Article:
Tonge, J.J., Keevil, B.G., Craig, J.N. et al. (3 more authors) (2022) Main salivary steroid collection in children under conditions replicating home sampling. Journal of Clinical Endocrinology and Metabolism, 107 (11). dgac419. pp. 3128-3136. ISSN 0021-972X

https://doi.org/10.1210/clinem/dgac419

Main Title: Salivary steroid collection in children under conditions replicating home sampling

Short title: Salivary steroid collection in young children

Key Words: Adrenal insufficiency, Salivary, Cortisol, Cortisone, Stability, Collection device

Joseph J. Tonge BSc [1], Brian G. Keevil PhD [2], Jessica N. Craig MBChB [1], Martin J. Whitaker PhD[3], Richard J. Ross MD[3], Charlotte J. Elder PhD [3,4*]

[1] Academic Unit of Medical Education, Medical School, University of Sheffield, Sheffield, UK

[2] Department of Clinical Biochemistry, University Hospital of South Manchester NHS Trust, Manchester, UK

[3] Department of Oncology & Metabolism, University of Sheffield, Sheffield, UK

[4] Department of Endocrinology, Sheffield Children's NHS Foundation Trust, Sheffield, UK

* Corresponding author: c.j.elder@sheffield.ac.uk, +44 114 305 3282. Room 10, The Damer Street Building, Sheffield Children's Hospital, Sheffield, UK. ORCID ID: 0000-0003-2390-5593.

Funding: CJE and BK were supported by funding from UKRI Biomedical Sciences Innovation Scholar secondments references MR/W002795/1 and 75781 respectively. We are grateful to the Society for Endocrinology who part funded this with a student research grant for JJT.

Disclosure summary: There is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported. JJT, BGK, JNC, MJW, RJR and CJE have nothing to declare

ClinicalTrails.gov Registration Number: NCT05350982

Abstract:

Objective: Measurement of salivary glucocorticoids is an accepted method for testing adrenal function but there is little data on stability during home collection. Current salivary collection techniques require active participation or present a choking hazard and are unsuitable for young children. We sought to: compare different salivary collection methods; assess stability of salivary glucocorticoids under conditions replicating home collection; assess patient tolerability and caregiver acceptability of a salivary collection device for young children, a swab encased in an infant pacifier (SaliPac™).

Methods: Six healthy adults collected salivary samples using a Salivette® Cortisol, passive drool and SalivaBio at night, waking and 3pm for five days. Time to collect 1ml saliva using the SalivaBio and SaliPac and caregiver acceptability were assessed in 30 children <6 years. Saliva was stored at 4°C, room temperature and 50°C for 24, 48, 72 hours and a week to replicate potential postage conditions. Salivary cortisol and cortisone concentrations were measured by mass spectrometry.

Results: There was no difference in salivary glucocorticoid concentrations using the three collection methods. Salivary cortisol and cortisone were stable for 72 hours at room temperature and 4°C, and repeated freeze-thaw cycles did not cause significant degradation. In children <6 years the SalivaBio and SaliPac were well tolerated and collected sufficient saliva for salivary steroid analysis in <4 minutes.

Conclusions: Salivette, passive drool and SalivaBio collect samples with comparable salivary cortisol and cortisone concentrations, which are stable under conditions replicating home collection. SaliPac is an acceptable device for salivary sampling in young children.

Introduction:

The measurement of glucocorticoids in salivary samples is becoming an accepted method for diagnosing adrenal insufficiency and Cushing's syndrome (1, 2, 3). In the salivary gland, plasma free cortisol is rapidly converted to inactive cortisone by 11ß-hydroxysteroid dehydrogenase-type-2 and there is a close correlation between biologically active free serum cortisol and salivary cortisol and cortisone (4, 5). Salivary sampling offers a non-invasive collection technique, enabling home sampling with postage to the laboratory, facilitating tailored capture of steroid circadian rhythm and an improved patient experience (6). Salivary measurement allows assessment of adrenal status in patients with altered cortisol binding proteins, where total serum cortisol is difficult to interpret, such as pregnancy, liver disease and oral contraceptive pill users (1).

There are numerous salivary collection techniques using both absorptive methods (cotton buds, filter paper, eye sponges, cotton dental rolls, generic swabs and specialised swabs) and aspiration (pipette and syringe connected to tubing). The Salivette® (Sarstedt, Germany) and passive drool are widely used (7, 8), with Salivette® Cortisol (Sarstedt, Germany) developed in response to studies demonstrating that cotton significantly compromises salivary cortisol samples (6, 9, 10). The Salivette presents a choking hazard and both Salivette and passive drool require active patient participation, making them unsuitable for young children. Aspiration is reported to result in high rates of insufficient salivary volumes for analysis and risks damaging delicate mucous membranes leading to blood contaminated samples (6, 11). Our experience is that children younger than six years are unlikely to comply with passive drooling. The SalivaBio Infants and Children's swabs (Salimetrics, USA) are synthetic and

intended for children aged under six months and six months to six years respectively; however, there is a paucity of data on their efficacy, reliability, and tolerability (6, 12).

Accurate diagnostic screening for hypo- and hyper-cortisolism requires sampling outside of traditional working hours, either first thing in the morning for adrenal insufficiency or last thing at night for Cushing's. Early morning serum cortisol as a screening test for adrenal insufficiency is flawed as there is a requirement for the patient to attend a healthcare setting for testing, by which time the natural cortisol peak experienced soon after waking is waning. This results in poor predictive value, leading to recommendations for time- and resource-consuming invasive diagnostic tests (13). It is therefore impractical to use venous sampling in many settings and home collection of salivary glucocorticoids, with postage of samples to the laboratory, addresses this issue. Previously published work has reported no significant change in salivary cortisol concentration when stored at 4^{o}C or 20^{o}C for up to 84 days, but that salivary steroids are more stable when stored refrigerated compared to room temperature (14, 15). To our knowledge there are no publications reporting salivary cortisone sample stability. To facilitate the global utility of salivary steroid measurement, with collection in rural and remote settings, data are required on the stability of both salivary glucocorticoids stored under different conditions and temperatures.

There is increasing recognition of the risk of adrenal insufficiency due to glucocorticoid suppression in neonates and children (16, 17, 18). Furthermore, with both steroid prescriptions and requests for adrenal function testing rising in the paediatric population (19, 20), there is a requirement for a simple, effective and patient friendly screening test for adrenal insufficiency suitable for infants and young children. Lack of a robust validated

salivary collection technique for this cohort has hampered salivary steroid research, specifically, the construction of paediatric normative reference data. The ability to measure salivary steroids in this young cohort would facilitate diagnosis of adrenal insufficiency, but a safe and reliable method for collecting saliva is required.

To support the development of paediatric salivary steroid measurement, we have investigated different collection methods and salivary glucocorticoid stability under conditions simulating home collection.

Methods

Salivary steroid collection techniques studied: We studied three salivary collection techniques: Salivette Cortisol (Figure 1a), passive drool (Figure 1b) and SalivaBio (Figure 1c). The Salivette® Cortisol swab (Sarstedt, Germany) is a biocompatible synthetic fibre roll chewed for two minutes then placed in a transportation tube. In the laboratory, the Salivette is centrifuged in the transportation tube at 1,500-3,000g for ten minutes at 4°C. The saliva is frozen, preventing bacterial growth, until analysis (21). Passive drool uses a straw or collection aid to guide saliva into a receptacle with volume markers (22). Saliva is viscous and contains air bubbles, making it difficult to gauge the exact volume collected by passive drool (23). The SalivaBio (Salimetrics, USA), is a 12cm synthetic non-cotton polymer swab. Approximately 2cm is placed in the mouth with the other end held by the patient or caregiver until the lower third is saturated, taking on average 2.5 minutes (24). The SalivaBio is centrifuged after collection in a 'swab basket' allowing drainage of saliva through the bottom of the tube.

Salivary collection technique comparison study: To compare the different techniques we conducted a prospective study in six healthy adult volunteers. The exclusion criteria were: pregnancy; known alcohol or drug misuse; current or recent (within six months) smoker or vaper; symptoms of uncontrolled infection, past or present history of salivary gland or oral mucosal pathology; protein losing disorders; liver disease; current or recent treatment with any formulation of glucocorticoids or drugs known to affect cortisol binding globulin including oestrogen, loperamide and opiates; recent liquorice ingestion; or history of hypothalmo-pituitary-adrenal axis pathology. We provided participants with Salivette Cortisol swabs, SalivaBio swabs, passive drool kits (SaliCap (IBL international, Germany)) and written instructions. Participants provided salivary samples using each of the techniques, in a randomised pre-determined order, at three specified time points: awakening, 3pm and on retiring for bed, on five days (45 samples per participant). We asked participants not to eat, drink, brush or floss their teeth for 60 minutes prior to sampling. Samples collected at home were immediately refrigerated, then centrifuged and the saliva frozen in the laboratory the following day until thawing for batch analysis.

Development of a new salivary collection technique for young children – SaliPac™: We modified the SalivaBio swab to create an alternative collection method for use in neonates, infants and young children, the SaliPac™ (Figure 2). Using sterile scissors, we cut 8cm of the swab and made a 1cm incision in the pacifier teat, securing the swab within the teat with 5mm protruding to facilitate salivary absorption. An indicator line was added at 6cm using a chromatography pen providing a visual prompt at ~1mL of saliva collected. In a pilot study we compared the salivary cortisol and cortisone levels after collection with the SaliPac and

SalivaBio and found SalivaBio modification and introduction of the pacifier did not impact salivary glucocorticoid recovery.

Salivary steroid stability and freeze-thaw studies: To mimic postage of salivary samples to a central laboratory we examined the effects of time and temperature on salivary steroid stability. We provided the same six healthy adult volunteers with SaliCaps to collect saliva by passive drool at 9am or 4pm. Saliva was separated into 16 aliquots by pipetting a minimum of 250ul onto a SalivaBio swab in a transportation tube. Four reference samples from each participant were immediately frozen at -80°C (time zero) and other samples stored at either 4°C, room temperature or 50°C for 24 hours, 48 hours, 72 hours or one week and then frozen. Additionally, we examined salivary glucocorticoids stability after one, two, three and four freeze-thaw cycles. Each thaw was for one hour and each freeze cycle lasted one hour at -80°C.

Salivary cortisol and cortisone assay: We quantified salivary cortisol and cortisone in all the salivary samples by liquid chromatography-tandem mass spectrometry (LCMS) using a Waters Xevo TQ-MS mass spectrometer and a Waters Aquity LC system with electrospray source operated in positive-ionization mode. For both salivary glucocorticoids lower limits of quantitation were 0.83 nmol/L. Inter-assay coefficient of variability (CV) were 9.7% and 10.3% and intra-assay CV were 9.3% and 7.9% at <2.76-52.42 nmol/L of salivary cortisol and 2.76-96.57 nmol/L of cortisone, respectively (25, 26).

SalivaBio and SaliPac feasibility study: A prospective single site, feasibility study in young children to assess the utility and tolerability of the SalivaBio and SalivaBio + pacifier (SaliPac)

was performed. Our objectives were to determine whether the devices can collect 0.2ml of saliva, the minimum salivary volume required for LCMS analysis, in an acceptable time frame; and determine participant acceptability and ease of use for caregivers. We recruited 30 children under six years from the outpatient department or inpatient wards at Sheffield Children's NHS Foundation Trust, UK; six from each age band: <6 months, 6-12 months, 1-2 years, 2-3 years and 3-5 years. Participants were excluded only if they had a mouth pathology precluding painless salivary collection. Data were collected on previous or current pacifier use, finger/thumb sucking, breastfeeding and whether the participant was being fed by nasogastric tube. Participants sampled using the SalivaBio and SaliPac in a pre-determined randomised order. The salivary samples were collected by the caregiver with a member of the study team timing the collection and noting any difficulties. We recorded two timings: first, the total time taken from the swab entering the child's mouth to the indicator line moving (i.e., completion of the 1ml sampling) which we have called "real-world collection time". This represents the length of time for a salivary collection when undertaken at home, inclusive of any additional time e.g., when the child spits out the swab or refuses to have it back in their mouth. The second timing was the time taken for the indicator line to move, but with the clock stopped each time the device was expelled from the mouth. We called this "actual collection time" as it reflects solely the time taken for the swab to absorb 1mL of saliva. The difference in real-world and actual collection times was used as a surrogate marker of device tolerability. We randomly selected ten participants to simulate home conditions by carrying out sampling using only our instruction leaflet, with no guidance from the study team. Following collection, we reweighed the swabs (on a single set of calibrated three decimal place scales), allowing volume of saliva to be calculated from the difference in mass of the tube before and after collection. Caregivers were asked to complete a simple 21-

question questionnaire developed in-house [(supplementary material(27)] to assess the acceptability of the collection techniques.

Ethics: The studies were approved by the Yorkshire and Humber Sheffield Research Ethics committee and written informed consent obtained from all caregivers. ClinicalTrails.gov NCT05350982.

Statistical analysis: The data were analysed using IBM® SPSS® Statistics (version 27). Differences in collection techniques were quantified by pairwise comparison, using a one samples t-test on both pairs. Statistical significance was set at 5% ($p<0.05$). Mean, standard deviation (SD) and range for binary outcomes and medians in cases of skewed distribution were calculated. Percentage median differences were used to compare samples collected in the stability study. A practical limit of >10% of baseline concentration was considered as clinically significant degradation as this exceeds the inter-assay CV for the assays. Percentage median difference was calculated for non-binary results requiring comparison to baseline concentration. Paired samples t-test were used to compare effect of storage conditions on baseline salivary steroid concentration.

Results

Salivary collection technique comparison study: The six healthy adult volunteers (2F) who participated in the cross validation and stability studies had a mean age of 46 years (range 32-63). A total of 270 salivary samples, 45 from each of the six participants, were collected. We compared salivary cortisol and cortisone values from the three techniques (Table 1) and found

no significant difference. When comparing SalivaBio and Salivette there was a mean difference of -0.226 nmol/L (95% CI -0.957 to 0.410) and -1.516 nmol/L (95% CI -4.242 to 1.210) in the measurement of salivary cortisol and cortisone, respectively. The comparison between SalivaBio and passive drool produced a mean difference of -0.751 nmol/L (95% CI -2.928 to 1.425) and 0.871 nmol/L (95% CI -3.001 to 4.745) in salivary cortisol and cortisone, respectively. The mean differences for salivary cortisol and cortisone when collected using the Salivette and passive drool were 0.214 nmol/L (95% CI -1.087 to 1.729) and 1.029 nmol/L (95% CI -2.280 to 4.338) respectively.

Salivary steroid stability and freeze-thaw studies: We collected 96 samples in total; 24 stored for each of the following times: 24, 48, 72 hours and one week; and 32 at each of the following conditions: room temperature (RT); refrigerated or 50°C. The mean salivary cortisol and cortisone concentration of the reference samples frozen immediately after collection were 3.675 nmol/L (SD 1.914) and 21.70 nmol/L (SD 8.21) respectively. Overall time and temperature increased degradation of salivary glucocorticoids (Figure 3). A significant difference in mean salivary cortisol, indicating statistically significant degradation, was observed in samples stored at both RT (p=0.041) and 50°C (p=0.044) for one week (Table 2). Salivary cortisone showed significant degradation at all time points at 50°C (p=0.048 at 24 hours, p=0.002 at 48 hours, p=0.009 at 72 hours and p=0.012 at one week) and when stored at RT for a week (p=0.012) (Table 3). There was a less than 10% median change in salivary cortisol out to a week for samples kept at RT (% median change (95% CI) -2.02% (1.61-9.75)) and refrigerated (-8.82% (-2.22 to -22.99)). This was similar for samples stored at 50°C until 72 hours (-6.54% (-0.10 to -20.62)), but by a week there was significant degradation (-20.99% (-2.5 to -31.14)). Salivary cortisone degraded faster than salivary cortisol at 50°C with samples

having a greater than 10% median change by 48 hours (-11.74% (-7.54 to -16.62)) and showing marked degradation by one week (-25.62% (-19.80 to -42.39)). Salivary cortisone samples showed minimal degradation when refrigerated out to a week (-3.40 (-0.63 to -12.32) and at RT for 72 hours (-5.27 (-3.08 to -13.02)) but had degraded by just over 10% median change by one week (-10.83% (-8.02 to -14.23)). Repeated freeze-thaw cycles did not result in significant degradation with the maximum median change in cortisol -2.69% (-18.5 to -3.29) and in cortisone -1.69% (-12.21 to -3.45), both following four cycles (Figure 4).

SalivaBio and SaliPac feasibility study: All 30 children (13F) recruited to our paediatric feasibility study successfully completed their visits. Participants had a mean age of 1.98 years (SD 1.6 years, range 11 days to 5 years 10 months). Half of the participants had used a pacifier (30% currently) for an average of 14 months. Two children were currently finger or thumb suckers (none with a past history). Breastfed children made up 40% of the cohort (15% currently) with a mean duration of 6.9 months (range 11 days to 19 months). One child had a nasogastric tube *in situ*. Four caregivers did not wish their child to trial the SaliPac, all from the group who had never used a pacifier, resulting in data from 30 samplings using the SalivaBio and 26 with the SaliPac. The required minimum volume of 0.2ml of saliva was collected in all 56 samplings. The full 1ml was collected in 47 (84%) samplings, >0.8ml in 52 (93%) with the smallest volume, 0.34 ml, collected from a sleeping child. Previous or current pacifier use significantly shortened time to collect 1ml of saliva (p=0.021), but a history of breastfeeding or finger/thumb sucking did not have an impact. The mean time to collect ~1ml of saliva (time to indicator change) using the SalivaBio was 122 seconds (SD 37, range 42-210) and 132 seconds (SD 31, range 73-229) using the SaliPac. There were no significant differences

in collection times between the two devices overall (p=0.319) or using the “real-world” versus “actual” collection times (p=0.146) (Table 4).

Questionnaire analysis demonstrated that all caregivers thought the salivary collection time was acceptable for both devices (mean score 10/10). Of the 26 who sampled using both, 21 stated they preferred the SaliPac, three the SalivaBio and two reported no preference. Three commented that the SaliPac “looked safer”. Caregivers reported a high level of acceptability for both SalivaBio (mean score of 8.2/10 (range 7-10)) and SaliPac (9.8/10 (range 8-10)) and collection to be easy using both devices (SalivaBio mean score 8.2/10 (range 6-10), SaliPac 9.2 (range 8-10)) (Table 5). All caregivers felt confident overseeing a salivary collection from their child at home using our Patient Information Leaflet.

Discussion

Our studies have demonstrated no significant difference in salivary cortisol or cortisone when collected using the commonly used techniques: Salivette, passive drool, and SalivaBio. We found salivary glucocorticoids degrade over time at the highest temperature studied (50°C) but demonstrate good stability of up to a week when kept at room temperature or refrigerated. Salivary glucocorticoids were unaffected by repeated freeze-thaw cycles. The SalivaBio swab, and our modified SaliPac, collected sufficient saliva for glucocorticoid analysis in under four minutes in all participants, and in two minutes in the majority. Both were well tolerated by the children and acceptable to caregivers, who felt confident that they could successfully perform home salivary collection.

Stability of salivary cortisol collected by Salivette, and passive drool has been demonstrated previously under a variety of storage conditions (laboratory and domestic freezer, refrigerated and room temperature), with the optimum storage condition room temperature (typically 20-23 ^{o}C) (14, 15). Salivary cortisol remains stable for at least 72 hours, with some studies reporting minimal degradation out to a week and far beyond (14, 15, 28, 29). To our knowledge there are no studies reporting stability of salivary cortisone, saliva collected using a SalivaBio swab or salivary glucocorticoids analysed by gold standard LCMS methodology. Cortisone is emerging as the preferred salivary biomarker in adrenal insufficiency, as it better reflects plasma free cortisol, is the more abundant salivary glucocorticoid and demonstrates greater sensitivity at low serum cortisol levels (4, 5). Our work has demonstrated salivary cortisone stability, especially when refrigerated, or for at least 72 hours at room temperature, providing reassurance that patients being investigated for hypo- or hypercortisolism can collect samples at home and post them back to the laboratory for analysis. Previous studies have shown repeated freeze-thaw cycles do not accelerate degradation of salivary cortisol, although good laboratory practice dictates these should be kept to a minimum (18,20). We have demonstrated this for salivary cortisol and cortisone facilitating global utility of salivary glucocorticoid use. Samples taken in rural, remote or resource poor settings can be posted to the local healthcare provider, frozen, then posted on to a central laboratory, where they may be refrozen before LCMS analysis.

Other groups have recognised the requirement for a simple, patient friendly and effective salivary screening test for adrenal insufficiency suitable for infants and young children. A recent study described a pacifier based salivary collection device but it was unable to collect saliva successfully from children under five months of age (15). Sorbettes (eye sponges) were

used previously for paediatric salivary collection, however the cotton material was found to bind salivary cortisol (30). Pipettes have been trialled but either required a salivary stimulant or failed to collect successfully in neonates (31, 32). One study investigated a pipette for home use but 16% of participants disliked the technique and no one collected the required 0.5mL (33). Universal cotton swabs have been used in clinical studies with varying success. One study tested them in 65 extreme premature infants, however the method required four swabs placed sequentially in the mouth for 1-2 minutes and 15% collected insufficient volumes for analysis (34). Cotton is now widely acknowledged to be an inappropriate material for salivary glucocorticoid collection (6, 9, 10). The SalivaBio and our modification, the SaliPac, provide easy, efficient and well tolerated methods to collect saliva samples from neonates, infants and young children addressing the unmet needs of current collection techniques. They could also have utility in adult cohorts where salivary collection may be challenging e.g., patients with altered cognition such as those with dementia or learning difficulties; or reduced conscious level such as those on intensive care.

Home salivary collection can reduce the need for hospital visits and facilitate collection of multiple timed samples. For the widespread clinical adoption of salivary glucocorticoids in the assessment of adrenal function, patients and their caregivers need to be able to comply with home testing protocols and post the samples back to the hospital/laboratory. A recent study of 19 healthy adults (mean age 42 years, 50% female) required to collect four salivary samples per day over three consecutive days and at two time points, reported high levels of adherence; with 95% adhering to the correct number of samples in the protocol and 84% to the specified timings (three on or soon after awakening and one on retiring for bed) (35). Other studies of home salivary collection in adults have shown similar results, with good

337 compliance and high rates of acceptability (36, 37) and studies in children have reported levels
338 of compliance above 90% in participants as young as 3.5 years (38, 39).
339
340 There are limitations of our studies. We analysed the impact of temperature and time on
341 salivary steroid stability, but did not combine storage conditions to mimic real life, with
342 different conditions experienced during the journey from patient to assay platform. A
343 previous study reported no impact of combining different storage conditions (28). We chose
344 the extreme temperature of 50°C to investigate the effect of heat on salivary glucocorticoid
345 stability and extrapolate to the utility of posting samples in tropical climes. Future studies
346 should include more commonly encountered temperatures e.g., 30°C, 35°C and 40°C under
347 controlled conditions with a larger sample size. We excluded volunteers with conditions
348 known to alter cortisol measurements (e.g., liver disease and pregnancy). High sensitivity and
349 specificity have been demonstrated between salivary and serum cortisol levels in pregnancy
350 and in those taking oestrogen containing contraceptive medication, and it is generally
351 acknowledged that salivary measurement facilitates adrenal assessment in patients with
352 altered cortisol binding proteins (1, 40, 41, 42). However we acknowledge that to assess the
353 future utility of salivary glucocorticoids in the screening and diagnosis of adrenal dysfunction
354 these groups would need to be studied. Although we attempted to recreate home conditions
355 for a cohort of participants and sought information about caregiver confidence performing
356 collections using the SalivaBio and SaliPac at home, caregiver numbers were small and all
357 visits were conducted in hospital. Whilst this detailed study on practicalities and techniques
358 provides the evidence that home measurement is possible, a further study involving a larger
359 number of caregivers, and conducted in the home setting, is required to test instructions to
360 caregivers and establish whether “real home” conditions yield similar results.

In conclusion, we have demonstrated no difference in salivary glucocorticoid concentrations collected using devices designed for very young children compared with the standard salivary collection techniques of Salivette and passive drool. Both salivary cortisol and cortisone are stable refrigerated and at room temperature to 72 hours and beyond, but salivary cortisone rapidly degrades at extreme high temperatures. The SalivaBio alone and contained in our novel modification, the SaliPac™, collect sufficient salivary volumes for steroid analysis in under four minutes, are easy to use and acceptable to children and caregivers. Our studies provide data to support the use of these salivary collection techniques in patients under six years of age, both for clinical indications and in research settings, enabling the construction of normative reference ranges in young children and the study of paediatric adrenal function in disease states.

Author Contributions: JJT and CJE designed the SaliPac device. JJT, BGK and CJE conducted the studies. BGK performed the salivary glucocorticoid analysis. JJT analysed the data. CJE and JJT wrote the paper and all authors designed the studies, contributed to reviewing protocols, results, analysis and editing the manuscript.

Data availability: Some or all datasets generated during and/or analysed during the current study are not publicly available but are available from the corresponding author on reasonable request.

References

1. Raff H. Utility of salivary cortisol measurements in Cushing's syndrome and adrenal insufficiency. *The Journal of clinical endocrinology and metabolism* 2009 **94** 3647–3655. (doi:10.1210/JC.2009-1166)
2. Blair J, Adaway J, Keevil B, & Ross R. Salivary cortisol and cortisone in the clinical setting. *Current Opinion in Endocrinology, Diabetes and Obesity*2017. pp 161–168. . (doi:10.1097/MED.0000000000000328)
3. Nieman LK, Biller BMK, Findling JW, Newell-Price J, Savage MO, Stewart PM, Montori VM, & Edwards H. The Diagnosis of Cushing's Syndrome: An Endocrine Society Clinical Practice Guideline. *The Journal of Clinical Endocrinology and Metabolism* 2008 **93** 1526. (doi:10.1210/JC.2008-0125)
4. Perogamvros I, Keevil BG, Ray DW, & Trainer PJ. Salivary Cortisone Is a Potential Biomarker for Serum Free Cortisol. *The Journal of Clinical Endocrinology & Metabolism* 2010 **95** 4951–4958. (doi:10.1210/JC.2010-1215)
5. Debono M, Harrison RF, Whitaker MJ, Eckland D, Arlt W, Keevil BG, & Ross RJ. Salivary Cortisone Reflects Cortisol Exposure Under Physiological Conditions and After Hydrocortisone. *The Journal of clinical endocrinology and metabolism* 2016 **101** 1469–1477. (doi:10.1210/JC.2015-3694)
6. Tryphonopoulos PD, Letourneau N, & Azar R. Approaches to Salivary Cortisol Collection and Analysis in Infants. *Biological Research for Nursing* 2014 **16** 398–408. (doi:10.1177/1099800413507128)
7. Adam EK & Kumari M. Assessing salivary cortisol in large-scale, epidemiological research. *Psychoneuroendocrinology* 2009 **34** 1423–1436. (doi:10.1016/J.PSYNEUEN.2009.06.011)

8. Dimolareva M, Gee NR, Pfeffer K, Maréchal L, Pennington K, & Meints K. Measuring cortisol in the classroom with school-aged children—a systematic review and recommendations. *International Journal of Environmental Research and Public Health* 2018 **15** . (doi:10.3390/IJERPH15051025)

9. Kidd S, Midgley P, Lone N, Michael Wallace A, Nicol M, Smith J, & McIntosh N. A re-investigation of saliva collection procedures that highlights the risk of potential positive interference in cortisol immunoassay. *Steroids* 2009 **74** 666–668. (doi:10.1016/J.STEROIDS.2009.02.009)

10. Shirtcliff EA, Allison AL, Armstrong JM, Slattery MJ, Kalin NH, & Essex MJ. Longitudinal stability and developmental properties of salivary cortisol levels and circadian rhythms from childhood to adolescence. *Developmental Psychobiology* 2012 **54** 493–502. (doi:10.1002/dev.20607)

11. Harrison D, Johnston L, Spence K, Gillies D, & Nagy S. Salivary cortisol measurements in sick infants: A feasible and objective method of measuring stress? *Journal of Neonatal Nursing* 2005 **11** 10–17. (doi:10.1016/J.JNN.2005.04.001)

12. Zwissig M, Rio L, Roth-Kleiner M, & Ramelet ASS. Measurement of stress in stable neonates during ambulance transportation: A feasibility study. *Australian Critical Care* 2018 **32** 28–33. (doi:10.1016/j.aucc.2018.06.006)

13. Sbardella E, Isidori AM, Woods CP, Argese N, Tomlinson JW, Shine B, Jafar-Mohammadi B, & Grossman AB. Baseline morning cortisol level as a predictor of pituitary-adrenal reserve: a comparison across three assays. *Clinical endocrinology* 2017 **86** 177–184. (doi:10.1111/CEN.13232)

14. Toone RJ, Peacock OJ, Smith AA, Thompson D, Drawer S, Cook C, & Stokes KA. Measurement of steroid hormones in saliva: Effects of sample storage condition.

http://dx.doi.org/10.3109/00365513.2013.835862 2013 **73** 615–621. (doi:10.3109/00365513.2013.835862)

15. Nalla AA, Thomsen G, Knudsen GM, & Frokjaer VG. The effect of storage conditions on salivary cortisol concentrations using an Enzyme Immunoassay. *Scandinavian Journal of Clinical and Laboratory Investigation* 2015 **75** 92–95. (doi:10.3109/00365513.2014.985252)

16. Ford LR, Willi SM, Hollis BW, & Wright NM. Suppression and recovery of the neonatal hypothalamic-pituitary-adrenal axis after prolonged dexamethasone therapy. *The Journal of pediatrics* 1997 **131** 722–726. (doi:10.1016/S0022-3476(97)70100-8)

17. Blair J, Lancaster G, Titman A, Peak M, Newlands P, Collingwood C, Chesters C, Moorcroft T, Wallin N, Hawcutt D, Gardner C, Didi M, Lacy D, & Couriel J. Early morning salivary cortisol and cortisone, and adrenal responses to a simplified low-dose short Synacthen test in children with asthma. *Clinical endocrinology* 2014 **80** 376–383. (doi:10.1111/CEN.12302)

18. Broersen LHA, Pereira AM, Jørgensen JOL, & Dekkers OM. Adrenal Insufficiency in Corticosteroids Use: Systematic Review and Meta-Analysis. *The Journal of Clinical Endocrinology & Metabolism* 2015 **100** 2171–2180. (doi:10.1210/JC.2015-1218)

19. Tran TN, King E, Sarkar R, Nan C, Rubino A, O'Leary C, Muzwidzwa R, Belton L, & Quint JK. Oral corticosteroid prescription patterns for asthma in France, Germany, Italy and the UK. *European Respiratory Journal* 2020 **55** . (doi:10.1183/13993003.02363-2019)

20. Ross C, Rab E, Colyer S, & Elder C. A cost-benefit analysis of the routine measurement of ACTH as part of the Short Synacthen Test. *Endocrine Abstracts* 2021 **78** . (doi:10.1530/ENDOABS.78.P3)

21. Gomar-Vercher S, Simón-Soro A, Montiel-Company JM, Almerich-Silla JM, & Mira A.

Stimulated and unstimulated saliva samples have significantly different bacterial profiles. *PLOS ONE* 2018 **13** e0198021. (doi:10.1371/JOURNAL.PONE.0198021)

22. Fernandes A, Skinner ML, Woelfel T, Carpenter T, & Haggerty KP. Implementing Self-collection of Biological Specimens With a Diverse Sample. *Field methods* 2013 **25** 58–73. (doi:10.1177/1525822X12453526)

23. Bäumler D, Kirschbaum C, Kliegel M, Alexander N, & Stalder T. The cortisol awakening response in toddlers and young children. *Psychoneuroendocrinology* 2013 **38** 2485–2492. (doi:10.1016/j.psyneuen.2013.05.008)

24. Novak D. A Novel Saliva Collection Method among Children and Infants: A Comparison Study between Oral Swab and Pacifier-based Saliva Collection. *Journal of Contemporary Dental Practice* 2021 **22** 9–12. (doi:10.5005/jp-journals-10024-3028)

25. Chen B, Lyu H, Xu X, & Wang C. Simultaneous Quantification of Cortisol and Cortisone in Serums and Saliva from Depressive Patients by Supported Liquid Extraction Coupled to HPLC-MS/MS. *Acta Chromatographica* 2020 **32** 269–275. (doi:10.1556/1326.2020.00733)

26. Jones RL, Owen LJ, Adaway JE, & Keevil BG. Simultaneous analysis of cortisol and cortisone in saliva using XLC–MS/MS for fully automated online solid phase extraction. *Journal of Chromatography B* 2012 **881**–**882** 42–48. (doi:10.1016/J.JCHROMB.2011.11.036)

27. Tonge JJ, Keevil BG, Craig JN, Whitaker MJ, Ross RJ, & Elder CJ. Supplementary data for: Salivary steroid collection in children under conditions replicating home sampling. *Zenedo, dataset*. 2022 . (doi:10.5281/ZENODO.6800272)

28. Clements AD & Richard Parker C. The relationship between salivary cortisol concentrations in frozen versus mailed samples. *Psychoneuroendocrinology* 1998 **23**

478 613–616. (doi:10.1016/S0306-4530(98)00031-6)
479 29. Garde AH & Hansen ÅM. Long-term stability of salivary cortisol. *Scandinavian Journal*
480 *of Clinical and Laboratory Investigation* 2005 **65** 433–436.
481 (doi:10.1080/00365510510025773)
482 30. Donzella B, Talge NM, Smith TL, & Gunnar MR. To spear or not to spear: Comparison
483 of saliva collection methods. *Developmental Psychobiology* 2008 **50** 714–717.
484 (doi:10.1002/dev.20340)
485 31. Soliman H, Elsharkawy A, & Abdel-Hady H. Does topical lidocaine reduce the pain
486 associated with the insertion of nasal continuous positive airway pressure prongs in
487 preterm infants?: A randomized, controlled pilot trial. *Clinical Journal of Pain* 2016 **32**
488 948–954. (doi:10.1097/AJP.0000000000000341)
489 32. Mohanan A, Varma B, Kumar S, Kumaran P, & Xavier A. Assessment of Salivary Flow
490 Rate and Antioxidant Levels in Ectodermal Dysplasia Patients: An In vivo Study.
491 *Contemporary Clinical Dentistry* 2019 **10** 74. (doi:10.4103/CCD.CCD_358_18)
492 33. Riedy CA, Milgrom P, Ly KA, Rothen M, Mueller G, Hagstrom MK, Tolentino E, Zhou L,
493 & Roberts MC. A surrogate method for comparison analysis of salivary concentrations
494 of Xylitol-containing products. *BMC Oral Health* 2008 **8** 1–8. (doi:10.1186/1472-6831-
495 8-5/TABLES/2)
496 34. Ng SM & Drury JA. Correlation of Plasma and Salivary Cortisol in Extremely Premature
497 Infants Telomerase and telomere biology in normal endometrial function and in
498 pathological conditions View project Donatella Pintus View project Ng et al. *Article in*
499 *Journal of Neonatal Biology* 2017 **6** 3. (doi:10.4172/2167-0897.1000260)
500 35. Sørensen SO, Pedersen J, Rasmussen MG, Kristensen PL, & Grøntved A. Feasibility of
501 home-based sampling of salivary cortisol and cortisone in healthy adults. *BMC*

research notes 2021 **14** . (doi:10.1186/S13104-021-05820-4)

36. Valentine-Graves M, Hall E, Guest JL, Adam E, Valencia R, Shinn K, Hardee I, Sanchez T, Siegler AJ, & Sullivan PS. At-home self-collection of saliva, oropharyngeal swabs and dried blood spots for SARS-CoV-2 diagnosis and serology: Post-collection acceptability of specimen collection process and patient confidence in specimens. *PLOS ONE* 2020 **15** e0236775. (doi:10.1371/JOURNAL.PONE.0236775)

37. Emami A, Hallinder H, Theorell T, Kim H, & Engström G. The Feasibility and Acceptability of In-Home Saliva Collection for Stress in Persons With Dementia and Their Family Caregivers. *Biological research for nursing* 2022 **24** . (doi:10.1177/10998004221076554)

38. Roth R, Baxter J, Vehik K, Hopkins D, Killian M, Gesualdo P, Melin J, Simell B, Strauss E, Lernmark Å, & Johnson SB. The Feasibility of Salivary Sample Collection in an International Pediatric Cohort: the TEDDY Study. *Developmental psychobiology* 2017 **59** 658. (doi:10.1002/DEV.21523)

39. Mandrell BN, Avent Y, Walker B, Loew M, Tynes BL, & Crabtree VML. In-home salivary melatonin collection: Methodology for children and adolescents. *Developmental Psychobiology* 2018 **60** 118–122. (doi:10.1002/DEV.21584)

40. Qureshi AC, Bahri A, Breen LA, Barnes SC, Powrie JK, Thomas SM, & Carroll P V. The influence of the route of oestrogen administration on serum levels of cortisol-binding globulin and total cortisol. *Clinical endocrinology* 2007 **66** 632–635. (doi:10.1111/J.1365-2265.2007.02784.X)

41. Manetti L, Rossi G, Grasso L, Raffaelli V, Scattina I, Sarto S Del, Cosottini M, Iannelli A, Gasperi M, Bogazzi F, & Martino E. Usefulness of salivary cortisol in the diagnosis of hypercortisolism: comparison with serum and urinary cortisol. *European Journal of*

Endocrinology 2013 **168** 315–321. (doi:10.1530/EJE-12-0685)

42. Wilson M & Thayer Z. Maternal salivary cortisone to cortisol ratio in late pregnancy: An improved method for predicting offspring birth weight. *Psychoneuroendocrinology* 2017 **78** 10–13. (doi:10.1016/J.PSYNEUEN.2016.12.018)

Figure Legends:

Figure 1. Salivary collection techniques: 1a. Salivette® Cortisol (Sarstedt, Germany), 1b. Passive drool (Salicap, IBL International, Germany) and 1c. SalivaBio swab (Salimetrics, USA).

Figure 2. **SaliPac™ salivary collection device: (a) SalivaBio swab (*Salimetrics, California, USA*); (b) indicator line and (c) plastic back cover.** Indicator line drawn to allow approximately 1mL of salivary collection.

Figure 3. Median % change in salivary cortisol (a) and salivary cortisone (b) concentration when stored refrigerated, frozen and at room temperature over the one-week study period. Glucocorticoid concentration measured in nmol/L. Changes in concentration are compared to the mean values from the reference samples frozen immediately after collection (salivary cortisol 3.675 nmol/L and salivary cortisone 21.7 nmol/L). The dashed lines at 10% degradation indicate the cut off set as acceptable stability.

Figure 4. Median % change in salivary cortisol (a) and salivary cortisone (b) concentration following four freeze-thaw cycles. Glucocorticoid concentration measured in nmol/L. Changes in concentration are compared to the mean values from the reference samples frozen immediately after collection (salivary cortisol 3.675 nmol/L and salivary cortisone 21.7 nmol/L). The dashed lines at 10% degradation indicate the cut off set as acceptable stability.

Tables:

Table 1. Comparison of salivary glucocorticoid levels following collection using three salivary collection techniques: Salivette® Cortisol (Sarstedt, Germany), passive drool (Salicap, IBL International, Germany) and SalivaBio (Salimetrics, USA). N=270 salivary samples, 90 collected using each collection technique, taken from six healthy adult volunteers.

Salivary Glucocorticoid (nmol/L)	Saliva collection techniques compared	Mean Difference	Standard Deviation	95% Confidence Interval
Cortisol	SalivaBio vs Salivette	-0.226	0.915	-0.957 to 0.410
	SalivaBio vs Passive Drool	-0.751	2.834	-2.928 to 1.425
	Salivette vs Passive Drool	0.214	1.301	-1.087 to 1.729
Cortisone	SalivaBio vs Salivette	-1.516	3.548	-4.242 to 1.210
	SalivaBio vs Passive Drool	0.871	5.140	-3.001 to 4.745
	Salivette vs Passive Drool	1.029	3.309	-2.280 to 4.338

Table 2. Salivary cortisol concentrations following storage under different time and temperature conditions. Salivary cortisol stored on a SalivaBio swab (Salimetrics, USA). N=96 salivary samples taken from six healthy adult volunteers. Baseline denotes the mean salivary cortisol value from the reference samples, 3.675 nmol/L. *indicates a statistically significant difference.

		Storage temperature		
Time at storage temperature	**Salivary cortisol (nmol/L)**	**4°C**	**Room temperature**	**50°C**
24 hours	**Mean ± SD**	3.523 ± 1.866	3.662 ± 2.099	3.545 ± 1.855
	% Difference from baseline	-2.90%	-3.51%	-5.77%
	p-value	0.086	0.928	0.318
48 hours	**Mean ± SD**	3.465 ± 2.005	3.430 ± 1.890	3.435 ± 1.999
	% Difference from baseline	-6.33%	-6.94%	-5.58%
	p-value	0.083	0.060	0.055
72 hours	**Mean ± SD**	3.577 ± 1.902	3.470 ± 1.877	3.362 ± 1.847
	% Difference from baseline	-3.71%	-5.66%	-6.54%
	p-value	0.352	0.066	0.090
1 week	**Mean ± SD**	3.357 ± 2.009	3.597 ± 1.932	2.962 ± 1.469
	% Difference from baseline	-8.82%	-2.02%	-20.09%
	p-value	0.041*	0.458	0.044*

Table 3. Salivary cortisone concentrations following storage under different time and temperature conditions. Salivary cortisone stored on a SalivaBio swab (Salimetrics, USA). N=96 salivary samples taken from six healthy adult volunteers. Baseline denotes the mean salivary cortisone value from the reference samples, 21.70 nmol/L. *indicates a statistically significant difference.

		Storage temperature		
Time in storage temperature	**Salivary cortisone (nmol/L)**	**4°C**	**Room temperature**	**50°C**
24 hours	**Mean ± SD**	21.040 ± 8.030	21.943 ± 9.436	20.430 ± 7.910
	% Difference from baseline	-2.03%	+1.27%	-7.43%
	p-value	0.232	0.737	0.048*
48 hours	**Mean ± SD**	21.208 ± 8.551	20.528 ± 8.377	19.227 ± 7.749
	% Difference from baseline	-4.24%	-7.37%	-11.74%
	p-value	0.399	0.770	0.002*
72 hours	**Mean ± SD**	20.903 ± 8.149	20.202 ± 7.762	18.963 ± 7.601
	% Difference from baseline	-5.10%	-5.27%	-14.12%
	p-value	0.236	0.080	0.009*
1 week	**Mean ± SD**	21.418 ± 8.609	19.575 ± 7.155	15.490 ± 5.950
	% Difference from baseline	-3.40%	-10.83%	-25.62%
	p-value	0.415	0.012*	0.012*

Table 4. Comparison of the time taken to collect sufficient saliva for glucocorticoid analysis from infants and young children using the SalivaBio (Salimetrics, USA) alone and within the SaliPac, under hospital and simulated home conditions. "Real world time" describes the total time taken in seconds from the swab entering the child's mouth to the caregiver completing the sampling and "actual collection time" the time taken to collect 1mL of saliva, with the timer stopped if the SaliPac or SalivaBio is expelled from the mouth. *Denotes a statistically significant difference.

		SalivaBio N=30	**SaliPac** N=26	**p-value**
Overall	**Mean Real world collection time (seconds)**	122.30 ± 37.33 (42 – 210)	131.58 ± 30.70 (73 – 229)	0.319
	Mean Actual collection time (seconds)	118.87 ± 32.42 (42 – 210)	131.38 ± 30.72 (73 – 229)	0.146
Hospital Condition	**Mean Real world collection time (seconds)**	120.50 ± 33.04 (42 - 189)	135.19 ± 32.01 (84 - 210)	0.321
	Mean Actual collection time (seconds)	118.25 ± 29.32 (42 - 189)	133.87 ± 30.08 (84 - 210)	0.716
Simulated home Condition	**Mean Real world collection time (seconds)**	125.90 ± 46.52 (89 - 188)	125.80 ± 29.15 (81 - 172)	0.459
	Mean Actual collection time (seconds)	124.88 ± 45.58 (89 - 188)	122.78 ± 28.06 (81 - 172)	0.952

603 **Table 5. Acceptability of salivary collection from infants and young children using the**
604 **SalivaBio (*Salimetrics, USA*) alone and within the SaliPac™, under hospital and stimulated**
605 **home conditions assessed by caregiver questionnaire.** Ease of use assessed caregivers'
606 ability to complete salivary collection independently using a patient information leaflet.
607 *denotes a statistically significant difference.

		SalivaBio N=30	**SaliPac** N=26	**p-value**
Caregiver Questionnaire	**Ease of use score α**	8.2	9.8	0.012*
	Acceptability score α	8.2	9.8	0.012*
	Parent/carer preference	3 (15%)	22 (85%)	0.001*
	Child preference	1 (4%)	25 (96%)	0.001*

608 ***α = Mean score out of 10.***