Plasma steroid profiling and response to trophins to illustrate intra-adrenal dynamics

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Abstract

The importance of corticosteroids in cardiovascular and other chronic disease is recognised. In addition, plasma steroid precursor-to-product ratios are useful and convenient indirect indicators of efficiency of key steroidogenic enzymes (aldosterone synthase, 11β-hydroxylase and 17α-hydroxylase). The use of liquid chromatography–tandem mass spectrometry (LC–MS/MS) has enabled measurement of numerous corticosteroid compounds simultaneously. However, normal responses to trophins and variation in salt intake are not well described. This study examined these parameters in a large group of healthy volunteers. Sixty normotensive volunteers were recruited and underwent infusion of angiotensin II (AngII) and ACTH, following low- and high-salt diet. Measurement of plasma steroids at baseline and 30 min after infusion of trophin was carried out by LC–MS. As expected, plasma mineralocorticoid levels increased in response to salt restriction and were suppressed with salt loading; ACTH infusion increased all corticosteroids, while AngII increased mineralocorticoids and suppressed glucocorticoid production. ACTH increased S:F but decreased DOC:B, thus the S:F ratio is a more appropriate index of 11β-hydroxylase efficiency. The B:F ratio increased following ACTH treatment and salt restriction. A larger proportion of plasma B than generally accepted may be derived from the zona glomerulosa and this ratio may be most informative of 17α-hydroxylase activity in salt-replete subjects. Although DOC:aldosterone, B:aldosterone and 18-hydroxyB:aldosterone should provide indices of aldosterone synthase efficiency, responses of individual compounds to trophins suggest that none of them accurately reflect this. Based on these data, aldosterone synthase activity is most accurately reflected by aldosterone concentration alone.

Key Words

- adrenal
- corticosteroid
- enzyme

Introduction

Corticosteroids are key regulators of the cardiovascular system. It has increasingly been accepted that aberrant blood pressure regulation and other cardiovascular consequences can be associated with altered production of the end products of the corticosteroid pathways, aldosterone and cortisol. For example, autonomous production of aldosterone results in hypertension with an excess of left ventricular hypertrophy, renal dysfunction and arrhythmia (Milliez et al. 2005), while cortisol excess, or Cushing’s syndrome, causes, among other features, high blood pressure, obesity, coagulopathy and dysglycaemia (Arnaldi et al. 2003).
Cortisol and aldosterone are the final products of specific pathways (Fig. 1), each comprising a series of enzyme-catalysed reactions. The efficiency of each enzyme may vary, for example, in a genetically determined manner, so that each step in the pathway may become rate limiting to a greater or lesser extent. This phenomenon is well documented in a series of rare monogenic disorders of corticosteroid synthesis (congenital adrenal hypertrophy (CAH)), where abnormal levels of intermediate compounds can be both pathogenic and diagnostic (Miller & Auchus 2011). Importantly, qualitatively similar but more subtle variations in corticosteroid production have been detected in normal and hypertensive subjects (Imrie et al. 2006, Freel et al. 2007, Freel et al. 2008) and related to polymorphic variation within the genes encoding enzymes that catalyse the final steps in the biosynthesis of aldosterone (aldosterone synthase, CYP11B2 and 11β-hydroxylase, CYP11B1). In these studies, secretion of the pathway end products, aldosterone and cortisol, was normal and only analysis of the pattern of intermediate steroid levels revealed these interesting and potentially important differences.

To date, most studies demonstrate variations in corticosteroid metabolism analysed using urinary metabolites and there are many metabolites for each compound. Plasma concentrations afford a more direct index of secretion and allow characterisation of short-term changes, for example, the response to acute alteration in angiotensin II (AngII) or adrenocorticotropic hormone (ACTH) drive, which may alter both absolute secretion rates of aldosterone and cortisol, respectively, and their relationship to their respective precursors. Indeed, the short-term response to stimulation may be the only way to discern a potentially altered enzyme efficiency and steroid pattern (as is the case with the plasma 17α-hydroxyprogesterone response to ACTH in ‘non-classic’ CAH; White & Speiser 2000). To assess the clinical significance of profile differences, reliable normal levels and responses to stimulants of glucocorticoids and mineralocorticoid production are necessary: in recent years, the technique of liquid chromatography–tandem mass spectrometry (LC–MS/MS) has largely replaced multiple RIA and has made larger studies of plasma steroid concentrations more achievable, thus making such an approach feasible.

Figure 1
Biosynthetic pathway of mineralocorticoids and glucocorticoids within the adrenal cortex. The dashed line denotes zona glomerulosa activity and the solid line, biosynthetic reactions occurring within the zona fasciculata. 11β-HSD, 11β-hydroxysteroid dehydrogenase; 18-hydroxyDOC, 18-hydroxydeoxycorticosterone.
A full spectrum of compounds can be assessed simultaneously with high precision accuracy and specificity (McDonald et al. 2011). The following study examined the modulation of relative corticosteroid product flow through three pathways, namely, the aldosterone pathway in the zona glomerulosa and the 17α-hydroxysteroid and 17-deoxysteroid pathways in the zona fasciculata, in a large group of normal subjects under different physiologically relevant environmental conditions. Its principal aim was to provide a normal basis for investigating the clinical significance of steroid-related enzyme polymorphisms.

Materials and methods

Patient selection and protocols

Normal volunteers were recruited by advertising in the local media and were required to be in good health, between the ages of 18–70 and not on any antihypertensive or steroid-containing medication at the time of recruitment. All subjects underwent dietary manipulation to standardise their salt intake to either ‘high-salt’ or ‘low-salt’ states. Briefly, subjects adhered to a modest salt-restricted diet for 5 days and were randomised to receive supplementation with either slow-sodium tablets, in order to achieve a salt intake of 200 mmol/day (200 mmmol diet), or placebo, in order to achieve a salt intake of 100 mmol/day (100 mmmol diet). On day 3, 24 h urine was collected for determining urinary sodium excretion to assess compliance and salt status. In the morning of day 4, subjects attended the Clinical Research Facility of the University of Glasgow where they were cannulated via a peripheral vein. After 30 min of recumbent rest, blood was drawn for steroid measurements. I.v. ACTH was administered at a rate of 1 ng/kg per min and, after 30 min, the infusion was stopped for further blood sampling. The volunteers returned on day 5 to be infused with AngII, administered at a rate of 3 ng/kg per min (BAChem, Weil am Rhein, Germany) under the same conditions. After a period of at least 2 weeks, the process was repeated so that all subjects were studied under both salt conditions.

Infusions were prepared by the Western Infirmary Pharmacy Production Unit, Glasgow. Blood pressure was monitored at 10 min intervals throughout both infusions. Blood samples were drawn using the Vacutainer System (BD, Oxford Science Park, Oxford, UK) at baseline before the infusion and at completion of the infusion. This study was approved by the West of Scotland Research Ethics Committee.

Plasma measurements of adrenal corticosteroids using LC–MS/MS

Samples were stored at room temperature and centrifuged at 25 °C for 15 min at 3000 g. Plasma samples containing 60 ng of internal standard (6β-methylprednisolone) were applied to Chem Elut SPE cartridges (Varian, Inc., Palo Alto, CA, USA) and the corticosteroids eluted with dichloromethane (HPLC grade). The eluates were evaporated to dryness under nitrogen and the residue re-dissolved in 10% acetonitrile (60 μl; HPLC grade). An aliquot (20 μl) was applied to an HPLC column (Polaris, 5 μ 18-A, 150×20 mm) coupled to a mass spectrometer (Varian 1200L with triple quadrupole detector). The corticosteroids were serially eluted using an acetonitrile:water gradient containing 2 mmol ammonium acetate. The performance criteria and normal ranges for each corticosteroid are summarised in Table 1. The internal standard was 16β-methylprednisolone (Ingram & Fraser 2010).

Plasma renin concentration was measured by the Diasorin Liaison immunochemiluminometric analyser (DiaSorin Ltd, Wokingham, Berkshire, UK).

Statistical analysis

Statistical analysis was performed using Minitab (State College PA, USA, version 15). Where steroid data could be normalised by transformation (log10), they were analysed using Student’s t-test or paired t-test where appropriate; alternatively, nonparametric methods of hypothesis testing were utilised as described.

Results

Baseline characteristics and efficacy of dietary salt manipulation.

Sixty subjects (33 women and 27 men) participated with a mean (± s.d.) blood pressure at recruitment of 126.7

<table>
<thead>
<tr>
<th>Compound</th>
<th>Mean morning level (normal range) (nmol/l)</th>
<th>Between batch CV (‰ (concentration; nmol/l))</th>
</tr>
</thead>
<tbody>
<tr>
<td>18OHB</td>
<td>4.1 (0.04–13.85)</td>
<td>18.7 (0.02)</td>
</tr>
<tr>
<td>Aldosterone</td>
<td>0.22 (0.04–0.94)</td>
<td>13.4 (0.1)</td>
</tr>
<tr>
<td>Cortisol</td>
<td>248.6 (30.3–933.7)</td>
<td>9.1 (182.3)</td>
</tr>
<tr>
<td>Corticosterone (B)</td>
<td>6.28 (0.27–45.6)</td>
<td>8.2 (6.6)</td>
</tr>
<tr>
<td>11-deoxycortisol (S)</td>
<td>0.3844 (0–2.02)</td>
<td>14.5 (0.49)</td>
</tr>
<tr>
<td>Deoxycorticosterone (DOC)</td>
<td>0.0212 (0–0.07)</td>
<td>21.1 (0.02)</td>
</tr>
</tbody>
</table>
(±13.9)/76 (±10.3) mmHg and a mean age of 50.1 (±17.1) years. There was no significant blood pressure or age difference between men and women. Compliance to the sodium-rich/restricted diet was checked by the urine sodium excretion rate and plasma renin concentration measurements. Following the 100 mmol/day diet, the mean urinary sodium excretion rate was 97.1 (±39.5 mmol/24 h and plasma renin concentration 19.1 (±13.9 mU/ml. Following the 200 mmol/day diet, the mean urinary sodium excretion rate was 199.8 (±64.6 mmol/24 h and plasma renin concentration 9.7 (±5.9 mU/ml. (P < 0.001).

### Effect of variation in salt intake on corticosteroid production

Individual corticosteroid concentrations in the two dietary regimes are compared in Table 2. Predictably, aldosterone concentration was significantly lower during the higher salt intake. In addition, concentrations of aldosterone precursors, corticosterone (B) and 18-hydroxycorticosterone (18OHB) were slightly but significantly suppressed by the higher salt intake. The levels of glucocorticoid compounds were unaffected. Under both conditions, the concentration of aldosterone and 18OHB are surprisingly low when compared with previously published results, and while the reasons for this are not clear, it does serve to highlight the variation in results with different methodologies.

Precursor-to-product ratios according to salt status are summarised in Table 3. Data were not normally distributed and so were log transformed (after ratios were calculated) to allow for parametric hypothesis testing using a paired t-test. The ratios of 11-deoxycortisol to cortisol (S:F) and 11-deoxycorticosterone to B (DOC:B) were not significantly affected by salt intake. The ratio of 18OHB to aldosterone was significantly lower during the lower salt intake regime. B:F, in contrast, was significantly lower during higher salt intake. DOC:18OHD was unaffected by salt regime.

### Influence of ACTH on corticosteroid production

The effects of ACTH on individual steroid concentrations according to dietary sodium intake are summarised in Table 4. Predictably, the levels of all compounds increased significantly during ACTH infusion, but the proportionate responses were different. The highest fold-increase (Table 4) was observed in B concentration and the fold increases in S and 18OHDOC all exceeded those in F. Alteration in sodium status did not affect the pattern of steroid response.

Log-transformed precursor-to-product ratios according to salt intake are shown in Table 5. S:F rose

<table>
<thead>
<tr>
<th>Table 2</th>
<th>Plasma corticosteroids measured under variations in salt intake. Data shown are medians and interquartile ranges (IQR) (n=60)</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>200 mmol diet</td>
</tr>
<tr>
<td>18OHB (nmol/l)</td>
<td>0.03 (0.01–0.05)</td>
</tr>
<tr>
<td>Aldosterone (nmol/l)</td>
<td>0.07 (0.02–0.13)</td>
</tr>
<tr>
<td>Cortisol (F) (nmol/l)</td>
<td>201.4 (139.9–274.8)</td>
</tr>
<tr>
<td>18OHD (nmol/l)</td>
<td>0.10 (0.04–0.25)</td>
</tr>
<tr>
<td>Corticosterone (B) (nmol/l)</td>
<td>3.17 (2.14–6.26)</td>
</tr>
<tr>
<td>11-deoxycortisol (S) (nmol/l)</td>
<td>0.33 (0.17–0.53)</td>
</tr>
<tr>
<td>Deoxycorticosterone (nmol/l)</td>
<td>0.02 (0.01–0.04)</td>
</tr>
</tbody>
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<table>
<thead>
<tr>
<th>Table 3</th>
<th>Log10-transformed corticosteroid ratios (indirect marker of enzyme efficiencies) under conditions of high and low dietary salt intake (n=60)</th>
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<tbody>
<tr>
<td></td>
<td>200 mmol diet</td>
</tr>
<tr>
<td>S:F</td>
<td>11β-hydroxylase</td>
</tr>
<tr>
<td>DOC:B</td>
<td>11β-hydroxylase/aldosterone synthase</td>
</tr>
<tr>
<td>18OHB:Aldo</td>
<td>Aldosterone synthase</td>
</tr>
<tr>
<td>B:F</td>
<td>17α-hydroxylase</td>
</tr>
<tr>
<td>DOC:18OHD</td>
<td>11β-hydroxylase</td>
</tr>
</tbody>
</table>
significantly in response to ACTH, but DOC:B was significantly reduced while DOC:18OHDc was slightly lower (this did not reach statistical significance in the high salt group). There was a large increase in B:F while 18OHB:aldosterone was unaffected by ACTH.

**Effect of AngII on corticosteroid production during different salt intakes**

The effects of AngII infusion on plasma steroids according to salt intake are shown in Table 6. In the low dietary salt group, plasma aldosterone and 18OHB concentrations rose significantly, while those of the glucocorticoids, F and S, fell significantly. The corticosteroid response to AngII stimulation in the higher salt regimen cohort was similar to that in the lower salt regimen cohort (Table 6). The only difference in steroid response to AngII according to sodium intake was in the corticosterone concentration, which did not significantly change under low-salt conditions, and 18OHDc, which demonstrated no significant change under high-salt conditions but fell slightly, but significantly, under low-salt conditions.

Precursor-to-product ratios according to salt intake in response to AngII are shown in Table 7. Some differences in the response of these ratios to AngII stimulation according to salt intake were observed. Most notably, the ratio of B:F rose slightly in the low salt group \((P = 0.05)\), but this was not replicated under high-salt conditions. In addition, during higher salt intake, the ratio of S:F fell in response to AngII \((P = 0.02)\), while no significant difference in this ratio was found during low salt intake. Under both salt intake conditions, the ratio 18OHB:aldosterone fell significantly and there was no change in the DOC:18OHDc or DOC:B ratios.

**Discussion**

The data generated from this study of normotensive, healthy volunteers provide a valuable reference resource detailing plasma steroid profiles (as measured by LC–MS/MS) at baseline and in response to major agonists of adrenal corticosteroid production. Variation in the efficiency (that is, Km and Vmax) of the individual enzymes comprising the biosynthetic pathways to the principal bioactive corticosteroids, cortisol, corticosterone and aldosterone, will alter the overall efficiency of their synthesis, response to agonists and the concurrent levels of their precursor compounds (Fig. 1) in the plasma. Efficiency is determined genetically through inherited
differences in protein structure. Major changes from normal activity are associated with the rare but well-characterised metabolic disruptions of congenital adrenal hyperplasia, but there is abundant evidence that much less severe polymorphic variations within CYP11B1 (11β-hydroxylase), CYP11B2 (aldosterone synthase) and CYP17A1 (17α-hydroxylase) also affect efficiency to an extent that is probably clinically significant (Barr et al. 2007, Connell et al. 2008, Freel et al. 2008) (L Diver, J M C Connell & E Davies 2014, unpublished observations). Thus, a robust and convenient means of assessing the quantitative effect and clinical relevance of a modest polymorphic variation in steroid biosynthetic genes as well as the acute changes in response to conventional tropins of adrenal steroid synthesis would be valuable. Plasma steroid profiling by means of LC–MS provides such a means and the study of a large group of normal subjects described herein provides a yardstick with which clinical cases can be compared and the influence of genetic variation assessed.

The use of the ratio of the concentration of an end product corticosteroid in plasma to that of its precursor is an established index of enzyme deficiency and has most recently been evaluated in neonates (Hicks et al. 2014). In the presence of efficiency-altering polymorphisms, basal hormone levels will be within the normal range but kinetics demand that, to achieve this, precursor (i.e. substrate) levels will vary. The lower the efficiency of the enzyme, the higher the ratio of precursor to product. Adrenal stimulation will emphasise this difference, as the reaction becomes rate limiting.

For each of the key adrenal biosynthetic enzymes, there is more than one possible index ratio. Thus, both ratios of S:F and DOC:B could be used to assess 11β-hydroxylase efficiency (Connell et al. 1996). Similarly, either B:F or DOC:S could indicate the efficiency of 17α-hydroxylase, and 18-OHB:aldosterone, B:aldosterone or DOC:aldosterone could indicate that of aldosterone synthase (Kater & Biglieri 1994). This simple study, which used zone-specific stimulation at intensities commensurate with quotidian experience, has attempted to select the most reliable indices of enzyme efficiency and these are discussed in more detail later in this study.

11β-hydroxylase

11β-hydroxylase catalyses the conversion of S to F and DOC to B. The activity of an exclusive zona fasciculata enzyme should be ACTH dependent and salt status/AngII independent (Nishimoto et al. 2010). Therefore, these agonists should affect S:F and DOC:B similarly, if they convincingly represent 11β-hydroxylation in the 17α-hydroxysteroid and 17-deoxysteroid pathways respectively. This was not the case. ACTH increased S:F but decreased DOC:B, reflecting the much greater relative response of B than F, perhaps reflecting superior kinetics for the DOC to B conversion (Barr et al. 2007). Moreover, some plasma B and DOC are derived from the zona glomerulosa aldosterone pathway, although this zone probably has a lower synthetic capacity (Miller & Auchus 2011). The results also indicated that B levels significantly responded to mild salt restriction and AngII (under high-salt-intake conditions), whereas DOC levels were unaffected by these conditions. Thus, S:F remains the index of choice to reflect 11β-hydroxylase efficiency in the zona fasciculata.

The balance of 11β- and 18-hydroxylation activities of this enzyme may be different in different forms of experimental hypertension and this has been explored in rat models of hypertension (Rapp & Dahl 1971, 1976). As far as we are aware, this has not been examined in relation to human 11β-hydroxylase polymorphisms. In this study, ACTH slightly altered DOC:18OHDOC in favour of the latter compound (in the low salt group only), but DOC:B was even more markedly reduced and uninfluenced by
dietary sodium (Table 5). The significance of the DOC:B:18OHDOC relationship requires more study.

17α-hydroxylase

17α-hydroxylase determines the relative production rates of 17α-hydroxysteroids and 17-deoxycorticosteroids. It also functions as a lyase, releasing C19 compounds; this aspect was not studied herein (Zuber et al. 1986). The ratios B:F and DOC:S should equally represent its activity and, as it is expressed only in the zona fasciculata, ACTH but not salt status or AngII should increase the ratios as the enzyme efficiency becomes limiting. There was a marked increase in B:F following ACTH treatment (Table 5), suggesting that, even under basal conditions, the enzyme's capacity is close to the limiting point. However, it is striking that mild salt restriction also significantly increased B:F (Table 3), although the effect of AngII was marginal or absent (Table 7). This suggests that a larger proportion of plasma B than generally accepted may be derived from the zona glomerulosa in this circumstance and that the ratio may be most informative in salt-replete subjects.

Aldosterone synthase

Aldosterone synthase catalyses a three-stage conversion of DOC to aldosterone and is expressed exclusively in the zona glomerulosa (Kawamoto et al. 1992). Intermediate stages are B and 18-hydroxyB, but DOC is the most efficient substrate in vitro (Fisher et al. 1998). All these precursor compounds also originate from the zona fasciculata. Salt status and AngII are specific agonists but aldosterone levels also respond acutely to ACTH (Arvat et al. 2000). In theory, DOC:aldosterone, B:aldosterone and 18-hydroxyB:aldosterone should provide indices of efficiency. ACTH did not alter 18-hydroxyB:aldosterone under either dietary regime (Table 5), possibly because this agonist acts primarily on the early aldosterone pathway while salt status and AngII act more specifically on the late aldosterone pathway (Lantos et al. 1967). Mild salt restriction (Table 3) and AngII (Table 7) under both dietary regimes significantly lowered this ratio, although both its individual components increased. This may suggest that under baseline conditions, this enzyme is operating significantly below its limits of efficiency and is easily responsive to stimulation. However, the relative increase in plasma 18-hydroxyB concentration was much greater during ACTH stimulation than during salt status/AngII stimulation suggesting significant zona fasciculata origin. The highly significant changes in DOC:aldosterone and
B: aldosterone may be misleading, as the concentrations of neither DOC nor B changed across AngII treatment. Taken together, these data indicate that none of these three ratios is an unambiguous index of aldosterone synthase efficiency; aldosterone concentration itself, possibly under mild salt restriction, is to be preferred.

**Effect of agonists on corticosteroid production**

The principal agonists of adrenal corticosteroid production (ACTH, AngII and sodium) demonstrated predictable effects; ACTH increased production of all steroids (Table 4) and salt restriction increased mineralocorticoid levels (Table 2). As expected, AngII infusion stimulated aldosterone and its precursors, but glucocorticoid production fell significantly (Table 6). The effect of AngII on glucocorticoid production is controversial and these results appear to be at odds with in vitro and animal data. For example, previous studies have shown that AngII stimulates cortisol production from bovine adrenal cells and that this effect is mediated through a phospholipase C-coupled angiotensin type 1 (AT1) receptor (Rabano et al. 2004). In addition, earlier cell-based studies demonstrated that AngII could both stimulate and suppress B production from adrenal cells depending upon the origin of such cells; the stimulatory effect was observed in ‘fasciculata-rich’ cells and not in total adrenal cells (Spinelli et al. 1989). However, a previous small study of human volunteers infused with AngII demonstrated temporary ACTH suppression, which could be in keeping with our results (Semple et al. 1979). However, in the latter study, ACTH levels eventually recovered and thus we might have observed a rise in glucocorticoid levels in our subjects if we had extended the sampling time after cessation of infusion. This is worthy of further investigation. However, we acknowledge that the apparent ‘suppressive’ effect of AngII could simply reflect an initial stress response of glucocorticoids to cannulation, which settled with time; the lack of a placebo infusion arm makes this theory difficult to disprove and is an acknowledged weakness of this study.

Whatever the influence of AngII on glucocorticoid production, our data demonstrate the advantage of the use of LC–MS technology and human volunteers to measure plasma corticosteroids and explore the influence of variation in hypothalamic–pituitary–adrenal axis activity in order to demonstrate short-term, but immediate, alterations in plasma concentrations.

A potential weakness of our study is the possibility of poor adherence of study subjects to dietary sodium manipulation, particularly among a population in whom there is often a high baseline dietary salt intake. Although there was a clear difference in mean urinary sodium excretion according to dietary intake, the relatively large S.D. in each case introduces the potential for ‘overlap’ in sodium status between groups and obfuscation of results. To address this, we reanalysed to include only patients with extremes of sodium excretion (<100 and >150 mmol/24 h) and were reassured to find no major changes in levels of plasma steroids or precursor:product ratios but a considerable reduction in subject numbers (22 per group). Furthermore, alteration in dietary salt produced the appropriate alteration in plasma renin concentration (which was unaltered when limited to the smaller cohort) as well as plasma mineralocorticoid levels.

In summary, the results from this study illustrate that S:F and B:F remain the indices of choice for monitoring the efficiencies of 11β-hydroxylase and 17α-hydroxylase respectively, and their usefulness is enhanced by low-dose ACTH stimulation and salt repletion. Aldosterone synthase, because of its complex triple reaction sequence and confinement to a single zone, is best monitored by aldosterone concentration alone, if possible with mild AngII stimulation/salt restriction. These data, from a large group of healthy human volunteers, should provide robust baseline information to help inform subsequent studies exploring...
the effect of minor polymorphic variation within steroidogenic enzymes in healthy and chronic disease states.

Declaration of interest
The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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