crossover, pharmacodynamic studies, we found 500 µg Nasacthin003 to demonstrate an equivalent plasma cortisol response to the IV SST at 60 minutes. Our repeatability study demonstrated between- and within-individual reproducibility of the test and the glucocorticoid response in children was indistinguishable from that of adults. Our early studies tested 6 different doses from 4 formulations of tetracosactide. The response threshold required for maximal adrenal stimulation was not reached with intranasal doses derived from the commercially available IV formulation at 25 and 100 µg. The current 250 µg SST is widely recognized to deliver a supraphysiological adrenal stimulus. Studies attempting to quantify the ACTH level required to maximally stimulate the adrenal gland are decades old and hampered by assay issues but quote between 60 and 80 pg/mL, approximately one-half of the endogenous peak values seen in healthy subjects in the early morning (9, 22). This is considerably lower than the levels generated following IV administration with 250 µg Synacthen (1580-66,000 pg/mL) but closer to those measured following administration of 1 µg (3.5-1920 pg/mL), leading some to advocate the 1-µg test as more physiological (9, 22). Groups comparing the low- and high-dose SST have demonstrated an equivalent cortisol response at 30 minutes, indicating that 1 µg is adequate to achieve maximal adrenal stimulation, but responses diverge

thereafter, thought to be due to continued adrenocortical stimulation with higher doses (8-10, 14). In our studies, the addition of the nasal drug enhancer, chitosan, and dose escalation both significantly improved absorption of tetracosactide and the resultant glucocorticoid response, yielding plasma tetracosactide levels sufficient to maximally stimulate the adrenal cortex. Additionally, our PK analysis demonstrated tetracosactide levels in excess of those seen after stimulation with 1 µg of IV Synacthen. The 500 µg Nasacthin003 dose was chosen for further study because it demonstrated that it exceeded the threshold for maximal adrenal stimulation, with no difference in plasma cortisol levels at 60 minutes when compared with the 250-µg IV test.

The SDs and CVs for the cortisol response at 60 minutes for 500 µg Nasacthin003 were higher than for 250-µg IV Synacthen. To our knowledge between-participant repeatability has not previously been reported for the 250-µg IV SST. We demonstrated CVs at 60 minutes after stimulation, both within and between participants, for the intranasal preparation, which were better than that previously published for low-dose IV Synacthen. Within-participant CVs ranged from 3.0% to 16.4% in 1 study and 46.7% to 57.2% in another, with between-participant being 28.0% to 48.6% (23, 24).

Analogues of ACTH, including ACTH(1-24) (Synacthen/Cosyntropin), have been administered nasally in historical studies for treatment, but the investigation for diagnostic purposes is novel (18, 19). Synacthen is inactivated in the gastrointestinal tract by proteolytic enzymes and therefore needs parenteral administration. It is a large polar molecule with a molecular mass of 2932 g/mol. These are not ideal properties for a nasal drug, which tend to be small and lipophilic. Drugs with a molecular weight below 1000 g/mol generally do not require adjuvants for effective absorption, but larger peptide molecules benefit from the increased drug residence times by slowing mucociliary clearance and modifying transmembrane transportation (17). The nasal route for proteins of a similar size has been previously investigated. For example, desmopressin (1069 g/mol) has a bioavailability of ~ 10% of the IV route and salmon calcitonin (3455 g/mol) has a relative bioavailability of between 3.9% and 7.9% when delivered with the enhancer sodium tauro-24,25-dihydrofusidate (25). Improved Synacthen absorption with the addition of nasal drug enhancers (sodium glycocholate and bacitracin) has been demonstrated in a murine study, but the combination of tetracosactide and chitosan to promote nasal absorption is novel (20).

The method of diagnosis of AI by the SST has been much debated. Points of discussion include: what dose of Synacthen to administer, which cortisol assay platform to analyze on, and what sampling times and diagnostic criteria to use. Our recently published international survey on SSTs reported widespread variation, including cortisol sampling timings and interpretative thresholds (3). Our noninvasive test affords the opportunity for research in large cohorts where controversies exist over diagnosis and management, in particular monitoring and testing of patients on glucocorticoids at risk of AI. Termed tertiary AI, this is now thought to be the most common cause of AI in the Western world (2). Nearly 1% of the UK adult population are on oral glucocorticoids at any 1 time and 4.5% of UK children are prescribed inhaled glucocorticoids, 10% above the recommended dose (26, 27). Biochemical evidence of AI has been reported in up to 40% of children taking inhaled glucocorticoids (28-30). The potential global clinical utility of a test that can be administered in the outpatient setting or the community offers the potential for cost savings by negating the need for day case admission to hospital.

The relationship between plasma cortisol and salivary cortisol and cortisone has been widely published (13-15). Salivary glucocorticoid estimation is growing in popularity because salivary glucocorticoids reflect plasma cortisol and sampling is more pleasant for patients. Late night salivary cortisol testing is a first-line investigation in Cushing syndrome (11). Cortisone is the more abundant glucocorticoid in saliva as plasma free cortisol and salivary cortisol are rapidly converted to inactive cortisone by 11ß-hydroxysteroid dehydrogenase type 2, making it better suited for the diagnosis of AI (13, 15, 16). Sixty minutes is the optimal timing of salivary glucocorticoid sampling following stimulation with IV Synacthen (14). The salivary cortisol and cortisone responses in children were similar between Nasacthin003 and IV Synacthen but slightly lower in adults. This may represent a dose effect in adults although, as discussed previously, the evidence suggests Nasacthin003 exceeds the threshold for maximal stimulation. It will be important, when introducing the noninvasive intranasal test into clinical practice, to perform clinical trials to define normal ranges for salivary cortisol and cortisone and document the sensitivity and specificity to diagnose adrenal insufficiency.

There are a number of limitations of our studies. All our studies were conducted in healthy adult and child volunteers who were dexamethasone suppressed. However, this conferred considerable advantage: uniformity between subjects and a clean baseline from which to assess response to tetracosactide formulations and perform PK modelling. We acknowledge the lack of a patient cohort, although our intention was to demonstrate equivalence with a diagnostic agent that has been in widespread use for more than 30 years, not redefine the SST. We used different assay platforms for plasma

cortisol (immunoassay) and the salivary glucocorticoids (liquid chromatography MS). This was pragmatic and reflected analytical techniques employed in clinical practice. We used an in-house assay for plasma cortisol analysis and sent salivary samples to a laboratory with significant expertise in salivary steroid analysis, as this was not available at the time in our institution. To be able to combine the results the same assays were used for all samples across the 5 studies.

In conclusion, we have developed a noninvasive alternative to the SST, with the administration of intranasal 500 µg Nasacthin003 and measurement of either salivary cortisone or cortisol at baseline and 60 minutes. Nasacthin003 was well tolerated and in 70 doses administered to 46 participants, only minor, short-lived, and anticipated adverse events were experienced. The new test could positively affect the growing demand for adrenal function testing. It could be conducted globally in community and outpatient settings, with potential cost savings, and reduced health care burden to patients.

Acknowledgments

We thank the research nurses, laboratory staff, clinical trial pharmacy staff, and Research and Development staff at both sites for their support of the studies. We are grateful to the data monitoring committee, study sponsors, and in particular the volunteers who participated in these studies. We especially acknowledge the support of Dr. Peter Watts (Phormulate Consulting Ltd) for his expert advice on the manufacture of nasal drug formulations and Dr. Jane Dalley, who assisted in the initial setting up of the studies, but died before she could see the fruits of her labor in print.

Financial Support: The Children's Hospital Charity (CA09018, CA110916), Medical Research Council (CiC round 4, CiC round 6), Academy of Medical Sciences (SGCL15), and British Society for Paediatric Endocrinology and Diabetes (Annual Research Award 2015).

Clinical Trial Information: The studies were registered with EudraCT (2009-013355-29 and 2012-003241-15).

Author Contributions: C.J.E., N.P.W., T.N.J., and R.J.R. designed the studies. C.J.E., R.V., and A.S.C. enrolled participants and conducted the studies. E.H.K. validated and performed the tetracosactide assay. B.K. performed the salivary glucocorticoid analysis. T.N.J., R.N.T., C.J.E., R.V., and A.S.C. analyzed the data. C.J.E. wrote the paper and all authors contributed to reviewing and editing the manuscript.

Additional Information

***Correspondence and Reprint Requests*:** Charlotte Elder, Academic Unit of Child Health, Sheffield Children's Hospital, Western Bank, Sheffield S10 2TH, United Kingdom. Email: c.j.elder@sheffield.ac.uk.

Disclosure Summary: C.J.E. and N.P.W. have a patent application for Nasacthin003. R.J.R. is a director of Diurnal Group Plc. and holds shares. T.N.J. holds shares in Diurnal Group Plc. R.V., R.N.T., E.H.K., B.K., and A.S.C. report no conflicts of interest in this work.

Data Availability: All data generated or analyzed during this study are included in this published article or in the data repositories listed in References.

References

1. Bornstein SR, Allolio B, Arlt W, et al. Diagnosis and treatment of primary adrenal insufficiency: an Endocrine Society Clinical Practice Guideline. *J Clin Endocrinol Metab*. 2016;**101**(2):364-389.
2. Charmandari E, Nicolaides NC, Chrousos GP. Adrenal insufficiency. *Lancet*. 2014;**383**(9935):2152-2167.
3. Cross AS, Helen Kemp E, White A, et al. International survey on high- and low-dose synacthen test and assessment of accuracy in preparing low-dose synacthen. *Clin Endocrinol (Oxf)*. 2018;**88**(5):744-751.
4. Elder CJ, Sachdev P, Wright NP. The short Synacthen test: a questionnaire survey of current usage. *Arch Dis Child*. 2012;**97**(10):870-873.
5. Bleicken B, Hahner S, Ventz M, Quinkler M. Delayed diagnosis of adrenal insufficiency is common: a cross-sectional study in 216 patients. *Am J Med Sci*. 2010;**339**(6):525-531.
6. Kazlauskaite R, Evans AT, Villabona CV, et al. Corticotropin tests for hypothalamic-pituitary-adrenal insufficiency: a metaanalysis. *J Clin Endocrinol Metab*. 2008; **93**(11): 4245-4253.
7. Ospina NS, Al Nofal A, Bancos I, et al. ACTH stimulation tests for the diagnosis of adrenal insufficiency: systematic review and meta-analysis. *J Clin Endocrinol Metab*. 2016;**101**(2):427-434.
8. Alía P, Villabona C, Giménez O, Sospedra E, Soler J, Navarro MA. Profile, mean residence time of ACTH and cortisol responses after low and standard ACTH tests in healthy volunteers. *Clin Endocrinol (Oxf)*. 2006;**65**(3):346-351.
9. Darmon P, Dadoun F, Frachebois C, et al. On the meaning of low-dose ACTH(1-24) tests to assess functionality of the hypothalamic-pituitary-adrenal axis. *Eur J Endocrinol*. 1999;**140**(1):51-55.
10. Dickstein G, Shechner C, Nicholson WE, et al. Adrenocorticotropin stimulation test - effects of basal cortsiol level, time of day, and suggested new sensitive low-dose test. *J Clin Endocrinol Metab*. 1991; **72**(4): 773-8.
11. Nieman LK, Biller BM, Findling JW, et al. The diagnosis of Cushing's syndrome: an Endocrine Society Clinical Practice Guideline. *J Clin Endocrinol Metab*. 2008;**93**(5):1526-1540.
12. Mak IYF, Au Yeung BYT, Ng YW, et al. Salivary cortisol and cortisone after low-dose corticotropin stimulation in the diagnosis of adrenal insufficiency. *J Endocr Soc*. 2017;**1**(2):96-108.
13. Perogamvros I, Keevil BG, Ray DW, Trainer PJ. Salivary cortisone is a potential biomarker for serum free cortisol. *J Clin Endocrinol Metab*. 2010;**95**(11):4951-4958.
14. Elder CJ, Harrison RF, Cross AS, et al. Use of salivary cortisol and cortisone in the high- and low-dose synacthen test. *Clin Endocrinol (Oxf)*. 2018;**88**(6):772-778.
15. Debono M, Harrison RF, Whitaker MJ, et al. Salivary cortisone reflects cortisol exposure under physiological conditions and after hydrocortisone. *J Clin Endocrinol Metab*. 2016;**101**(4):1469-1477.
16. Blair J, Lancaster G, Titman A, et al. Early morning salivary cortisol and cortisone, and adrenal responses to a simplified low-dose short Synacthen test in children with asthma. *Clin Endocrinol (Oxf)*. 2014;**80**(3):376-383.
17. Davis SS, Illum L. Absorption enhancers for nasal drug delivery. *Clin Pharmacokinet*. 2003;**42**(13):1107-1128.

18. Hiroi N, Ichijo T, Ueshiba H, Miyachi Y. Intranasal administration of adrenocorticotropin-(1–24) stimulates adrenocortical hormone secretion. *Journal of Clinical Endocrinology & Metabolism* 2002; **87**(4): 1750-3.
19. Jeffcoate WJ, Phenekos C, Ratcliffe JG, Williams S, Rees L, Besser GM. Comparison of the pharmacokinetics in man of two synthetic ACTH analogues: alpha1-24 and substituted alpha1-18 ACTH. *Clin Endocrinol (Oxf)*. 1977;**7**(1):1-11.
20. Wüthrich P, Martenet M, Buri P. Effect of formulation additives upon the intranasal bioavailability of a peptide drug: tetracosactide (ACTH1-24). *Pharm Res*. 1994;**11**(2):278-282.
21. European Medicines Agency. Committee for Medicine Products for Human Use (CHMP) - July 2010 plenary meeting monthly report. London, UK https://www.ema.europa.eu/en/events/committee-medicinal-products-human-use-chmp-19-22-july-2010. Published July 29, 2010. Accessed March 12, 2020.
22. Oelkers W, Boelke T, Bahr V, Exner P, Faust B, Harendt H. Dose-response relationships between plasma adrenocortictropin (ACTH), cortisol, aldosterone and 18-hydroxycorticosterone after injection of ACTH-(1–39) or human corticotropin-releasing hormne in man. *J Clin Endocrinol Metab*. 1988; **66**(1): 181-6.
23. Bridges NA, Hindmarsh PC, Pringle PJ, Honour JW, Brook CGD. Cortisol, androstenedione (A4), dehydroepiandrosterone sulphate (DHEAS) and 17 hydroxyprogesterone (170HP) responses to low doses of (1–24)ACTH. *J Clin Endocrinol Metab*. 1998; **83**(10): 3750-3.
24. Park YJ, Park KS, Kim JH, Shin CS, Kim SY, Lee HK. Reproducibility of the cortisol response to stimulation with the low dose (1 microg) of ACTH. *Clin Endocrinol (Oxf)*. 1999;**51**(2):153-158.
25. Lee WA, Ennis RD, Longenecker JP, Bengtsson P. The bioavailability of intranasal salmon calcitonin in healthy volunteers with and without a permeation enhancer. *Pharm Res*. 1994;**11**(5):747-750.
26. Thomas M, Turner S, Leather D, Price D. High-dose inhaled corticosteroid use in childhood asthma: an observational study of GP prescribing. *Br J Gen Pract*. 2006;**56**(531):788-790.
27. van Staa TP, Leufkens HG, Abenhaim L, Begaud B, Zhang B, Cooper C. Use of oral corticosteroids in the United Kingdom. *QJM*. 2000;**93**(2):105-111.
28. Paton J, Jardine E, McNeill E, et al. Adrenal responses to low dose synthetic ACTH (Synacthen) in children receiving high dose inhaled fluticasone. *Arch Dis Child*. 2006;**91**(10):808-813.
29. Todd GR, Acerini CL, Ross-Russell R, Zahra S, Warner JT, McCance D. Survey of adrenal crisis associated with inhaled corticosteroids in the United Kingdom. *Arch Dis Child*. 2002;**87**(6):457-461.
30. Hawcutt DB, Jorgensen AL, Wallin N, et al. Adrenal responses to a low-dose short synacthen test in children with asthma. *Clin Endocrinol (Oxf)*. 2015;**82**(5):648-656.