CLINICAL STUDY

Evaluation of an enzyme immunoassay for plasma-free metanephrines in the diagnosis of catecholamine-secreting tumors

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Abstract

Objective: To define the test characteristics of an enzyme immunoassay (EIA) for plasma-free metanephrines (metanephrine and normetanephrine) in the diagnosis of pheochromocytoma and paraganglioma.

Design: Prospective observational design from a single University Hospital. Twenty-four hour urine for catecholamines and plasma for free metanephrines were collected from patients with a clinical suspicion of pheochromocytoma or paraganglioma. Patient records were reviewed for clinical data, follow-up, imaging and laboratory results to establish or exclude the diagnosis of pheochromocytoma.

Patients and methods: Out of 178 consecutive patients, 10 had a paraganglioma and 12 had a pheochromocytoma; 156 were finally judged not to harbour active tumors and were therefore considered as controls. The main outcome measure was the diagnosis or exclusion of paraganglioma or pheochromocytoma and test characteristics of plasma-free metanephrines measured by EIA.

Results: Urinary epinephrine had a sensitivity of 45.5% and norepinephrine a sensitivity of 75% (98.8% specificity) for the diagnosis of pheochromocytoma. Plasma-free metanephrine and normetanephrine both had a sensitivity of 66.7% and a specificity of 100%, but when combined (either positive) they demonstrated a 91.7% sensitivity with a preserved specificity of 100%. For the diagnosis of paraganglioma, urinary norepinephrine gave slightly better results than plasma-free metanephrines, but combined testing was of no additional value.

Conclusions: Plasma-free metanephrines measured by EIA have better diagnostic test characteristics than urinary catecholamines in the diagnosis of pheochromocytoma. The EIA offers a simple and effective measurement of plasma-free metanephrines.

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Introduction

Pheochromocytomas are rare neuroendocrine tumors arising from chromaffin cells of the adrenal medulla or extra-adrenal paraganglia. Tumors of extra-adrenal origin are now usually referred to as paragangliomas (1). Different tests are available to the clinician for their biochemical diagnosis: they are based on plasma or urinary measurement of the direct secretory products of the adrenomedullary-sympathetic system or their metabolites, specifically catecholamines or their metanephrine derivatives (2). In general, as the catecholamines epinephrine and norepinephrine may be secreted in an intermittent fashion, most authorities now tend to recommend assessment of their metabolites metanephrine and normetanephrine as these

provide a more robust measure of catecholamine output (3). According to the recommendations for the diagnosis of pheochromocytoma from the First International Symposium on Pheochromocytoma, the initial assessment of secretory capacity should include the measurements of fractionated metanephrines in urine or plasma, or both, as available (4). However, plasma-free metanephrines are currently analyzed by cumbersome and technically demanding techniques such as HPLC, or HPLC coupled with mass spectrometry, which limit their availability. We have therefore conducted a prospective study to define the test characteristics of a new enzyme immunoassay (EIA) for plasma-free metanephrines in the diagnosis of pheochromocytoma and paraganglioma in a cohort of patients from a single referral centre.

Patients and methods

Patients

Consecutive patients attending the Endocrine Clinic at St Bartholomew's Hospital (London, UK) for suspicion of catecholamine-secreting tumors were included in this study, which was agreed by the Institutional Review Board. The diagnosis of pheochromocytoma was suspected on clinical grounds (classical or suspicious symptoms, hypertension that was severe, resistant to multiple medications, or seen at a young age, etc.) or because of the incidental finding of an adrenal mass. A number of patients were tested as part of a surveillance program in the context of genetic syndromes. In the great majority of patients, the diagnosis was excluded on normal biochemistry, an absence of abnormal imaging, and a failure to progress on regular review. All patients included in the study were tested by undergoing a 24-h urinary collection for the measurement of catecholamines (epinephrine, norepinephrine, and dopamine), with a single fasting blood test for plasma-free metanephrines (metanephrine and normetanephrine) analysis by EIA. Specific dietary recommendations are not routinely given to our patients as catecholamines and metanephrines are not materially affected by dietary intake. No interrogation regarding paracetamol was undertaken as this agent, which may contaminate results, is included in a whole variety of remedies of which most patients are unaware, so specific questioning is of dubious value.

The clinician in charge of the patient was free to decide whether imaging was necessary (adrenal magnetic resonance imaging (MRI) or computed tomography (CT), plus radionuclide imaging with ^{123}I -meta-iodo benzylguanidine (^{123}I -MIBG) and the form of follow-up required. All clinicians were blinded to the plasma metanephrine results. At the end of the recruitment period, one of the authors (M P), blinded to the plasma metanephrine results, reviewed all patient records for the following information: working diagnosis made by the attending physician, follow-up results, imaging studies, and potential explanations for false positive urinary catecholamines results (such as obstructive sleep apnoea, medications, etc). This final diagnosis was concordant with the diagnosis made by the attending clinician in all cases, with the exception of a single patient. Following this analysis, a single patient with positive plasma metanephrines, initially considered not to have a pheochromocytoma, was reviewed and reclassified as showing a true pheochromocytoma (see Results).

A secretory pheochromocytoma or paraganglioma was diagnosed if the urinary catecholamines were positive (>99th centile) with positive adrenal or extra-adrenal imaging. The diagnosis was confirmed histologically in all patients subjected to operative removal. In the case of paragangliomas, the diagnosis of a non-secreting tumor was made in the presence of

typical radiological findings with normal urinary catecholamines results and positive surgical pathology. The diagnosis of a pheochromocytoma was considered to be excluded if biochemical testing was negative in patients with a low clinical probability, and no further evidence appeared during prolonged follow-up at a mean follow-up of 1.5 years (538 ± 210 days).

Where appropriate, negative cross-sectional imaging (CT or MRI) or negative ^{123}I -MIBG scanning was used as corroborative evidence. If the 24-h urinary catecholamines were positive (>99th centile), negative adrenal, abdominal, and, in some cases, thoracic, cross-sectional imaging, plus ^{123}I -MIBG scanning, were utilized to exclude the diagnosis. When the diagnosis was rejected in spite of positive urinary catecholamines, an alternate explanation for the false positive results was sought and obtained.

Urinary catecholamines analysis by HPLC with electrochemical detection

Twenty-four hour urinary specimens were collected from patients into acid (6 M HCl; pH < 3) bottles. No dietary restrictions were enforced (as noted above). Measurement of urinary catecholamines (norepinephrine, epinephrine, and dopamine) was performed by HPLC-electrochemical detection (ECD) following a technique previously described in detail (5).

Plasma-free metanephrines analysis by EIA

Plasma-free metanephrine and normetanephrine were determined using an EIA commercial kit manufactured by Labor Diagnostica Nord GmbH, Nordhorn, Germany, and supplied by Immunodiagnostic Systems Ltd (IDS), Tyne and Wear, UK.

Extraction Metanephrine and normetanephrine in calibrators, controls, and patient samples were precipitated following 60 min incubation at room temperature in tubes in which two precipitants were added then vortex mixed. Following centrifugation at 3000 g for 10 min, 100 μl of supernatant was used for the following acylation step.

Acylation For each supernatant, 100 μl was pipetted into the respective wells of the acylation plate. Addition of 25 μl of buffer, followed by 25 μl of freshly diluted acylation reagent was added to all wells. The acylation plate was then incubated for 15 min at room temperature on an orbital shaker (14 g).

EIA From each acylated supernatant, 50 μl was used in the metanephrine assay and 10 μl was used in the normetanephrine assay. Each supernatant volume was pipetted into the respective wells of each of the metanephrine and normetanephrine EIA plates, followed by 100 μl of each respective antiserum.

Each plate was covered with foil and left to incubate at 2–8 °C for >40 h. After this incubation time, each plate was washed and blotted three times before 100 µl of enzyme conjugate was added and incubated for 30 min at room temperature on an orbital shaker. There was further washing and blotting followed by the addition of 100 µl of substrate to all wells with incubation at room temperature for ~20 min. The final step was to add 100 µl of stop solution to all wells followed by absorbance reading of the solution using a microplate reader at 450 nm with reference wavelength between 620 and 650 nm. The absorbance reading took place within the 10 min allowed from the time the stop solution was added.

Assay sensitivity was 10 pg/ml for both plasma metanephrine and normetanephrine. Inter-assay coefficients of variation were 8.8% at 34 pg/ml and 9.4% at 339 pg/ml for plasma metanephrine, and 14.5% at 62 pg/ml and 8.55% at 625 pg/ml for plasma normetanephrine respectively. Quality control samples were added to each assay run ($n=10$) in order to compute the inter-assay coefficient of variation.

Institutional and manufacturers' normal limits for biochemical assays

The normative limits for 24-h urinary catecholamines were <144 nmol/24 h for epinephrine, <560 nmol/24 h for norepinephrine, and <3194 nmol/24 h for dopamine: these were based on a previous assessment of the 95th centile (5). According to the manufacturer's recommendations, normal values for plasma-free metanephrines are <90 pg/ml for metanephrine and <200 pg/ml for normetanephrine.

Statistical analysis

Figures with receiver-operating characteristic curves (ROC curves) and ROC curve calculations (area under ROC curve, 95% confidence interval, CI) were done with MedCalc (version 9.6.4.0). All other figures were drawn and calculations (mean, 95% CI, sensitivities, and specificities) were performed using GraphPad Prism 5 (GraphPad Software Inc., La Jolla, CA 92037, USA).

Results

The total cohort consisted of 236 samples. Out of these, 30 were taken from the same patients <12 months apart and were excluded from the analysis. In another 22 cases, urinary catecholamines were not available, thus leaving 184 separate samples for the analysis. These 184 samples came from 178 different patients (mean age 45.2 years, 95% CI, ± 18 years, 108 females and 70 males). Six patients were tested twice (more than 12 months apart). Of the total group, 156 patients

(161 samples) were finally judged not to harbour active tumors and were therefore considered as controls. Out of the 22 patients (23 samples) with catecholamine-secreting tumors, 10 were paragangliomas (Table 1) (one patient with two samples, only one sample was used for all calculations) and 12 were pheochromocytomas (Table 1). The final diagnosis was made after a mean follow-up of 1.5 years (538 ± 210 days).

In the 178 subjects, the reasons for testing were clinical symptoms or investigation for secondary hypertension in 78 (43.8%), a confirmed or suspected genetic syndrome in 55 (30.9%), follow-up after treatment for pheochromocytoma or paraganglioma in 23 (12.9%), and investigations after the discovery of an adrenal incidentaloma in 22 (12.4%). The genetic syndromes consisted of 15 patients with multiple endocrine neoplasia (MEN; three MEN1, nine MEN2A, and three MEN2B), 13 von Hippel-Lindau (VHL) patients, 4 patients with succinate dehydrogenase (SDH) mutations, 2 patients with neurofibromatosis type 1, and 5 patients with a positive family history for familial paragangliomas. In the 16 remaining subjects, a genetic syndrome was suspected in terms of the clinical background (ten MEN2, four VHL, and two SDH mutations).

Twenty-four hour urinary catecholamines

A total of 184 results from 178 different patients were available for urinary catecholamine analysis. The detailed results are given in Table 2 and displayed graphically in Fig. 1. In the 156 controls, the mean 24 h urinary epinephrine was 34.1 nmol/24 h, norepinephrine was 308.8 nmol/24 h, and dopamine was 1741 nmol/24 h. For the 12 pheochromocytomas, the mean 24 h urinary epinephrine was 786 nmol/24 h, norepinephrine 2407 nmol/24 h, and dopamine 2970 nmol/24 h. For one of the patients with a pheochromocytoma, the urinary epinephrine result was not available because of analytical interference probably due to a medication. For the ten paragangliomas, the mean 24 h urinary epinephrine was 36.5 nmol/24 h, norepinephrine was 1947 nmol/24 h, and dopamine 4358 nmol/24 h.

Using the institutional cut-offs (abnormal 24 h urinary catecholamines if >144 nmol/24 h for epinephrine, if >560 nmol/24 h for norepinephrine, and if >3194 nmol/24 h for dopamine), the sensitivities (specificities) were 45.5% (98.8%) for epinephrine, 75.0% (91.3%) for norepinephrine, and 16.7% (93.8%) for dopamine in the diagnosis of pheochromocytoma. In the diagnosis of paraganglioma, sensitivities (specificities) were 0% (98.8%) for epinephrine, 70.0% (91.3%) for norepinephrine, and 40% (93.8%) for dopamine. The confidence intervals for sensitivities and specificities, and ROC curves, are given in Table 3.

Table 1 Clinical, biochemical and imaging characteristics of patients with confirmed pheochromocytomas and paragangliomas.

Case	Age (years)	Sex	Norepi (nmol/24 h)	Epi (nmol/24 h)	Dopamine (nmol/24 h)	MN (pg/ml)	NormN (pg/ml)	Imaging	Tumor size and location	Genetic syndrome	Final diagnosis and comments
<i>Paragangliomas</i>											
1	43.8	M	181	30	1320	5	37	Neck ultrasound	3.6 × 1 cm mass adjacent to left carotid artery	VHL	Paraganglioma. Recurrent paraganglioma (VHL patient under follow-up)
2	35.7	M	890	95	2329	5	370	CT (liver lesions positive on MIBG)	Metastatic disease (liver, left para-aortic mass, and solitary lung mass)	No	Paraganglioma (follow-up patient with extensive metastatic disease)
3	41.6	M	7162	30	18 147	17	1406	MRI and MIBG positive	Metastatic disease (spine, retroperitoneal)	No	Paraganglioma (follow-up patient with extensive metastatic disease)
4	33.8	M	357	30	4405	5	10	CT, MRI, and MIBG positive	Residual and metastatic disease (paracaval, right adrenal bed, and bone)	No	Paraganglioma. Composite tumor (paraganglioma and ganglioblastoma) (follow-up patient with extensive metastatic disease)
5	44.1	F	858	30	1799	5	186	MIBG positive	Metastatic disease (liver)	No	Paraganglioma (follow-up patient with extensive metastatic disease)
6	42.1	F	2453	30	934	18	384	CT, MRI, and MIBG positive	Multiple 2–3 cm mass (mainly aortocaval)	VHL	Paraganglioma (follow-up patient with extensive disseminated disease)
7	68.4	F	3901	30	4757	117	3839	CT and MIBG positive	Multiple retroperitoneal lesions	No	Paraganglioma (follow-up patient with metastatic disease)
8	65.2	F	201	30	1480	30	10	CT and MIBG positive	Left neck mass 4.2 × 3.2 cm	No	Paraganglioma (recurrent disease 13 years after excision of a first paraganglioma)
9	51.2	M	5877	30	16 007	5	452	MRI positive (MIBG negative)	Diffuse periaortic metastatic lymphadenopathy		Paraganglioma. Metastatic paraganglioma (follow-up, initially presented with bladder paraganglioma 7 years earlier)
10	12.1	F	388	30	1305	17	129	MRI positive	2.5 cm mass adjacent to the right adrenal	VHL	Pheochromocytoma. Tumor detected during surveillance screening
<i>Pheochromocytomas</i>											
1	49.1	M	1546	238	2277	119	114	CT heterogeneous left adrenal mass, MRI and MIBG positive	Left adrenal 5 cm	No	Pheochromocytoma (initial presentation, investigated for hypertension). Surgical resection
2	52.5	M	664	169	1951	136	246	CT, MRI, and MIBG positive	Right adrenal 3.5 cm tumor	No	Pheochromocytoma (initial presentation). Surgical resection
3	70.7	M	4760	90	6191	284	6000	CT and MIBG positive	Multiple abdominal metastases	No	Pheochromocytoma (follow-up patient with metastatic disease, initial presentation 3 years earlier with right adrenal pho)

Table 1 Continued

Case	Age (years)	Sex	Norepi (nmol/24 h)	Epi (nmol/24 h)	Dopamine (nmol/24 h)	MN (pg/ml)	NorMN (pg/ml)	Imaging	Tumor size and location	Genetic syndrome	Final diagnosis and comments
4	80.4	F	362	68	453	275	247	CT appearance: right benign adrenal adenoma. MIBG positive during follow-up	2 cm right adrenal mass	No	Pheochromocytoma (initial presentation, incidental adrenal mass, on retrospect symptomatic and hypertensive). Poor surgical candidate, treated with alpha blocking agent. Diagnosis was reconsidered and MIBG requested after the initial work up when metanephrines results became available to the clinician
5	80.1	F	957	69	1080	31	264	CT, MRI, and MIBG positive	2 cm left adrenal mass	No	Pheochromocytoma (initial presentation, incidental adrenal mass). Surgical resection
6	16.2	M	7820	NA	2886	40	800	CT and MRI: right adrenal mass with a further left paraaortic mass. MIBG uptake for left mass	3.8×2.3 cm right adrenal mass and left paraaortic mass	No	Right pheochromocytoma + paraganglioma (initial presentation). Surgical resection of both lesions
7	35.1	M	9614	7499	12 687	3000	6000	CT and MIBG positive	10×12 cm right adrenal mass	No	Pheochromocytoma (initial presentation, investigated for severe hypertension and symptoms). Surgical resection
8	48.7	M	698	169	2087	167	193	CT and MIBG positive	Right adrenal 3.5 cm mass	SDHB mutation of unknown significance	Pheochromocytoma (initial presentation). Surgical resection
9	36.3	F	653	Analytical interference (no result)	1874	1687	1048	MRI and MIBG positive	Left adrenal mass	No	Pheochromocytoma (initial presentation in a pregnant woman). Surgical resection
10	61.9	F	1059	NA	1402	36	65	CT positive and MIBG negative	1.5 cm right adrenal mass	No	Pheochromocytoma (initial presentation, incidental adrenal mass). Surgical resection
11	58.1	M	323	252	1182	205	116	CT and MIBG positive	3.4 cm left adrenal mass	No	Pheochromocytoma (initial presentation, incidental adrenal mass). Surgical resection
12	68.8	F	432	30	1575	50	346	CT and MIBG positive	3.4 cm right adrenal mass	No	Pheochromocytoma (initial presentation, incidental adrenal mass). Surgical resection. Negative urinary catecholamines, preop diagnosis based on imaging

Nor epi, norepinephrine; Epi, epinephrine; MN, metanephrine; Nor MN, normetanephrine.

Table 2 Twenty-four hour urinary catecholamines and plasma-free metanephrines: mean values (lower and upper 95% CI of the mean).

	Pheochromocytoma (n=12)	Paragangliomas (n=10)	Controls (n=161)
24-h urinary catecholamines (nmol/24-h)			
Epinephrine	785.8 ^a (–711.0–2283)	36.5 (21.8–51.2)	34.1 (30.6–37.6)
Norepinephrine	2407 (371.8–4443)	1947 (330.3–3564)	308.8 (284.5–333.1)
Dopamine	2970 (821.7–5119)	4358 (609.9–8106)	1741 (1559–1924)
Plasma-free metanephrines (pg/ml)			
Metanephrine	502.5 (74.8–1080)	24.1 (0.06–48.3)	17.6 (15.2–20.0)
Normetanephrine	1287 (124.5–2698)	646.9 (208.8–1503)	43.1 (36.9–49.2)

^an=11 for 24-h epinephrine (see text).

Plasma-free metanephrines

In the same cohort of 178 patients, 184 results were available for plasma metanephrines analysis. The detailed results are given in Table 2 and displayed graphically in Fig. 1. In the 156 controls, the mean plasma metanephrine was 17.6 pg/ml and mean plasma normetanephrine 43.1 pg/ml. In our control population, the highest value for metanephrine was 74 and 180 pg/ml for normetanephrine. For the 12 pheochromocytomas, mean plasma metanephrine was 502.5 pg/ml and mean plasma normetanephrine was 1287 pg/ml. For the ten paragangliomas, mean plasma metanephrine was 24.1 pg/ml and mean plasma normetanephrine was 646.9 pg/ml. Using the manufacturer's cut-offs (abnormal plasma metanephrine if >90 pg/ml; abnormal normetanephrine if >200 pg/ml), the sensitivity (66.7%) and specificity (100%) were identical for metanephrine and for normetanephrine in the diagnosis of pheochromocytoma. In the diagnosis of paraganglioma, sensitivities (specificities) were 10% (100%) for metanephrine and 40% (100%) for normetanephrine. For the diagnosis of pheochromocytoma, ROC curves for 24-h urinary norepinephrine, plasma metanephrine, plasma normetanephrine, sum of plasma metanephrines, and sum of normalized ratios of plasma metanephrines are given in Fig. 2.

Test characteristics for combined urinary epinephrine and norepinephrine versus combined metanephrine and normetanephrine (for pheochromocytoma only)

We then assessed whether combining the results from the urinary epinephrine and norepinephrine, and plasma-free metanephrine and normetanephrine respectively, would change the test utility in the diagnosis of pheochromocytoma. For paragangliomas, this analysis was of no added value as urinary epinephrine was negative in all cases, and plasma-free metanephrine was positive in only a single case with a positive normetanephrine: thus, combined testing failed to improve the utility of either urinary catecholamines or plasma metanephrines.

For plasma normetanephrine and metanephrine (using the same manufacturer's cut-offs: abnormal metanephrine if >90 pg/ml; abnormal normetanephrine if >200 pg/ml), the combination of results (either

Figure 1 Test results for 24-h urinary catecholamines (norepinephrine, epinephrine, and dopamine) and plasma-free metanephrines (metanephrine and normetanephrine). Dashed lines are cut-off values.

Table 3 Test characteristics (sensitivity, specificity, and area under the curve for ROC) for 24-h urinary catecholamines and plasma-free metanephrines.

	Pheochromocytoma			Paraganglioma		
	Sensitivity % (95% CI)	Specificity % (95% CI)	Area under ROC curve	Sensitivity % (95% CI)	Specificity % (95% CI)	Area under ROC curve
24-h urinary catecholamines						
Epinephrine	45.5% (16.7–76.6%)	98.8% (95.6–99.8%)	0.844 (0.781–0.895)	0%	98.8% (95.6–99.9%)	0.517 (0.439–0.594)
> 144 nmol/24 h						
Norepinephrine	75.0% (42.8–94.5%)	91.3% (85.8–95.2%)	0.927 (0.877–0.961)	70.0% (34.8–93.3%)	91.3% (85.8–95.2%)	0.787 (0.718–0.845)
> 560 nmol/24 h						
Dopamine	16.7% (2.1–48.4%)	93.8% (88.9–97.0%)	0.616 (0.540–0.689)	40.0% (12.2–73.8%)	93.8% (88.9–97.0%)	0.689 (0.613–0.757)
> 3194 nmol/24 h						
Plasma-free metanephrines						
Metanephrine	66.7% (34.9–90.1%)	100% (97.7–100.0%)	0.964 (0.924–0.986)	10%	100% (97.7–100%)	0.525 (0.448–0.602)
> 90 pg/ml						
Normetanephrine	66.7% (34.9–90.1%)	100% (97.7–100.0%)	0.969 (0.931–0.981)	40.0% (12.2–73.8%)	100% (97.7–100.0%)	0.778 (0.708–0.838)
> 200 pg/ml						

one to be positive) retained a 100% specificity (i.e. not a single false positive result), but sensitivity was increased to 91.7% for the diagnosis of pheochromocytoma (a single pheochromocytoma would have been missed; Table 4 and Fig. 3). In order to construct a ROC curve with a single variable, we divided the metanephrine and normetanephrine values by their respective upper limit reference (90 pg/ml for free plasma metanephrine and 200 pg/ml for free plasma normetanephrine). Then, we added these two ratios and obtained a single variable integrating the two free metanephrines as a sum of normalized ratios. On the ROC curve, the cut-off with the highest accuracy was 1.48 (sensitivity 91.7% and specificity 100%). The area under the ROC curve had the highest value at 0.987 (95% CI: 0.956–0.998) compared with the ROC curves for the single results (see Table 3). The simple sum of metanephrine and normetanephrine gives basically the same results at a cut-off of 228 pg/ml, but the sum of normalized ratios allows easier comparisons with other methods if they have different reference values.

In comparison, the combination of urinary epinephrine and norepinephrine (either one to be positive above our institutional cut-offs) increased the sensitivity of urinary catecholamines to 83.3% but at the cost of a loss of specificity (90.1%). By raising the cut-offs for epinephrine to > 220 nmol/24 h and > 650 nmol/24 h for norepinephrine, the maximal specificity obtained was 95.6% with the same sensitivity of 83.3% (Table 4 and Fig. 4).

Discussion

In this study, we investigated the utility of a new EIA for plasma-free metanephrines in comparison with our current analytical technique, urinary catecholamines measured by HPLC-ECD. Ideally, the EIA should have been tested against metanephrines measurements by HPLC or gas chromatography coupled with tandem mass spectroscopy; however, at the time we performed our study, this technique for plasma or urinary metanephrines was not routinely available in our institution, and this is still the case in many other centres worldwide. We decided to analyze separately the results for paragangliomas and pheochromocytomas because of their different secretion patterns.

Our study shows that plasma-free metanephrines measured by EIA have better test characteristics in the diagnosis of pheochromocytoma than urinary catecholamines. Plasma metanephrine showed a sensitivity of 66.7% and a specificity of 100% at a cut-off level of 90 pg/ml. At a cut-off level of 200 pg/ml, plasma normetanephrine had similar characteristics. The sensitivity and specificity of plasma-free metanephrine was higher than its urinary catecholamine counterpart, epinephrine. The sensitivity of urinary norepinephrine was higher than that of plasma-free

Figure 2 Multiple comparison of ROC curves in the diagnosis of pheochromocytoma. U NE, 24-h urinary norepinephrine; MN, plasma metanephrine; NorMN, plasma normetanephrine; MN + NorMN, sum of plasma metanephrines; MN/90 + NorMN/200, sum of normalized ratios of plasma-free metanephrines.

normetanephrine but at the price of a lower specificity. The area under the ROC curve for plasma-free normetanephrine was similar to that for urinary norepinephrine, but assessment of either free metanephrine or normetanephrine had the highest sensitivity and specificity. The combination of both metanephrines results (either metanephrine or normetanephrine to be positive) still maintained a 100% specificity (i.e. not a single false positive result) with a sensitivity of 91.7% for the diagnosis of pheochromocytoma (one pheochromocytoma would be missed). In our cohort, lowering the metanephrine cut-offs to diagnose the one missed pheochromocytoma would lead to an unacceptable loss of specificity. Combined plasma metanephrines had higher sensitivity and specificity compared with combined urinary epinephrine and norepinephrine even if catecholamine cut-offs were raised to improve their specificity.

In the diagnosis of paraganglioma, urinary norepinephrine seemed to perform slightly better than plasma-free normetanephrine with a sensitivity of 70% (40% for plasma-free normetanephrine) and a specificity of 91.3% (100% for plasma-free normetanephrine) and an area under ROC curve of 0.787 (0.778 for plasma-free normetanephrine). Paragangliomas show a different catecholamine secretory pattern compared with pheochromocytomas, a fact that has been well established for many years. Extra-adrenal pheochromocytomas – paragangliomas – rarely secrete epinephrine, and paragangliomas are generally less often secretory compared with pheochromocytomas (6).

In the recommendations for the diagnosis of pheochromocytoma from the First International Symposium on pheochromocytoma, the authors stated that 'measurements of fractionated metanephrines (i.e. normetanephrine and metanephrine measured separately) in urine or plasma provide superior diagnostic sensitivity to measurements of the parent catecholamines' (4). Catecholamines and metanephrines are excreted by the kidneys and renal function impairment leads to variations in their levels. However, in patients with renal failure, total metanephrines levels are elevated but free metanephrines are not affected (7). Drug interference can cause false positive metanephrines results. Measurements of metanephrines by mass spectrometry should minimize potential analytical interference, but this technique is not widely available as yet (8). Metanephrines can also be elevated without pheochromocytoma in some situations of major physical stress (e.g. surgery, stroke, obstructive sleep apnoea, etc.) (8).

In an international study including the National Institutes of Health (NIH), plasma-free metanephrines and urinary fractionated metanephrines had the highest sensitivities (99 and 97% respectively) (3). The specificity was 86% for urinary catecholamines, 93% for urinary total metanephrines, and 89% for plasma-free metanephrines, but was the lowest for urinary fractionated metanephrines at 69%. Based on ROC curve analysis, plasma-free metanephrines had the best test characteristics when different cut-offs were used for sensitivity and specificity (3). In a study from the Mayo Clinic, fractionated plasma metanephrines still had the highest sensitivity (97%) compared with a sensitivity of

Table 4 Test characteristics (sensitivity and specificity) for combined 24-h urinary epinephrine and norepinephrine versus combined plasma-free metanephrines in the diagnosis of pheochromocytoma.

	Pheochromocytoma	
	Sensitivity % (95% CI)	Specificity % (95% CI)
24-h urinary catecholamines		
24-h urinary epinephrine > 144 and/or norepinephrine > 560 nmol/24 h	83.3% (51.6–97.9%)	90.1% (84.3–94.2%)
24-h urinary epinephrine > 220 and/or norepinephrine > 650 nmol/24 h	83.3% (51.6–97.9%)	95.6% (91.1–98.2%)
Plasma-free metanephrines		
Plasma-free metanephrine > 90 and/or plasma-free normetanephrine > 200 pg/ml	91.7% (61.5–99.8%)	100% (97.7–100%)

Figure 3 Test results for combined testing with plasma-free metanephrines in pheochromocytoma.

90% for urinary total metanephrines and catecholamines (either test positive), although the difference was not statistically significant. However, the specificity of fractionated plasma metanephrines was 85% compared with 98% for the combined urinary measurements (9). The authors' conclusions were that while plasma-free metanephrines offer the most sensitive test for a pheochromocytoma, because of low specificity its routine use in screening a low probability population might give rise to too many false positive results (9). Measurement of 24-h urinary total metanephrines and

Figure 4 Test results for combined testing with 24-h urinary norepinephrine and epinephrine in pheochromocytoma.

catecholamines would be more suitable for testing low-risk subjects (the most frequent situation), whereas plasma-free metanephrines should be reserved for selected high-risk patients (such as familial endocrine syndromes) (9). Plasma-free metanephrines in both studies were analyzed using HPLC. In an editorial commenting upon the latter study, Eisenhofer emphasized that 'assays of plasma free metanephrines are (...) not as easy to establish and run on a routine basis as conventional assays of urinary metanephrines and catecholamines'. Even at the NIH, transfer of the technology from the research to the routine laboratory environment was not without some difficulty, despite the presence of highly competent and skilled technical staff' (10). Thus, while either plasma-free or urinary fractionated metanephrines were considered to be the most accurate biochemical markers of catecholamine-secreting tumors, the technical complexity of the assay(s) involved rendered their wide-scale use problematic. Testing of plasma metanephrines with an EIA may represent an alternative for centres that do not have access to the most accurate tandem mass spectrometry for plasma metanephrines, particularly in smaller units.

A more recent study from a single French centre, also using HPLC, has compared the test characteristics of fractionated metanephrine measurements (urinary and plasma) to catecholamine measurements (urinary and plasma) (11). The assay characteristics of metanephrines were always better than those of the catecholamines from which they were derived. Urinary metanephrines yielded the highest specificity and plasma metanephrines the highest sensitivity, in concordance with the conclusions of Sawka *et al.* (9, 11). In a retrospective study from Scotland, urinary free metanephrines had a sensitivity of 100% (specificity of 94%) compared with the sensitivity of 84% for urinary catecholamines, but plasma metanephrines were not evaluated in that study (12). However, to date few studies have reported results using a technology other than HPLC to assay plasma-fractionated metanephrines. Unger and colleagues used an RIA developed by the same manufacturer as the EIA studied here (13). In their series of 24 sporadic pheochromocytomas, plasma normetanephrine was the best single parameter with a sensitivity of 91.7% and a specificity of 95.6%, using a normetanephrine threshold of >126 pg/ml derived from ROC curve analysis. We now show that a similar but simpler assay using EIA has very favorable characteristics for the investigation and diagnosis of catecholamine-secreting tumors. A recent study from China, using the same EIA as reported here, has reported that the combination of plasma-free metanephrine and normetanephrine (either positive) had a sensitivity of 96.7% and a specificity of 86.3% (14). They calculated their cut-off values as mean + 2 s.d. in controls ($n = 51$), which were 91.9 pg/ml for metanephrine and 134.5 pg/ml for

normetanephrine. However, their study had several limitations in that patients were recruited only through referral for ^{131}I -MIBG, the diagnosis of pheochromocytoma was based exclusively on ^{131}I -MIBG imaging, and no comparison with other biochemical tests was available in the relatively small control population.

In conclusion, our study is a prospective work conducted in a referral centre with experience in pheochromocytoma and paraganglioma management. Special care was taken in reviewing patient notes for the diagnosis of cases versus controls taking into account the clinical probability, urinary catecholamines results, cross-sectional and nuclear imaging when available, and follow-up. This study found that the diagnostic characteristics of fractionated plasma metanephrines (metanephrine and normetanephrine), as measured by EIA, are superior to the measurement of 24-h urinary catecholamines analyzed by HPLC-ECD. At the relevant cut-offs employed, the EIA for metanephrines showed a perfect specificity with a high sensitivity. While the total number of catecholamine-secreting tumors was still relatively small, we believe that our results are particularly robust regarding the specificity as we used as a control group not normal volunteers, but a patient population with a suspicion of pheochromocytoma. Thus, a positive result is almost certainly diagnostic of a catecholamine-producing tumor, either a pheochromocytoma or a paraganglioma. The absence of false positive results would be especially useful when testing a low probability population (9) where the test still provides high sensitivity, but criteria for further investigation may need to be modified where there is a high *a priori* suspicion of a tumor. In such cases, follow-up with urinary metanephrines and/or plasma chromogranin A has been suggested to be of value (15). Further evaluation of this EIA is required with a larger number of cases (pheochromocytomas and paragangliomas) for a better assessment of its sensitivity.

Declaration of interest

The authors have no conflict of interest to declare.

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References

- 1 Lenders JW, Eisenhofer G, Mannelli M & Pacak K. Pheochromocytoma. *Lancet* 2005 **366** 665–675.
- 2 Grossman A, Pacak K, Sawka A, Lenders JW, Harlander D, Peaston RT, Reznick R, Sisson J & Eisenhofer G. Biochemical diagnosis and localization of pheochromocytoma: can we reach a consensus? *Annals of the New York Academy of Sciences* 2006 **1073** 332–347.
- 3 Lenders JW, Pacak K, Walther MM, Linehan WM, Mannelli M, Friberg P, Keiser HR, Goldstein DS & Eisenhofer G. Biochemical diagnosis of pheochromocytoma: which test is best? *Journal of the American Medical Association* 2002 **287** 1427–1434.
- 4 Pacak K, Eisenhofer G, Ahlman H, Bornstein SR, Gimenez-Roqueplo AP, Grossman AB, Kimura N, Mannelli M, McNicol AM & Tischler AS. Pheochromocytoma: recommendations for clinical practice from the First International Symposium. *Nature Clinical Practice. Endocrinology and Metabolism* 2007 **3** 92–102.
- 5 Ross GA, Newbould EC, Thomas J, Bouloux PM, Besser GM, Perrett D & Grossman A. Plasma and 24 h-urinary catecholamine concentrations in normal and patient populations. *Annals of Clinical Biochemistry* 1993 **30** 38–44.
- 6 Erickson D, Kudva YC, Ebersold MJ, Thompson GB, Grant CS, Van Heerden JA & Young WF Jr. Benign paragangliomas: clinical presentation and treatment outcomes in 236 patients. *Journal of Clinical Endocrinology and Metabolism* 2001 **86** 5210–5216.
- 7 Eisenhofer G. Free or total metanephrines for diagnosis of pheochromocytoma: what is the difference? *Clinical Chemistry* 2001 **47** 988–989.
- 8 Kudva YC, Sawka AM & Young WF Jr. Clinical review 164: the laboratory diagnosis of adrenal pheochromocytoma: the Mayo Clinic experience. *Journal of Clinical Endocrinology and Metabolism* 2003 **88** 4533–4539.
- 9 Sawka AM, Jaeschke R, Singh RJ & Young WF Jr. A comparison of biochemical tests for pheochromocytoma: measurement of fractionated plasma metanephrines compared with the combination of 24-hour urinary metanephrines and catecholamines. *Journal of Clinical Endocrinology and Metabolism* 2003 **88** 553–558.
- 10 Eisenhofer G. Editorial: biochemical diagnosis of pheochromocytoma – is it time to switch to plasma-free metanephrines? *Journal of Clinical Endocrinology and Metabolism* 2003 **88** 550–552.
- 11 D'Herbomez M, Forzy G, Bauters C, Tierny C, Pigny P, Carnaille B, Pattou F, Wemeau JL & Rouaix N. An analysis of the biochemical diagnosis of 66 pheochromocytomas. *European Journal of Endocrinology* 2007 **156** 569–575.
- 12 Boyle JG, Davidson DF, Perry CG & Connell JM. Comparison of diagnostic accuracy of urinary free metanephrines, vanillyl mandelic acid, and catecholamines and plasma catecholamines for diagnosis of pheochromocytoma. *Journal of Clinical Endocrinology and Metabolism* 2007 **92** 4602–4608.
- 13 Unger N, Pitt C, Schmidt IL, Walz MK, Schmid KW, Philipp T, Mann K & Petersenn S. Diagnostic value of various biochemical parameters for the diagnosis of pheochromocytoma in patients with adrenal mass. *European Journal of Endocrinology* 2006 **154** 409–417.
- 14 Gao YC, Lu HK, Luo QY, Chen LB, Ding Y & Zhu RS. Comparison of free plasma metanephrines enzyme immunoassay with (131)I-MIBG scan in diagnosis of pheochromocytoma. *Clinical and Experimental Medicine* 2008 **8** 87–91.
- 15 Algeciras-Schimmich A, Preissner CM, Young WF Jr, Singh RJ & Grebe SK. Plasma chromogranin A or urine fractionated metanephrines follow-up testing improves the diagnostic accuracy of plasma fractionated metanephrines for pheochromocytoma. *Journal of Clinical Endocrinology and Metabolism* 2008 **93** 91–95.

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