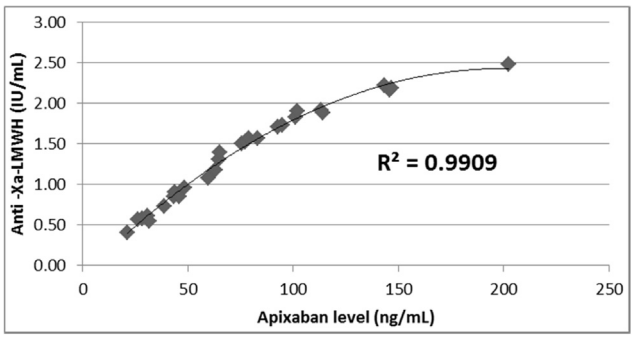

Fig. 1 Anti-Xa-LMWH versus apixaban level.

LMWH result of less than 0.40 IU/mL, for example in a patient going for emergency surgery, can be reassuring that the apixaban level is very low. Our findings are consistent with those of a recently published study.[7] Therefore, when no apixaban level is available it would be reasonable to use an anti-Xa-LMWH assay to exclude significant apixaban effect in emergency situations.

A similar approach has also been proposed for rivaroxaban.[8] As illustrated in the correlation curve, the relationship between anti-Xa-LMWH and apixaban levels becomes non-linear as apixaban concentration increases, and therefore anti-Xa-LMWH activity is not a suitable alternative for reliably quantitating apixaban. Furthermore, there is variability between absolute anti-Xa-LMWH activity reported with different commercial reagents and studies.[8,9] Ideally laboratories should develop their own cut-offs with their own reagents if using anti-Xa-LMWH to exclude apixaban. Importantly, it should also be confirmed that the patient has been receiving apixaban rather than rivaroxaban, as rivaroxaban will also be detected but may have a different cut-off point for exclusion using anti-Xa-LMWH. Conversely, both apixaban (Fig. 1) and rivaroxaban have greater anti-Xa activity than that seen with a similar therapeutic dose of LMWH,[1–3] so even a small amount of apixaban or rivaroxaban significantly interferes with attempts to measure enoxaparin activity by anti-Xa assay.

Conflicts of interest and sources of funding: This work was funded by Bristol Myers Squibb (BMS)/Pfizer. BMS/Pfizer were not involved in the design or conduct of the study.

J. Singh[1], D. M. Ong[1], A. Wallis[1], G. Kelsey[1,2], H. Tran[1,3]

[1]Laboratory Haematology, Alfred Hospital, Melbourne, Vic, Australia; [2]Laboratory Haematology, Royal Melbourne Hospital, Melbourne, Vic, Australia; [3]Australian Centre for Bleeding Disorders, Monash University, Melbourne, Vic, Australia

Contact Dr Jasmine Singh.
E-mail: snghjsmn07@gmail.com

1. Becker RC, Yang H, Barrett Y, *et al*. Chromogenic laboratory assays to measure the factor Xa-inhibiting properties of apixaban–an oral, direct and selective factor Xa inhibitor. *J Thromb Thrombolysis* 2011; 32: 183–7.
2. Beyer J, Trujillo T, Fisher S, *et al*. Evaluation of a heparin-calibrated antifactor Xa assay for measuring the anticoagulant effect of oral direct Xa inhibitors. *Clin Appl Thromb Hemost* 2016; 22: 423–8.
3. Bonar R, Favaloro E, Mohammed S, *et al*. The effect of the direct factor Xa inhibitors apixaban and rivaroxaban on haemostasis tests: a comprehensive assessment using in vitro and ex vivo samples. *Pathology* 2016; 48: 60–71.
4. Dale BJ, Chan NC, Eikelboom JW. Laboratory measurement of the direct oral anticoagulants. *Br J Haematol* 2016; 172: 315–36.
5. Gosselin RC, Adcock M. The laboratory's 2015 perspective on direct oral anticoagulant testing. *J Thromb Haemost* 2016; 14: 886–93.
6. Samuelson BT, Cuker A, Siegal DM, *et al*. Laboratory assessment of the anticoagulant activity of direct oral anticoagulants: a systematic review. *Chest* 2017; 151: 127–38.
7. Billoir P, Barbay V, Joly LM, *et al*. Anti-Xa oral anticoagulant plasma concentration assay in real life: rivaroxaban and apixaban quantification in emergency with LMWH calibrator. *Ann Pharmacother* 2019; 53: 341–7.
8. Gosselin RC, Francart SJ, Hawes EM, *et al*. Heparin-calibrated chromogenic anti-Xa activity measurements in patients receiving rivaroxaban: can this test be used to quantify drug level? *Ann Pharmacother* 2015; 49: 777–83.
9. Sabor L, Raphaël M, Dogné JM, *et al*. Heparin-calibrated chromogenic anti-Xa assays are not suitable to assess the presence of significant direct factor Xa inhibitors levels. *Thromb Res* 2017; 156: 36–8.

DOI: https://doi.org/10.1016/j.pathol.2019.07.012

ACTH measurements in Cushing's syndrome: the need for caution and communication

Sir,

Adrenocorticotropic hormone (ACTH) concentration levels are key in determining the cause of Cushing's syndrome. However, the assay may be vulnerable to interference. This report discusses the case of a 40-year-old woman with cortisol excess and unilateral adrenal lesion but elevated ACTH concentration suggestive of ACTH-dependent Cushing's syndrome. Through close collaboration with the chemical pathology team, it was determined that assay interference was likely leading to a falsely elevated ACTH concentration. The patient underwent a successful unilateral adrenalectomy and avoided further unnecessary testing.

A 40-year-old Chinese woman was referred to the endocrinology outpatient clinic for investigation of an incidentally discovered left adrenal mass and elevated random serum cortisol. On questioning, she reported 2 years of lethargy, central adiposity, and easy bruising as well as amenorrhoea for the previous 3 months. She had no headaches or visual changes. She had suffered from low back pain. She had no relevant family history and took no prescribed or alternative medications. She was a life-long non-smoker and worked in an administrative role.

On clinical examination, her weight was 67 kg and she was normotensive (BP 120/80 mmHg). She had the classical Cushingoid appearance of moon facies, buffalo hump and central adiposity and when compared to photos from 5 years prior, the physical changes were marked. There was no significant abdominal striae or thin skin.

Repeat computed tomography (CT) of her adrenal glands demonstrated a stable left 31 × 24 mm adrenal adenoma with heterogeneous contrast enhancement (non-contrast 22 and post-contrast 126 Hounsfield units) unchanged compared to imaging 18 months ago. Functional testing for primary hyperaldosteronism and phaeochromocytoma was negative.

The patient had biochemical evidence of cortisol excess. Random serum cortisol was elevated at 656 and 706 nmol/L (reference interval 145−619) and failed to suppress with the 1 mg dexamethasone suppression test (cortisol 692−697 nmol/L). A pituitary screen demonstrated secondary hypogonadism (oestradiol <70 pmol/L, FSH 7I U/L, LH <1 IU/L). Prolactin [163 mIU/L (59−619)], TSH [0.76 mIU/L (0.5−4.0)] and free thyroxine [15.4 pmol/L (10−19)] concentrations were normal. A metabolic screen demonstrated dyslipidaemia (cholesterol 6.9 mmol/L, LDL 3.9 mmol/L, Tg 1.5 mmol/L, HDL 2.2 mmol/L). HbA1c was 5.6%.

A high dose 4 mg intravenous dexamethasone suppression test was performed to help identify the source of cortisol excess.[1] Dexamethasone was infused at 1 mg/h for 4 h and the results are summarised in Table 1. The cortisol level failed to suppress, consistent with hypercortisolaemia. Serum dexamethasone concentrations were consistent with exogenous intravenous infusion. The ACTH was initially performed on the Immulite assay (Siemens, Germany) and remained detectable throughout, with a peak of 3.0 pmol/L at 4 h. An 8 mg high dose dexamethasone suppression test with the same assay confirmed an elevated ACTH before and after corticosteroid administration (2.8 pmol/L and 2.3 pmol/L, respectively).

This result was consistent with ACTH-dependent hypercortisolaemia, either from a pituitary or ectopic source. However, this was potentially discordant with the clinical picture of gradual onset hypercortisolaemia and known adrenal lesion. Further investigations were performed to validate the ACTH result. The samples from the 4 mg dexamethasone suppression test were analysed on separate assays (Cobas 602, Roche, Switzerland; and Liaison XL, Diasorin, Italy) and in contrast, returned undetectable and low ACTH concentrations, respectively (Table 1). Testing with heterophile antibody blocking tube (HBT) (Scantibodies Laboratory, USA) resulted in reduction of Siemens Immulite ACTH results by around 50% compared with a control patient with a similar initial ACTH concentration (Supplementary Table 1, Appendix A). These findings raised the possibility of potential positive interference with Immulite ACTH assay.

The patient did not use biotin supplements and rheumatoid factor was negative. She owned a pet rabbit but had no other significant contact with animals.

This case was discussed at a departmental meeting and it was thought that the investigations were most consistent with Cushing's syndrome from a cortisol secreting adrenal adenoma with falsely elevated ACTH concentration on the Siemens Immulite method due to assay interference. The patient underwent a successful laparascopic retroperitoneal left adrenalectomy and histopathology revealed a 35 mm cortical adrenal adenoma with benign features. Post-surgical ACTH concentration from the Siemens Immulite assay remained elevated at 1 h (ACTH 2.1 pmol/L, cortisol 106 nmol/L) and 8 h (ACTH 3.3 pmol/L, cortisol 55 nmol/L). However, in contrast, the Roche ACTH assay (ACTH 0.26 pmol/L at 1 h, ACTH 1.5 pmol/L at 8 h) and Diasorin ACTH assay (ACTH 1.19 pmol/L at 1 h, ACTH 3.07 pmol/L at 8 h) showed an increase over time that would be expected in response to the removal of the source of excess cortisol. She was subsequently commenced on hydrocortisone replacement therapy with a plan to wean this as her remaining adrenal gland recovered.

The diagnosis of Cushing's syndrome requires the confirmation of excess cortisol concentration and loss of diurnal secretion pattern demonstrated by an elevated 24 h urine free cortisol, midnight salivary cortisol or low dose dexamethasone suppression test (1 mg overnight, or 2 mg over 48 h).[2] Once cortisol excess is established, the ACTH concentration is key to determine the likely source of pathology to guide further investigations and management. If the ACTH is elevated then an ACTH secreting pituitary adenoma or ectopic ACTH source should be considered. If the ACTH is suppressed, then the cause of excess cortisol is likely to be from the adrenal gland or an exogenous source.[3]

ACTH concentrations are measured by two-site immunometric 'sandwich' assays. Increasingly, assay interference is recognised to cause false positive and negative results. This can lead to unnecessary and invasive investigations or surgery with associated morbidity and cost.[4,5] There are numerous case reports and recent case series highlighting this problem.[5–8] The erroneous ACTH results were performed with the Siemens Immulite assay in all the above cases,[5–8] including one patient who underwent an unnecessary hypophysectomy for an incorrect diagnosis of Cushing's disease.[8]

Immunoassay interference can be caused by substances that alter the measurable concentration of analyte or the

Table 1 4 mg dexamethasone infusion results

	Time	Cortisol (nmol/L) Siemens Centaur (RI: AM: 145−619; PM: 85−460)	ACTH (pmol/L) Siemens Immulite (RI 1.1−10 pmol/L)	ACTH (pmol/L) Roche Cobas 602 (RI 1.6−14 pmol/L)	ACTH (pmol/L) Diasorin Liaison XL (RI 1.0−10.6 pmol/L)
Day 1					
−60 min	0915	722	1.6	<0.22	
−60 min	0915	538			
−5 min	1010	644	2.0		
	0	Infusion commenced			
+3 h	1315	684	2.5		0.66
+4 h	1415	669	3.0	<0.22	
		Infusion completed			
+5h	1515	702	1.9		0.95
Day 2	0930	766	2.7	<0.22	0.65

RI, reference interval.

antibody binding crucial to the analysis.[9] Factors affecting the measurable analyte concentration include hormone-binding globulins, issues with sample collection or storage and autoanalyte antibodies. Factors that alter antibody binding include heterophile antibodies (HA), human anti-animal antibodies (HAAA) and other proteins that affect antibody binding such as complement and paraproteins. HA are generally polyreactive against heterogeneous antigens and interfere with immunoassays by a non-competitive mechanism. They can react differently for different antibody combinations giving rise to falsely high or low results. HAAA are polyclonal antibodies produced against specific animal immunogens. The human anti-mouse antibodies (HAMA) are the most common HAAA but may include antibodies against sheep, goat, rabbit, etc. These animals are used to produce antibodies used in commercial immunoassays. Therefore, presence of HAAA in a patient's blood can produce false results depending on the assay format and the nature of the antibodies.[9]

Interference should be considered if there is discordance between the laboratory results and the clinical picture. Strategies to detect immunoassay interferences include serial dilutions demonstrating lack of linearity, the elimination of HA using heterophile antibody blocking reagent or precipitation of interfering antibodies using polyethylene glycol (PEG), and use of different assay platforms with different capture or detection antibodies to negate the effect of interfering substances.[5]

In our case, after suspecting assay interference, we used a variety of analytical platforms to verify the ACTH result. The Siemens Immulite assay uses monoclonal mouse antibody as the solid phase and polyclonal rabbit antibody as the conjugate.[10] In contrast, Roche[11] and Liaison[12] assays use monoclonal mouse antibodies in both phases. Interestingly, despite their similarities, the results from the Roche and Liaison assays were similar but not identical. Dilution was performed (1 in 2) on the original Immulite ACTH concentration of 3.1 pmol/L. Linear dilution was observed to 1.2 pmol/L, which was inconsistent with an interfering substance.[5] We also used a commercially produced heterophile antibody blocking preparation (Scantibodies) that did not completely reduce the measured ACTH concentration on Immulite, which may reflect an incomplete blocking of interfering antibodies. This is consistent with other published experience with both lower and higher ACTH levels reported following the use of heterophile blocking agents.[5,6,8] The latter observation may relate to the commercial preparation's increased affinity for polyclonal IgG, leaving behind high affinity monospecific IgM antibodies to bind to the analyte antibodies, leading to high ACTH signal.[5,6]

This case highlights the importance of interpreting assay results within the clinical context. Where discrepancy exists, the laboratory results should be verified and interference excluded to determine the true result. Unfortunately, based on the heterogeneous nature of the antibodies and variations in assay platforms, there is no definitive way to confirm or eliminate assay interference. However, a combination of strategies can be used to better inform clinical decision-making. In this case, communication and cooperation between clinicians and chemical pathologists helped avoid invasive and costly investigations with risk of significant morbidity, while achieving an excellent outcome for the patient.

Conflicts of interest and sources of funding: The authors state that there are no conflicts of interest to disclose.

APPENDIX A. SUPPLEMENTARY DATA

Supplementary data to this article can be found online at https://doi.org/10.1016/j.pathol.2019.08.007.

Sarah Qian[1], Joel Smith[2], Nilika Wijeratne[2,3], Dev Kevat[1,4]

[1]Department of Endocrinology and Diabetes, Western Health, St Albans, Vic, Australia; [2]Department of Biochemistry, Dorevitch Pathology, Heidelberg, Vic, Australia; [3]Department of Medicine, Monash University, Clayton, Vic, Australia; [4]School of Public Health, Monash University, Melbourne, Vic, Australia

Contact Dr Dev Kevat.
E-mail: dev.kevat@monash.edu

1. Abou Samra AB, Dechaud H, Estour B, *et al*. Beta-lipoprotein and cortisol responses to an intravenous infusion dexamethasone suppression test in Cushing's syndrome and obesity. *J Clin Endocrinol Metab* 1985; 61: 116–9.
2. Nieman LK, Biller BMK, Findling JW, *et al*. The diagnosis of cushing's syndrome: an endocrine society clinical practice guideline. *J Clin Endocrinol Metab* 2008; 93: 1526–40.
3. Nieman LK, Biller BM, Findling JW, *et al*. Treatment of cushing's syndrome: an endocrine society clinical practice guideline. *J Clin Endocrinol Metab* 2015; 100: 2807–31.
4. Yener S, Demir L, Demirpence M, *et al*. Interference in ACTH immunoassay negatively impacts the management of subclinical hypercortisolism. *Endocrine* 2017; 56: 308–16.
5. Donegan DM, Algeciras-Schimnich A, Hamidi O, *et al*. Corticotropin hormone assay interference: a case series. *Clin Biochem* 2019; 63: 143–7.
6. Grasko J, Willliams R, Beilin J, *et al*. A diagnostic conundrum: heterophilic antibody interference in an adrenocorticotropic hormone immunoassay not detectable using a proprietary heterophile blocking reagent. *Ann Clin Biochem* 2013; 50: 433–7.
7. Choy KW, Teng J, Wijeratne N, *et al*. Immunoassay interference complicating management of Cushing's disease: the onus is on the clinician and the laboratory. *Ann Clin Biochem* 2017; 54: 183–4.
8. Greene LW, Geer EB, Page-Wilson G, *et al*. Assay-specific spurious ACTH results lead to misdiagnosis, unnecessary testing and surgical misadventure – a case series. *J Endocr Soc* 2019; 3: 763–72.
9. Tate J, Ward G. Interferences in immunoassay. *Clin Biochem Rev* 2004; 25: 105–12.
10. Siemens Healthcare Diagnostics. *Immulite 2000 ACTH. Package insert, PIEL2KAC-2{17},2015-07-16*. Gwynedd: Siemens Healthcare Diagnostics, 2015.
11. Roche Diagnostics. *Cobas Adrenocorticotropic Hormone. Package insert, version 8.0, 2013*. Mannheim: Roche Diagnostics, 2013.
12. Diasorin. *Liaison ACTH. Package insert*. Saluggia: Diasorin, 2016.

DOI: https://doi.org/10.1016/j.pathol.2019.08.007