Catecholamine metabolomic and secretory phenotypes in phaeochromocytoma

Graeme Eisenhofer[1,2], Karel Pacak[3], Thanh-Truc Huynh[3], Nan Qin[1], Gennady Bratslavsky [4], W Marston Linehan[4], Massimo Mannelli[5], Peter Friberg[6], Stefan K Grebe[7], Henri J Timmers[8], Stefan R Bornstein[2] and Jacques W M Lenders[2,9]

[1]Institute of Clinical Chemistry and Laboratory Medicine and [2]Department of Medicine, University of Dresden, 01307 Dresden, Germany

[3]Reproductive and Adult Endocrinology Program, Eunice Kennedy Shriver National Institute of Child Health and Human Development and [4]Urologic Oncology Branch, Center for Cancer Research, National Cancer Institute, National Institutes of Health, Bethesda, 20892 Maryland, USA

[5]Department of Clinical Pathophysiology, University of Florence, 50139 Florence, Italy

[6]Department of Clinical Physiology, the Sahlgrenska Academy and University Hospital, University of Gothenburg, SE 41345 Gothenburg, Sweden

[7]Department of Laboratory Medicine and Pathology, Mayo Clinic, Rochester, 55905 Minnesota, USA

Departments of [8]Endocrinology and [9]Internal Medicine, Radboud University Nijmegen Medical Center, 6525 GA Nijmegen, The Netherlands

(Correspondence should be addressed to G Eisenhofer, Institute of Clinical Chemistry and Laboratory Medicine and the Department of Medicine, University of Dresden, Fetscherstrasse 74, 01307 Dresden, Germany;
Email: graeme.eisenhofer@uniklinikum-dresden.de)

Abstract

Phaeochromocytomas and paragangliomas (PPGLs) are highly heterogeneous tumours with variable catecholamine biochemical phenotypes and diverse hereditary backgrounds. This analysis of 18 catecholamine-related plasma and urinary biomarkers in 365 patients with PPGLs and 846 subjects without PPGLs examined how catecholamine metabolomic profiles are impacted by hereditary background and relate to variable hormone secretion. Catecholamine secretion was assessed in a subgroup of 156 patients from whom tumour tissue was available for measurements of catecholamine contents. Among all analytes, the free catecholamine *O*-methylated metabolites measured in plasma showed the largest tumour-related increases relative to the reference group. Patients with tumours due to multiple endocrine neoplasia type 2 and neurofibromatosis type 1 (NF1) showed similar catecholamine metabolite and secretory profiles to patients with adrenaline-producing tumours and no evident hereditary background. Tumours from these three patient groups contained higher contents of catecholamines, but secreted the hormones at lower rates than tumours that did not contain appreciable adrenaline, the latter including PPGLs due to von Hippel–Lindau (*VHL*) and succinate dehydrogenase (*SDH*) gene mutations. Large increases of plasma dopamine and its metabolites additionally characterised patients with PPGLs due to the latter mutations, whereas patients with NF1 were characterised by large increases in plasma dihydroxyphenylglycol and dihydroxyphenylacetic acid, the deaminated metabolites of noradrenaline and dopamine. This analysis establishes the utility of comprehensive catecholamine metabolite profiling for characterising the distinct and highly diverse catecholamine metabolomic and secretory phenotypes among different groups of patients with PPGLs. The data further suggest developmental origins of PPGLs from different populations of chromaffin cell progenitors.

Endocrine-Related Cancer (2011) **18** 97–111

Introduction

Phaeochromocytomas and paragangliomas (PPGLs) are heterogeneous tumours with highly variable signs and symptoms and diverse clinical presentations (Manger 2009). Much of the heterogeneity is due to wide-ranging variations in the types and relative amounts of catecholamines produced by the tumours and differences in episodic versus continuous hormonal

secretion (Feldman *et al*. 1979, Feldman 1981, Ito *et al*. 1992, Eisenhofer *et al*. 2001). This in turn appears to reflect differences in expression of genes regulating catecholamine biosynthetic and secretory processes (Isobe *et al*. 2000, Eisenhofer *et al*. 2008, Timmers *et al*. 2008).

Synthesis of catecholamines is primarily regulated by the cytoplasmic enzyme, tyrosine hydroxylase, which converts tyrosine to 3,4-dihydroxyphenylalanine (DOPA; Nagatsu & Stjarne 1998). DOPA is then converted to dopamine, which is translocated from the cytoplasm into catecholamine storage vesicles. The presence of dopamine-β hydroxylase within storage vesicles of noradrenergic nerves and chromaffin cells leads to conversion of dopamine to noradrenaline. In adrenal medullary chromaffin cells, noradrenaline is metabolised to adrenaline by phenylethanolamine-*N*-methyltransferase (PNMT). Since that enzyme is located in the cytoplasm, production of adrenaline depends on leakage of noradrenaline from storage vesicles by a continuous process that is counterbalanced by the actions of vesicular monoamine transporters to return cytoplasmic catecholamines back into storage vesicles.

Metabolism of catecholamines occurs by a plethora of pathways resulting in numerous metabolites, but primarily first occurs in the same cells where the catecholamines are synthesised (Eisenhofer *et al*. 2004*b*). The presence of monoamine oxidase in noradrenergic neurons of the central and sympathetic nervous systems means that most noradrenaline produced at these sites is deaminated to 3,4-dihydroxyphenylglycol (DHPG) following neuronal reuptake or leakage of the transmitter from storage vesicles into the neuronal cytoplasm. Additional presence of catechol-*O*-methyltransferase within adrenal chromaffin cells means that the noradrenaline and adrenaline produced there are respectively metabolised to normetanephrine and metanephrine. This depends on leakage of the catecholamines from storage vesicles into the cytoplasm. Metabolism of dopamine can also occur by multiple pathways leading to production of the deaminated metabolite, dihydroxyphenylacetic acid, or the *O*-methylated metabolite, methoxytyramine.

The present analysis utilised a dataset from a large population of patients, linked to a tumour tissue bank, to comprehensively characterise the catecholamine metabolite profiles of patients with PPGLs, and any relationships of metabolomic signatures to different catecholamine secretory phenotypes and hereditary backgrounds. A total of 18 catecholamine-related plasma and urinary analytes were examined in 365 patients with PPGLs and 846 subjects without PPGLs

who served as a reference group. The primary aim was to characterise the presence of distinct catecholamine biochemical and secretory phenotypes among PPGLs as a basis for future studies exploring the pathogenetic characteristics and developmental origins of the tumours from different populations of chromaffin cell progenitors.

This manuscript is the third in a series utilizing data from a single cohort of 1211 subjects. Originally, all manuscripts in the series were compiled as part of a single article totaling over 12,500 words. That article was determined to be unsuitable for publication. Consequently the original article was split into four separate manuscripts, each focusing on a separate topic area. All effort has been made to avoid presentation of duplicate or redundant material in each of the four manuscripts. The data in the present manuscript are focused on catecholamine metabolomic and secretory profiles that are not presented elsewhere.

Subjects and methods

Subjects

The study involved retrospective analysis of data from 1211 subjects, including 365 patients with pathologically confirmed PPGLs and 846 subjects without PPGLs. The latter group was included for comparative purposes to establish in patients with PPGLs the relative increases of plasma and urine tumour biomarkers above normal. Subjects were investigated under multicentre clinical protocols, based mainly at the National Institutes of Health (NIH) in Bethesda (MD, USA), but which also included several European Centres. The latter included Radboud University Medical Center (Nijmegen, The Netherlands), the University of Florence (Florence, Italy), Gothenburg University Hospital (Gothenburg, Sweden) and Dresden University Hospital (Dresden, Germany). Written informed consent was obtained from patients enrolled into intramural review board-approved studies at the NIH, which also allowed for collection of patient samples at offsite centres.

Patients with PPGLs had a mean age of 40 years (range 6–83 years) at initial diagnosis of tumours and included 190 males and 175 females (Table 1). Among these patients, there were 173 patients with clearly identified hereditary syndromes or gene mutations and 192 patients in whom there was no clearly identified mutation or evidence of an established hereditary syndrome. Among the 192 patients with no evidence of an established hereditary syndrome, gene testing failed to confirm the presence of mutations of *VHL*, *RET*,

Table 1 Characteristics of patients and their tumours according to the presence or absence of an established specific mutation or hereditary syndrome

	Established mutation or hereditary syndrome					No mutation or syndrome
	VHL	**MEN 2**	**NF1**	**SDHB**	**SDHD**	
N	66	38	10	48	11	192
Age (mean ± s.d.)	31 ± 17	40 ± 13	42 ± 13	31 ± 15	31 ± 16	47 ± 16
Gender (M/F)	37/29	17/21	6/4	32/16	6/5	92/100
Tumour location (A/E/B)[a]	58/5/3	38/0/0	9/1/0	2/43/3	1/5/5	149/40/3

[a]Tumour locations indicate adrenal (A), extradrenal (E) or both adrenal and extra-adrenal (B) primary tumours.

succinate dehydrogenase subunit D (*SDHD*) or SDH subunit B (*SDHB*) genes in genomic DNA available from 94 patients.

The high proportion of patients with hereditary syndromes largely reflects disproportionate referral of those patients to the participating specialist centres. Among this group, there were 66 patients with von Hippel–Lindau (VHL) syndrome, 38 patients with multiple endocrine neoplasia type 2 (MEN 2), 10 patients with neurofibromatosis type 1 (NF1) and 59 patients with mutations of the *SDH* genes, including 48 patients with mutations of the *SDHB* gene and 11 patients of the *SDHD* gene (Table 1). Three of the 38 patients with MEN 2 had the MEN 2B form of the syndrome.

Adrenal and extra-adrenal locations of tumours were determined using results of imaging studies and surgical and pathological records. Most patients with VHL syndrome, MEN 2 and NF1 had adrenal tumours (Table 1). In contrast, most patients with mutations of *SDH* genes had extra-adrenal tumours, including eight patients with multifocal tumours at both adrenal and extra-adrenal locations.

The 846 subjects without PPGLs included 379 males and 467 females. Subjects had a mean age of 41 years (range 6–84 years) and included 175 normotensive volunteers, 94 hypertensive volunteers and 577 patients in whom testing for PPGLs was carried out and tumours were excluded by previously described criteria (Lenders *et al*. 2002). The use of medications known to cause false-positive elevations of plasma or urinary catecholamines and metanephrines (e.g. tricyclic antidepressants and phenoxybenzamine) constituted additional exclusion criteria.

Collections of blood, urine and surgical specimens

Blood samples from all 1211 study participants were obtained with subjects supine for at least 20 min before blood collection. Subjects were instructed to fast and abstain from caffeinated and decaffeinated beverages overnight and avoid taking acetaminophen for 5 days before blood sampling. Samples of blood were transferred into tubes containing heparin as anticoagulant and immediately placed on ice until centrifuged (4 °C) to separate the plasma. Plasma samples were stored at −80 °C until assayed.

Twenty-four hour urine samples were collected from 338 of the 365 patients with PPGLs and 513 of the 847 subjects without tumours. Samples were collected with hydrochloric or another acid as a preservative, total urine volume was determined and aliquots were kept at 4 °C until assayed.

Samples of tumour tissue were procured from 156 patients with PPGLs, generally within 1 h of surgical resection of tumours. The dimensions of tumours were recorded. Small samples of each tumour (10–50 mg) were dissected from the mass, frozen on dry ice and stored at −80 °C. As part of further processing, tissue samples were weighed frozen and then homogenised in at least 5 volumes of 0.4 M perchloric acid containing 0.5 mM EDTA. Homogenised samples were centrifuged (1500 *g* for 15 min at 4 °C), and supernatants were collected and stored at −80 °C until assayed for catecholamines.

Laboratory analyses

Plasma, urinary and tissue catecholamines (noradrenaline, adrenaline and dopamine) and plasma and urinary fractionated metanephrines (normetanephrine and metanephrine) were quantified by liquid chromatography with electrochemical detection. Concentrations of catecholamines were determined after extraction from plasma or perchloric acid tissue supernatants using alumina adsorption as described previously (Eisenhofer *et al*. 1986). The assays of catecholamines in plasma also included measurements of three other catechols: DOPA, the precursor of dopamine and product of the rate-limiting step in catecholamine biosynthesis; DHPG, a deaminated

metabolite of noradrenaline and adrenaline produced principally within sympathetic nerves; and 3,4-dihydroxyphenylacetic acid, the deaminated metabolite of dopamine.

Plasma and urinary fractionated metanephrines (normetanephrine and metanephrine) were estimated using different liquid chromatographic methods as described elsewhere (Lenders *et al.* 1993, 2002). The assays in plasma also allowed determination of methoxytyramine, the *O*-methylated metabolite of dopamine. Assays of plasma concentrations of metanephrine, normetanephrine and methoxytyramine were principally directed to measurements of the free metabolites (i.e. free metanephrines and methoxytyramine). However, additional measurements of the much higher concentrations of deconjugated metanephrines were also carried in 192 and 789 respective subjects with and without PPGLs. These latter measurements were carried out after incubating 200 µl aliquots of plasma with sulphatase over 30 min for 37 °C and reflect concentrations of both free and sulphate-conjugated metabolites, similar to the measurements of urinary fractionated metanephrines.

Data analyses

Differences in signal strengths of the 18 plasma and urinary catecholamine-related analytes were assessed from comparisons of their relative increases in patients with PPGLs above mean values in the reference population. The 95% confidence intervals of values in the reference group were also calculated for provision of lower and upper limits of normal. Values from the reference group were used for comparisons of catecholamine-related analytes in subgroups of patients with PPGLs, as outlined below.

Patients identified with disease-causing mutations or hereditary syndromes were divided into five subgroups (VHL, MEN 2, NF1, SDHB and SDHD) according to the nature of the syndrome or gene mutation that was detected. Patients without evidence of a hereditary syndrome or mutation were divided into three subgroups according to the noradrenergic, adrenergic or dopaminergic phenotypes of their tumours, as based on previous findings relating tumour tissue contents of the different catecholamines to increases in plasma concentrations of their respective *O*-methylated metabolites (Eisenhofer *et al.* 2005*b*). For this, tumour-derived increments of plasma normetanephrine, metanephrine and methoxytyramine were established by subtracting the concentration of each metabolite in each patient

with a PPGL from the mean concentration in the reference group. Noradrenergic tumours were defined as those with predominant increases of only normetanephrine, accompanied by either normal plasma concentrations of metanephrine and methoxytyramine (below the upper reference intervals) or by increases of <5% for metanephrine and 10% for methoxytyramine relative to the sum of increments for all three metabolites. Conversely, adrenergic and dopaminergic tumours were defined as those characterised by respective increases of plasma metanephrine and methoxytyramine above the upper reference intervals and associated increments, relative to the combined increments of all three metabolites, of larger than 5% for metanephrine and 10% for methoxytyramine.

For estimations of tumour-derived catecholamine secretion into plasma or excretion into urine, differences in plasma concentrations (nmol/l) or urinary outputs (µmol/day) of catecholamines in patients with PPGLs compared with mean values in the reference population of subjects without tumours provided estimates of increases in the amines due to tumours. Rates of catecholamine secretion from tumours into plasma were estimated using the formula, $S = P \times C \times 1.44$, where S is the rate of catecholamine secretion (µmol/day), P is the plasma concentration of catecholamines due to the tumour (nmol/l), C is the circulatory clearance of catecholamines from plasma (l/min) and where the value, 1.440, was used to convert secretion rates of nmol/min to µmol/day, the same units as for urine. The formula was based on that described elsewhere (Eisenhofer *et al.* 2008).

Rates of catecholamine secretion into plasma or excretion into urine (µmol/day) were divided by estimates of tumour volume to normalise for differences in tumour size and to derive final rates in units of µmol/day per cubic cm of tumour. Volumes of tumours (V) in cubic cm were estimated using the formula for the volume of a sphere, $V = 4/3 \, \pi r^3$, where r, the radius in cm, was derived from estimated mean diameters (the latter was calculated from the cubed roots of rectangular volumes).

Rate constants for catecholamine secretion into plasma or excretion into urine (per day) – representing the proportions of total catecholamines in a tumour secreted into plasma or excreted into urine over a day – were estimated by dividing rates of catecholamine secretion into plasma or excretion into urine (µmol/day) by total tumour catecholamine contents (µmol). Total tumour catecholamine contents were estimated from the product of tissue

catecholamine concentrations and tumour mass (the latter was derived from tumour volume, assuming a specific gravity of 1.0).

Statistical analysis

Owing to the skewed distributions of plasma concentrations and urinary outputs of catecholamines and their metabolites, statistical significance of differences in neurochemical data was determined in all cases after logarithmic transformation. Comparisons of data among groups were by ANOVA with the Tukey–Kramer or Scheffe's tests used for *post hoc* comparisons. Patients were clustered using an unsupervised hierarchical clustering method to delineate distinctions and similarities among different groups of patients. This analysis utilised sets of analytes that most clearly differed among groups.

Results

Tumour-related increases in plasma and urine catecholamines and metabolites

Increases in plasma concentrations and urinary outputs of the three endogenous catecholamines, their *O*-methylated and deaminated metabolites and their amino acid precursor, DOPA, showed considerable variability among the 365 patients with PPGLs relative to the reference population of 846 subjects (Fig. 1). Among the principal metabolites of noradrenaline, DHPG in plasma and VMA in urine showed the lowest signal strengths in patients with PPGLs. Plasma-free normetanephrine showed the highest signal strength among all 18 catechol-related analytes profiled, with a 12.2-fold increase above the reference population that significantly ($P < 0.0004$) surpassed all other analytes, including deconjugated normetanephrine in plasma (7.4-fold increase) and urine (6.7-fold increase).

Among the *O*-methylated metabolites of adrenaline, there were no differences in signal strengths of free or deconjugated metanephrine in urine or plasma, but relative increases of these metabolites were larger ($P < 0.05$) than those for plasma and urinary adrenaline (Fig. 1). Among the dopamine-related analytes, plasma concentrations of free methoxytyramine showed the largest signal with 3.3-fold increases above the reference population, surpassing ($P < 0.006$) all other dopamine-related analytes. Plasma DOPA, DOPAC and urinary dopamine were increased by only 10–38% above mean levels of the reference population.
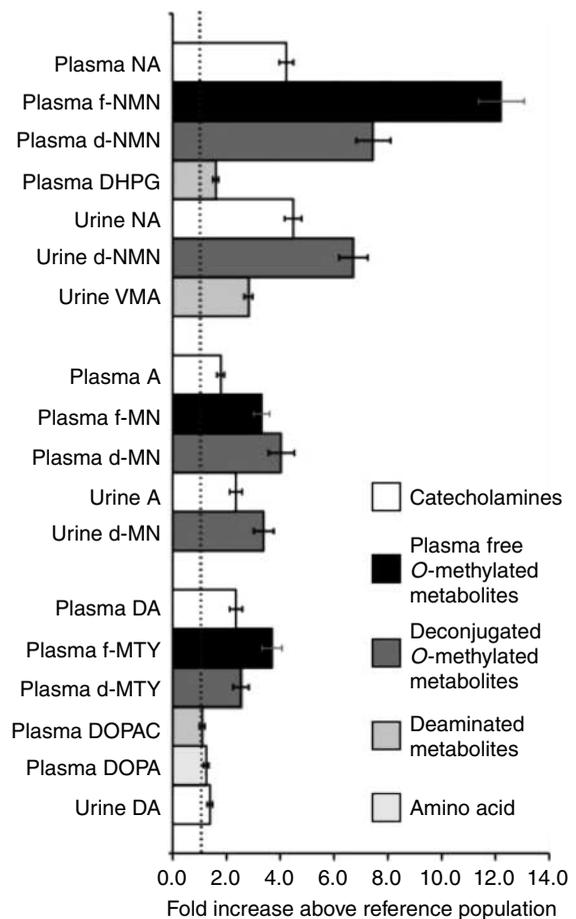

Figure 1 Plasma and urinary catecholamine metabolomic profiles in patients with PPGLs relative to reference. Data are shown as fold increases (means ± s.e.m.) of values in patients with PPGLs above mean values for the reference population. Data for the catecholamines, including noradrenaline (NA), adrenaline (A) and dopamine (DA), are shown by the empty bars; for the free *O*-methylated catecholamine metabolites, including normetanephrine (f-NMN), metanephrine (f-MN) and methoxytyramine (f-MTY), by the black bars; for the deconjugated *O*-methylated catecholamine metabolites, including deconjugated normetanephrine (d-NMN), deconjugated metanephrine (d-MN) and deconjugated methoxytyramine (d-MTY), by the dark grey bars; for the deaminated catecholamine metabolites, including dihydroxyphenylglycol (DHPG), vanillylmandelic acid (VMA) and dihydroxyphenylacetic acid (DOPAC), by the medium grey bars; and for the amino acid precursor of the catecholamines, dihydroxyphenylalanine (DOPA), by the light grey bar.

Plasma and urine catecholamine metabolomic profiles

Distinct patterns in the profiles of the 18 catechol-related analytes emerged after patients with PPGLs were grouped according to hereditary syndromes or, in patients without hereditary syndromes, according to catecholamine phenotype (Table 2). Among the latter patients, there were 87 patients with predominantly

Table 2 Plasma and urine catecholamine metabolomic profiles in patients with phaeochromocytomas and paragangliomas (PPGLs) according to the presence or absence of an established mutation or hereditary syndrome

| | Reference population | | Established mutation or hereditary syndrome | | | | | No established mutation or hereditary syndrome[a] | | |
| | No tumour (n=846) | | VHL (n=66) | MEN 2 (n=38) | NF1 (n=10) | SDHB (n=48) | SDHD (n=11) | NA (n=87) | A (n=95) | DA (n=10) |
Analyte	Mean ± s.e.m.	95% CI	Mean ± s.e.m.	Mean ± s.e.m.	Mean ± s.e.m.	Mean ± s.e.m.	Mean ± s.e.m.	Mean ± s.e.m.	Mean ± s.e.m.	Mean ± s.e.m.
Noradrenaline and metabolites										
Plasma NA (nmol/l)	1.70±0.03	(0.51–4.18)	8.64±1.50*,†	4.19±0.64*	7.35±1.21*	26.50±5.04*,†	9.20±2.61*,†	23.49±3.10*,†	9.90±1.54*	41.01±16.48*,†
Plasma f-NMN (nmol/l)	0.33±0.01	(0.11–0.74)	4.38±1.09*,†	6.34±1.58*	6.13±1.97*	13.15±2.88*,†	3.37±0.90*,†	14.18±2.69*,†	9.74±1.59*	9.92±3.80*,†
Plasma d-NMN (nmol/l)	11.92±0.25	(3.85–28.12)	87.57±21.61	132.20±45.78	117.51±62.16	281.15±109.63	62.94±33.62	304.92±66.01	209.42±61.38	135.60±36.67
Plasma DHPG (nmol/l)	5.12±0.06	(2.69–8.88)	6.59±0.29	8.53±0.94	18.41±3.83	10.16±1.00	9.31±1.07	10.21±0.87	12.07±1.62	10.89±2.37
Urine NA (µmol/day)	0.25±0.01	(0.08–0.61)	1.46±0.23	0.96±0.19	1.54±0.43	3.50±0.75	2.08±1.12	4.20±0.67	1.92±0.33	4.80±1.82
Urine d-NMN (µmol/day)	1.61±0.05	(0.45–4.14)	8.26±1.81	12.45±2.81	14.65±4.85	32.40±6.53	10.85±4.20	32.25±5.91	24.33±5.14	27.86±9.42
Urine VMA (µmol/day)	19.43±0.44	(7.06–43.39)	41.96±4.43	70.69±13.48	126.96±4.80	81.01±15.09	42.53±10.12	112.96±15.51	108.38±16.96	112.39±33.59
Adrenaline and metabolites										
Plasma A (nmol/l)	0.15±0.01	(0.01–0.67)	0.17±0.03†	0.97±0.22‡	1.77±0.71‡	0.21±0.04‡	0.20±0.07†	0.23±0.03†	2.15±0.32‡	0.34±0.17†
Plasma f-MN (nmol/l)	0.15±0.00	(0.04–0.45)	0.16±0.01†	4.54±0.75‡	3.35±0.88‡	0.21±0.02†	0.14±0.03†	0.40±0.09†	5.71±0.70‡	0.19±0.05†
Plasma d-MN (nmol/l)	4.58±0.09	(1.00–14.90)	5.60±0.73†	111.50±22.37‡	78.97±28.67‡	5.86±0.84†	4.30±1.02†	13.38±2.72†	222.92±43.15‡	5.29±0.86†
Urine A (µmol/day)	0.02±0.00	(0.01–0.09)	0.03±0.00†	0.46±0.10‡	0.69±0.27‡	0.02±0.00†	0.01±0.00†	0.05±0.01†	0.85±0.16‡	0.03±0.01†
Urine d-MN (µmol/day)	0.49±0.02	(0.09–1.56)	0.52±0.04†	15.28±3.45‡	14.77±4.09‡	0.67±0.06†	0.55±0.14†	0.98±0.24†	27.75±7.89‡	1.07±0.44†
Dopamine, DOPA and metabolites										
Plasma DA (nmol/l)	0.08±0.00	(0.01–0.41)	0.17±0.05†	0.15±0.02†	0.24±0.04†	10.39±8.06‡	5.84±4.87‡	0.33±0.06†	0.17±0.02†	36.21±18.31‡
Plasma f-MTY (nmol/l)	0.03±0.00	(0.00–0.11)	0.06±0.01†	0.08±0.02†	0.10±0.03†	2.72±1.56‡	2.12±1.32‡	0.30±0.08†	0.18±0.03†	15.82±7.40‡
Plasma d-MTY (nmol/l)	1.75±0.07	(0.20–7.41)	3.62±0.77†	5.95±2.12†	2.15±1.67†	86.57±73.36‡	6.39±4.70†	23.20±9.09†	7.91±1.77†	322.36±320.54‡
Plasma DOPAC (nmol/l)	8.54±0.17	(3.78–16.59)	8.51±0.44	8.96±0.74	21.71±8.79	38.72±27.27	13.53±4.06	9.98±0.68	11.78±1.32	45.95±26.32
Plasma DOPA (nmol/l)	8.16±0.09	(4.98–12.63)	8.00±0.24	9.69±0.57	20.60±6.49	16.64±5.15	11.40±1.67	13.85±1.99	13.89±1.43	26.78±11.84
Urine DA (µmol/day)	1.37±0.10	(0.39–3.66)	1.55±0.08	2.04±0.17	3.64±1.47	3.91±1.16	4.57±2.39	2.44±0.29	2.13±0.19	11.46±4.56

*,†Differences in patterns of relative increases above reference values for plasma NA and f-NMN. †,‡Divergent patterns of increases above reference values for adrenaline or dopamine and their metabolites among the different groups of patients. NA, noradrenaline; f-NMN, free normetanephrine; d-NMN, deconjugated normetanephrine; DHPG, 3,4-dihydroxyphenylglycol; VMA, vanillylmandelic acid; A, adrenaline; f-MN, free metanephrine; d-MN, deconjugated metanephrine; DA, dopamine; f-MTY, free methoxytyramine; d-MTY, deconjugated methoxytyramine; DOPAC; 3,4-dihydroxyphenylacetic acid; DOPA, 3,4-dihydroxyphenylalanine.
[a]Patients without an established mutation were divided into three groups according to whether their tumours produced predominantly noradrenaline (NA) or significant amounts of adrenaline (A) or dopamine (DA).

noradrenaline-producing tumours (noradrenergic phenotype), 95 patients with tumours that produced significant amounts of adrenaline (adrenergic phenotype) and 10 patients with tumours characterised by significant dopamine production (dopaminergic phenotype).

The clearest pattern to emerge among patients with hereditary PPGLs was a divergence in increases of plasma concentrations and urinary outputs of adrenaline and its *O*-methylated metabolite, metanephrine, measured in both free and deconjugated forms (Table 2). Specifically, patients with *VHL*, *SDHB* and *SDHD* mutations showed no significant increases of plasma or urinary adrenaline and free or deconjugated metanephrine above reference levels. In contrast, patients with MEN 2 and NF1 showed highly significant ($P < 0.0001$) 7- to 31-fold increases of all adrenaline-related analytes, a pattern similar to that in patients with adrenergic tumours and no clear hereditary syndrome.

Unsupervised hierarchical cluster analysis utilising the plasma and urinary data for adrenaline and its metabolites identified two dominant expression clusters (Fig. 2). Cluster 1 comprised all patients with *VHL* and *SDH* mutations, whereas cluster 2 comprised MEN 2 and NF1 patients. The remaining patients with no evidence of an established hereditary syndrome or gene mutation were partitioned among the two clusters. Most of those with noradrenergic or dopaminergic tumours partitioned in cluster 1, whereas most of those with adrenaline-producing tumours partitioned in cluster 2.

Patients with NF1 were further distinguished from MEN 2 patients and other patient groups by higher ($P < 0.05$) plasma concentrations of DHPG (Table 2). Plasma concentrations of DOPAC were also higher ($P < 0.05$) in patients with NF1 than in patients with MEN 2 and VHL syndrome. Post-operative values were available in two of the ten patients with NF1. Both these two subjects showed pre-operative to post-operative decreases in plasma concentrations of DHPG (65 and 76% decreases) and DOPAC (32 and 53% decreases), indicating that the pre-operatively increased levels of the metabolites were derived from the tumours.

Larger ($P < 0.0001$) increases of plasma-free normetanephrine relative to noradrenaline in patients with adrenergic than noradrenergic or dopaminergic tumours represented another pattern in neurochemical profiles (Table 2). More specifically, increases of plasma normetanephrine above reference were 4.3- to 7.8-fold larger than those of noradrenaline in all patients with adrenaline-producing tumours, including
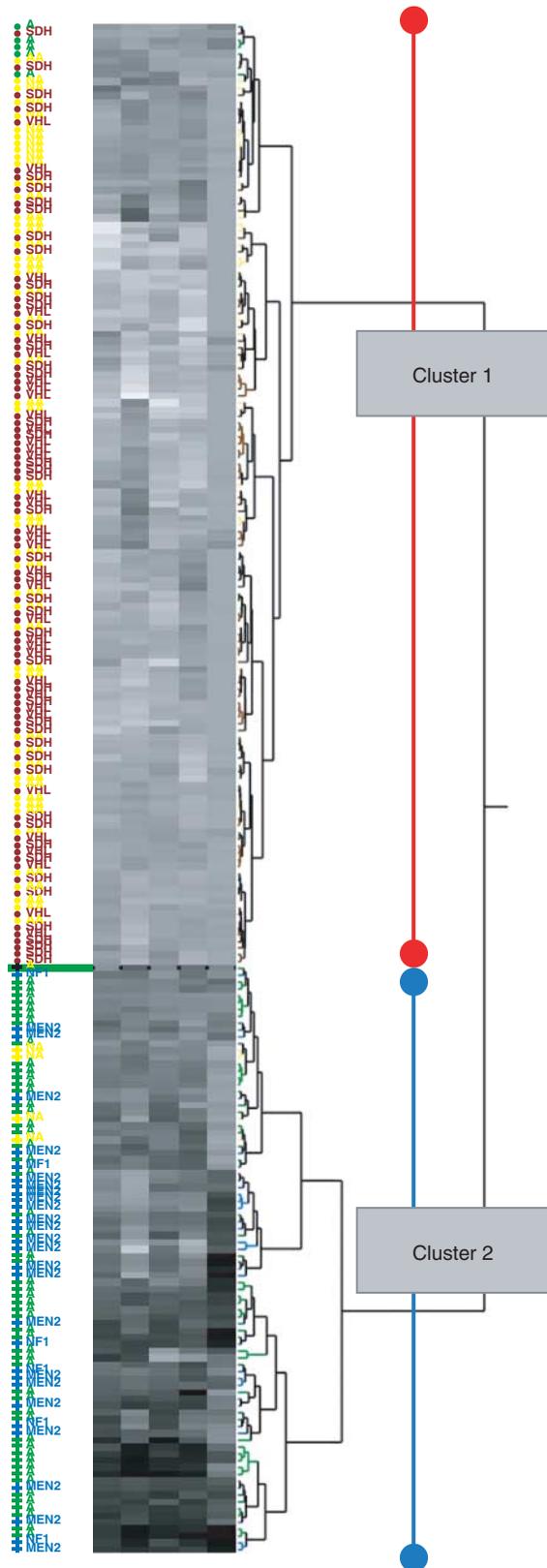
those with MEN 2 and NF1. This contrasted with patients who had noradrenergic or dopaminergic tumours, including patients with VHL, SDHB and SDHD mutations, in whom increases in plasma-free normetanephrine were only 1.8- to 3.0-fold larger than those of noradrenaline.

Distinctly larger increases of dopamine-related analytes in patients with tumours due to *SDHB* and *SDHD* mutations compared to other mutations represented another clear difference in profiles of catechol-related analytes among patient groups (Table 2). In particular, plasma concentrations of dopamine and free methoxytyramine were more than 90-fold higher ($P < 0.0001$) than reference in patients with *SDHB* mutations and more than 70-fold higher ($P < 0.001$) in patients with *SDHD* mutations. In contrast, these analytes showed less than fourfold increases above reference in VHL, MEN 2 and NF1 patients. Also in striking contrast to the more than 70-fold increases in plasma-free methoxytyramine and dopamine, urinary outputs of dopamine in patients with *SDHB* and *SDHD* mutations were respectively increased by only 2.9- and 3.3-fold above reference.

Tumour locations and catecholamine phenotypes

All patients with NF1 and MEN 2 showed increases in plasma metanephrine that characterised their tumours as adrenergic, and all except one patient had tumours at adrenal locations (Table 1). Remarkably, the single patient with NF1 who had a retroperitoneally located extra-adrenal tumour also showed substantial increases in plasma metanephrine (1.74 nmol/l), plasma adrenaline (1.08 nmol/l) and urinary metanephrine (7.78 µmol/day).

As indicated by the predominantly adrenal locations of PPGLs among patients with VHL syndrome (Table 1), the noradrenergic phenotype of tumours in these patients was independent of proximity to the adrenal cortex. Lack of an adrenergic phenotype in tumours due to *SDH* mutations was consistent with their mainly extra-adrenal locations. Nevertheless, 11 of these patients had adrenal tumours with normal plasma and urinary concentrations of metanephrine in all except two patients. One patient had a slightly increased plasma metanephrine (19% above the upper limit). That patient, however, had a substantially increased plasma normetanephrine (13-fold above the upper limit) and a normal urinary output of metanephrine indicating relatively little, if any, tumoural adrenaline production. The second patient had a normal plasma concentration of metanephrine, but

Figure 2 Unsupervised hierarchical cluster analysis of patients with and without an established mutation or hereditary syndrome. Patients with *VHL* or *SDH* mutations shown in red are confined to cluster 1, whereas patients with MEN 2 and NF1 in blue are confined to cluster 2. Patients without evidence of hereditary syndrome are illustrated in green for those with adrenaline-producing tumours and in yellow for noradrenergic or dopaminergic tumours. Clustering was based on measured values for adrenaline-related analytes in plasma and urine, with lower to higher analyte levels illustrated in grey scale by respective progression from lighter to darker heat map areas.

a slightly increased urinary output of metanephrine (18% increase above the upper limit) and a dramatically increased urinary output of normetanephrine (32-fold above the upper limit), again indicating negligible tumoural adrenaline relative to noradrenaline production.

Among the 87 patients with no hereditary syndrome who had PPGLs with an adrenergic phenotype, all except 4 patients had tumours with adrenal locations (96%). In contrast, among the 95 patients with noradrenergic PPGLs and the 10 patients with dopaminergic PPGLs, 62 and 40% respectively had tumours with exclusively adrenal locations.

Tumour catecholamines and secretory phenotypes

Tumour tissue concentrations of catecholamines showed considerable variability among the different groups of patients with and without established disease-causing mutations (Fig. 3A). The presence of markedly higher tumour tissue concentrations of adrenaline in patients with MEN 2 and NF1 than in those with mutations of *VHL*, *SDHB* and *SDHD* genes represented the clearest distinguishing feature among patients with hereditary PPGLs. Tumour tissue concentrations of noradrenaline also showed considerable differences among groups, with generally higher levels in tumours that produced adrenaline than those that did not. Consequently, total tissue concentrations of catecholamines (sum of dopamine, noradrenaline and adrenaline) were highest in adrenaline-producing tumours, and lowest in all other groups of tumours, regardless of the presence or absence of a hereditary syndrome.

Rates of tumour-derived catecholamine secretion into plasma (Fig. 3B) or excretion into urine (Fig. 3C), normalised to tumour volume, showed a reciprocal pattern compared to that for the differences of tumour tissue catecholamines among the groups (Fig. 3A). Specifically, while tissue concentrations of catecholamines were lowest in tumours with a noradrenergic or dopaminergic phenotype and highest in those with an

Figure 3 Bar graphs showing tumour contents of catecholamines (panel A), rates of tumour-derived catecholamine secretion into plasma (panel B) and excretion into urine (panel C), and rate constants for tumour-derived catecholamine secretion into plasma (panel D) and excretion into urine (panel E). Data are shown for tumours from patients with VHL syndrome ($n=44$), MEN 2 ($n=32$), NF1 ($n=6$) and with mutations of *SDHB* ($n=11$) and *SDHD* ($n=3$) genes. Data are also shown for noradrenergic (NA, $n=26$) and adrenergic (A, $n=34$) tumours from patients without evidence of an established hereditary syndrome or gene mutation. Different alphabetic characters indicate significant differences ($P<0.05$) between groups, while the presence of identical characters indicates lack of a significant difference.

adrenergic phenotype, the former tumours were characterised by high rates and the latter by low rates of catecholamine secretion into plasma or excretion into urine. Patients with VHL syndrome or with noradrenergic tumours and no identified hereditary syndrome showed particularly high rates of tumoural catecholamine secretion, averaging 5- to 7-fold higher than in patients with MEN 2 and NF1 or with adrenergic tumours and no hereditary syndrome.

The differences in tumour-derived catecholamine secretion and excretion among the different patient groups were particularly pronounced when assessed as rate constants for catecholamine secretion into plasma (Fig. 3D) or excretion into urine (Fig. 3E). The rate constants for catecholamine secretion into plasma indicated that adrenaline-producing sporadic and hereditary tumours released only 2–5% of their tumour tissue contents of catecholamines into the bloodstream per day, compared to 57% for sporadic noradrenergic tumours and 34, 46 and 15% respectively for tumours from patients with *VHL*, *SDHB* and *SDHD* gene mutations. Similarly, only between 0.1 and 0.4% of the catecholamine contents of adrenaline-producing sporadic and hereditary tumours were excreted into urine per day, compared to 3.0% for sporadic noradrenergic tumours and between 1.2 and 4.9% for tumours from patients with *VHL*, *SDHB* and *SDHD* gene mutations.

Discussion

This study involving a large cohort of well-characterised patients with PPGLs provides a comprehensive analysis of relative increases in catecholamines and catecholamine metabolites in patients with and without hereditary PPGLs. Our previous observations about catecholamine phenotypic differences in hereditary PPGLs were limited to patients with MEN 2 and VHL syndrome and did not include the comprehensive analysis of the catecholamine metabolome described here (Eisenhofer *et al*. 2001, 2004*a*, 2005*b*, 2008, Huynh *et al*. 2005, Cleary *et al*. 2007). Our other

findings suggesting tumoural production of dopamine in patients with *SDHB* mutations did not include comparisons with other patient groups or measurements of plasma methoxytyramine, tumour tissue dopamine or measured values for plasma dopamine (Timmers *et al*. 2007). The present more comprehensive dataset confirms many of the findings of these earlier studies, but more importantly considerably extends previous observations by establishing distinct catecholamine metabolomic and secretory phenotypes of PPGLs among multiple subgroups of patients, including those with tumours due to mutations of *SDHD*, *SDHB*, *VHL*, *NF1* and *RET* genes. These data have relevance to emerging concepts concerning the highly heterogeneous nature of PPGLs, how this heterogeneity relates to underlying germline mutations of tumour susceptibility genes and how this in turn explains distinct differences in gene expression profiles and pathways of development of tumours from different chromaffin cell progenitors.

Catecholamine metabolomic profiles explained

The profiling of catecholamine-related analytes in plasma and urine establishes that among all precursor amines and metabolites examined, the free *O*-methylated metabolites provide higher signal strengths for indicating the presence of PPGLs than their precursor catecholamines or corresponding deaminated and sulphate-conjugated metabolites. The higher signal strengths of the *O*-methylated metabolites than of their monoamine precursors have been amply demonstrated (Grossman *et al*. 2006), and are established to reflect continuous production of *O*-methylated metabolites within chromaffin tumour cells by processes that are independent of variations in exocytotic catecholamine secretion (Eisenhofer *et al*. 1998). The particularly low signal strength of DHPG is in agreement with previous findings (Brown 1984) and reflects the substantial and almost exclusive production of this metabolite within sympathetic nerves (Fig. 4), which considerably dilutes any additional contribution from chromaffin tumour cells. Formation of VMA within the liver, mainly after extraneuronal *O*-methylation of the DHPG formed within sympathetic neurons (Eisenhofer *et al*. 2004*b*), explains the relatively low diagnostic signal strength of this metabolic end product.

The higher diagnostic signal strengths of plasma free than of plasma and urinary deconjugated normetanephrine and methoxytyramine are also explained by different sources of the free compared to the sulphate-conjugated metabolites (Fig. 4). Sulphate conjugation occurs principally in gastrointestinal
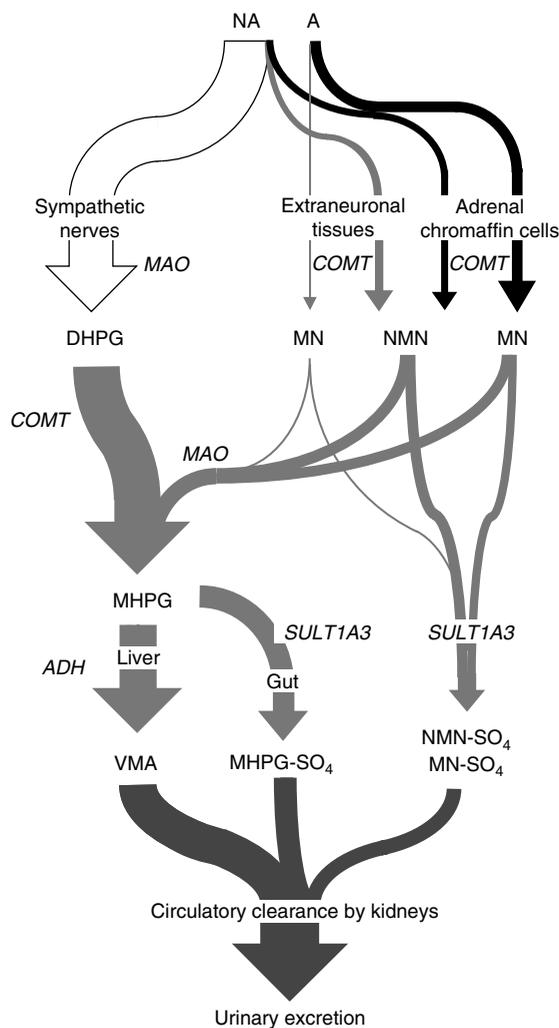

Figure 4 Neuronal, extraneuronal and adrenal pathways of metabolism for noradrenaline and adrenaline. NA, noradrenaline; A, adrenaline; DHPG, dihydroxyphenylglycol; MN, metanephrine; NMN, normetanephrine; MHPG, methoxyhydroxyphenylglycol; VMA, vanillylmandelic acid; MHPG-SO$_4$, methoxyhydroxyphenylglycol sulphate; NMN-SO$_4$, normetanephrine sulphate; MN-SO$_4$ metanephrine sulphate; MAO, monoamine oxidase; COMT, catechol-*O*-methyltransferase; ADH, alcohol dehydrogenase; SULT1A3, sulfotransferase type 1A3.

tissues as a mechanism for inactivating both dietary-derived monoamines and the substantial amounts of endogenous dopamine and noradrenaline produced by mesenteric organ catecholamine systems (Eisenhofer *et al*. 2004*b*). The contributions of these sources to levels of sulphate-conjugated normetanephrine and methoxytyramine thereby dilute the signals of the sulphate-conjugated metabolites derived from the free metabolites produced within chromaffin tumour cells.

Because almost all adrenaline is produced within adrenal chromaffin cells, there is little influence of

other sources of adrenaline to dilute any diagnostic signal of sulphate-conjugated metanephrine relative to its free metanephrine precursor; thus, in contrast to normetanephrine and methoxytyramine, both free and deconjugated metanephrine have similar diagnostic signal strengths. Nevertheless, since most of the signal strength of the *O*-methylated metabolites reflects that of normetanephrine, the free metabolites provide more sensitive and specific biomarkers for PPGLs compared to the deconjugated metanephrines (Lenders *et al.* 2002, Unger *et al.* 2009). They are also less susceptible to dietary influences (de Jong *et al.* 2009).

The much lower signal strength of urinary dopamine compared to plasma dopamine and plasma-free and deconjugated methoxytyramine is explained by the more than 90% of dopamine in urine that is derived from renal extraction and decarboxylation of circulating DOPA (Brown & Allison 1981). This large contribution of plasma DOPA to urinary dopamine considerably dilutes any signal of tumour-derived urinary dopamine. Thus, compared to plasma measurements of dopamine and methoxytyramine, measurements of urinary dopamine provide an insensitive biomarker of tumour dopamine production (Eisenhofer *et al.* 2005*a*).

Catecholamine metabolomic phenotypes

Our observations of distinct patterns in catecholamine metabolomic profiles among different groups of patients with hereditary PPGLs extend and bring together previous isolated observations about different catecholamine phenotypes and gene expression profiles. In particular, the present data showing distinct catecholamine metabolomic profiles in tumours from NF1 and MEN 2 patients, compared to patients with mutations of *VHL*, *SDHB* and *SDHD* genes, are consistent with patterns in gene expression profiles observed by two other groups (Dahia *et al.* 2005, Favier *et al.* 2009). Using unsupervised hierarchical cluster analysis, these groups established the presence of two dominant expression clusters, one that included tumours from NF1 and MEN 2 patients and the other tumours from patients with mutations of *VHL*, *SDHB* and *SDHD* genes. Similarly, here, we also show the presence of two dominant clusters comprised of the same patient groups, but in this instance established using unsupervised hierarchical cluster analysis of catecholamine metabolomic profiles.

The differences in gene expression profiles from the two earlier transcriptomic studies indicated two pathways of tumourigenesis: one pathway in VHL-, SDHB- and SDHD-related tumours involved activation of hypoxia- and angiogenesis-related genes, and the other pathway in tumours from MEN 2 and NF1 patients involved increased kinase signalling. The present data additionally suggest different origins of the two cluster groups from adrenergic and noradrenergic chromaffin cell precursors, or alternatively, downstream effects of the differentially activated signalling pathways on expression of PNMT, the enzyme that converts noradrenaline to adrenaline.

Interestingly, while tumours in NF1 and MEN 2 patients exhibited similar adrenergic phenotypes, large increases of both plasma DHPG and DOPAC provided an additional feature that distinguished tumours in NF1 patients from those of other groups. The substantial increases of these deaminated metabolites of noradrenaline and dopamine suggest higher activity of monoamine oxidase and a more neuronal-like metabolising phenotype in NF1-associated tumours than in other PPGLs.

Furthermore, while tumours in patients with *VHL* and *SDH* mutations all exhibited negligible adrenaline production, large increases of dopamine and dopamine-related metabolites in patients with *SDH* mutations represented an additional characteristic that distinguished this group from patients with tumours due to *VHL* mutations. Thus, while transcriptomic profiling studies indicate that PPGLs due to SDH and VHL mutations are closely linked (Dahia *et al.* 2005), the present catecholamine metabolomic profiling data clearly indicate different dopaminergic and noradrenergic neurochemical signatures among the two groups. These latter findings agree with a more recent transcriptomic profiling study that confirmed the presence of two distinct cluster groups, but also revealed further differences in gene expression between tumours due to *VHL* and *SDH* mutations (Favier *et al.* 2009). Other studies have established contrasting clinical manifestations of SDHB- and VHL-associated chromaffin cell tumours (Srirangalingam *et al.* 2009) that may also relate to differences in catecholamine metabolomic and secretory phenotypes observed here.

Catecholamine secretory phenotypes

In addition to establishing distinct catecholamine metabolomic phenotypes, this study also establishes for the first time that the various groups of patients develop tumours with distinct catecholamine secretory phenotypes. More specifically, adrenaline-producing tumours contain higher concentrations of catecholamines yet show lower rates of catecholamine

secretion into plasma and excretion into urine compared to tumours that do not produce appreciable adrenaline.

Differences in secretory characteristics and expression of secretory pathway components in different populations of adrenergic and noradrenergic chromaffin cells are well established (Marley & Livett 1987, Teraoka *et al*. 1993, Langley & Grant 1995, Aunis & Langley 1999). In particular, fractionation of bovine adrenal medullary cells into two populations of low density noradrenergic and high density adrenergic chromaffin cells revealed that the noradrenergic cells released a higher percentage of their catecholamine contents than adrenergic cells (Krause *et al*. 1996). Similarly in the present study, noradrenergic tumours released between 15 and 57% of their catecholamine stores each day, compared to <5% for adrenaline-producing tumours.

The divergent lower tissue concentrations but higher rates of catecholamine secretion in noradrenergic than adrenergic PPGLs extend findings of previous studies focusing on tumours in MEN 2 and VHL syndrome (Eisenhofer *et al*. 2001, 2008). These earlier studies showed that although tumours in VHL syndrome secrete noradrenaline at higher more continuous rates than those in patients with MEN 2, the latter tumours are more easily provoked to secrete catecholamines in episodic bursts with a resulting more symptomatic clinical presentation. These differences in secretory profiles reflect extensive differences in expression of numerous genes encoding components of the regulated secretory pathway, including enzymes regulating transmitter synthesis, vesicular proteins and their processing enzymes, exocytotic machinery components as well as receptors and other signal transduction factors responsible for excitation-secretion coupling (Eisenhofer *et al*. 2008). In general, noradrenergic tumours due to *VHL* mutations are characterised by immature constitutive secretory pathways, whereas mature regulated secretory pathways typical of fully differentiated adrenal medullary chromaffin cells characterise adrenergic tumours in MEN 2.

Similar differences in expression of secretory pathway components also likely extend to and explain the differences in tumour tissue contents and secretion of catecholamines among the various groups of patients of this study. The lower rates of catecholamine secretion from adrenergic than noradrenergic or dopaminergic tumours furthermore clarify other differences in the clinical presentation of the different groups. In particular, the larger relative increases of plasma normetanephrine than noradrenaline in patients with adrenaline-producing tumours than other groups reflect the lower rates of hormonal secretion in the former than latter groups. This also explains the strikingly much higher diagnostic sensitivity of metanephrines than catecholamines for detection of adrenaline- than noradrenaline-producing PPGLs (Eisenhofer *et al*. 2005*b*).

Perspective

The adrenergic phenotype of phaeochromocytomas in patients with MEN 2 and NF1 largely reflects their origins from adrenal chromaffin cells in which PNMT has been induced by exposure to high local concentrations of adrenal cortical steroids. Relative lack of adrenaline production in most extra-adrenal tumours similarly reflects their lack of proximity to adrenal sources of steroids. What remains unexplained is the relative lack of adrenaline production in a significant proportion of adrenal tumours, including all those in patients with *VHL* and *SDH* mutations.

Unlike the adrenal medulla of some animal species that contain separate populations of adrenergic and noradrenergic chromaffin cells, the adult human adrenal medulla is largely comprised of a single population of PNMT-positive adrenergic cells (Cleary *et al*. 2005). This observation argues against the development of phenotypically distinct adrenal phaeochromocytomas from separate populations of mature adrenergic and noradrenergic or dopaminergic chromaffin cells. Dedifferentiation provides another explanation. However, an alternative basis for the distinct phenotypes of PPGLs is suggested by the current view that some of the tumours develop from immature chromaffin cell progenitors that have escaped neuronal apoptosis during embryonic development (Lee *et al*. 2005, Dahia 2006, Tischler 2006, Woodward & Maher 2006).

Of relevance to the above alternative explanation, PPGLs with a noradrenergic phenotype overexpress the gene for hypoxia-inducible transcription factor 2α (HIF-2α; Eisenhofer *et al*. 2004*a*), a transcription factor with a central role in the development of PPGLs in patients with *VHL* and *SDH* mutations (Pollard *et al*. 2006, Favier *et al*. 2009). Of further relevance, an increasing body of evidence indicates that expression of HIF-2α and related genes is crucial for the development of embryonic tyrosine hydroxylase expressing sympathoadrenal progenitor cells (Tian *et al*. 1998, Favier *et al*. 1999, Bishop *et al*. 2008, Brown *et al*. 2009). Blocking this expression leads to impaired development of these cells and reduced catecholamine synthesis, presumably through

increased apoptosis of tyrosine hydroxylase expressing noradrenaline-producing chromaffin cells.

The above observations conversely also explain susceptibility of immature chromaffin progenitor cells to the tumourigenic influences of mutations that further increase expression of HIF-2α. In such exposed progenitor cells, failure of developmental culling and arrested differentiation may be expected to lead to the development of PPGLs with a retained immature dopaminergic or noradrenergic catecholamine phenotype at both extra-adrenal and adrenal locations. In contrast, tumours with a mature adrenergic phenotype would only be expected to develop after migration of neural crest chromaffin progenitors into the adrenal enlagen, and then only after induction of PNMT by locally produced steroids (Adams & Bronner-Fraser 2009). This concept is also consistent with other findings that adrenaline-producing tumours present at later ages than noradrenergic PPGLs (Eisenhofer *et al*. 2011).

In summary, previous findings of global differences in gene expression profiles among different groups of hereditary and sporadic PPGLs and the present findings of associated patterns in catecholamine metabolomic and secretory profiles together support the likelihood that many of the differences between the various groups of tumours reflect distinct origins from immature noradrenergic or dopaminergic chromaffin progenitors compared to the more highly differentiated adrenergic chromaffin cells of the adult adrenal medulla. The distinct catecholamine metabolomic and secretory signatures of different groups of PPGLs provide a new framework for future studies exploring the pathogenetic development of these tumours from different populations of chromaffin cell progenitors.

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.

Funding

References

Adams MS & Bronner-Fraser M 2009 Review: the role of neural crest cells in the endocrine system. *Endocrine Pathology* **20** 92–100. (doi:10.1007/s12022-009-9070-6)

Aunis D & Langley K 1999 Physiological aspects of exocytosis in chromaffin cells of the adrenal medulla. *Acta Physiologica Scandinavica* **167** 89–97. (doi:10.1046/j.1365-201x.1999.00580.x)

Bishop T, Gallagher D, Pascual A, Lygate CA, de Bono JP, Nicholls LG, Ortega-Saenz P, Oster H, Wijeyekoon B, Sutherland AI *et al.* 2008 Abnormal sympathoadrenal development and systemic hypotension in PHD3 −/− mice. *Molecular and Cellular Biology* **28** 3386–3400. (doi:10.1128/MCB.02041-07)

Brown MJ 1984 Simultaneous assay of noradrenaline and its deaminated metabolite, dihydroxyphenylglycol, in plasma: a simplified approach to the exclusion of phaeochromocytoma in patients with borderline elevation of plasma noradrenaline concentration. *European Journal of Clinical Investigation* **14** 67–72. (doi:10.1111/j.1365-2362.1984.tb00706.x)

Brown MJ & Allison DJ 1981 Renal conversion of plasma DOPA to urine dopamine. *British Journal of Clinical Pharmacology* **12** 251–253.

Brown ST, Kelly KF, Daniel JM & Nurse CA 2009 Hypoxia inducible factor (HIF)-2 alpha is required for the development of the catecholaminergic phenotype of sympathoadrenal cells. *Journal of Neurochemistry* **110** 622–630. (doi:10.1111/j.1471-4159.2009.06153.x)

Cleary S, Brouwers FM, Eisenhofer G, Pacak K, Christie DL, Lipski J, McNeil AR & Phillips JK 2005 Expression of the noradrenaline transporter and phenylethanolamine *N*-methyltransferase in normal human adrenal gland and phaeochromocytoma. *Cell and Tissue Research* **322** 443–453. (doi:10.1007/s00441-005-0026-y)

Cleary S, Phillips JK, Huynh TT, Pacak K, Fliedner S, Elkahloun AG, Munson P, Worrell RA & Eisenhofer G 2007 Chromogranin A expression in phaeochromocytomas associated with von Hippel–Lindau syndrome and multiple endocrine neoplasia type 2. *Hormone and Metabolic Research* **39** 876–883. (doi:10.1055/s-2007-993135)

Dahia PL 2006 Evolving concepts in pheochromocytoma and paraganglioma. *Current Opinion in Oncology* **18** 1–8. (doi:10.1097/01.cco.0000198017.45982.06)

Dahia PL, Ross KN, Wright ME, Hayashida CY, Santagata S, Barontini M, Kung AL, Sanso G, Powers JF, Tischler AS *et al.* 2005 A HIF1alpha regulatory loop links hypoxia and mitochondrial signals in pheochromocytomas. *PLoS Genetics* **1** 72–80. (doi:10.1371/journal.pgen.0010008)

Eisenhofer G, Goldstein DS, Stull R, Keiser HR, Sunderland T, Murphy DL & Kopin IJ 1986 Simultaneous liquid-chromatographic determination of 3,4-dihydroxyphenyl-glycol, catecholamines, and 3,4-dihydroxyphenylalanine in plasma, and their responses to inhibition of monoamine oxidase. *Clinical Chemistry* 32 2030–2033.

Eisenhofer G, Keiser H, Friberg P, Mezey E, Huynh TT, Hiremagalur B, Ellingson T, Duddempudi S, Eijsbouts A & Lenders JW 1998 Plasma metanephrines are markers of pheochromocytoma produced by catechol-*O*-methyltransferase within tumors. *Journal of Clinical Endocrinology and Metabolism* 83 2175–2185. (doi:10.1210/jc.83.6.2175)

Eisenhofer G, Walther MM, Huynh TT, Li ST, Bornstein SR, Vortmeyer A, Mannelli M, Goldstein DS, Linehan WM, Lenders JW *et al.* 2001 Pheochromocytomas in von Hippel–Lindau syndrome and multiple endocrine neoplasia type 2 display distinct biochemical and clinical phenotypes. *Journal of Clinical Endocrinology and Metabolism* 86 1999–2008. (doi:10.1210/jc.86.5.1999)

Eisenhofer G, Huynh TT, Pacak K, Brouwers FM, Walther MM, Linehan WM, Munson PJ, Mannelli M, Goldstein DS & Elkahloun AG 2004*a* Distinct gene expression profiles in norepinephrine- and epinephrine-producing hereditary and sporadic pheochromocytomas: activation of hypoxia-driven angiogenic pathways in von Hippel–Lindau syndrome. *Endocrine-Related Cancer* 11 897–911. (doi:10.1677/erc.1.00838)

Eisenhofer G, Kopin IJ & Goldstein DS 2004*b* Catecholamine metabolism: a contemporary view with implications for physiology and medicine. *Pharmacological Reviews* 56 331–349. (doi:10.1124/pr.56.3.1)

Eisenhofer G, Goldstein DS, Sullivan P, Csako G, Brouwers FM, Lai EW, Adams KT & Pacak K 2005*a* Biochemical and clinical manifestations of dopamine-producing paragangliomas: utility of plasma methoxytyramine. *Journal of Clinical Endocrinology and Metabolism* 90 2068–2075. (doi:10.1210/jc.2004-2025)

Eisenhofer G, Lenders JW, Goldstein DS, Mannelli M, Csako G, Walther MM, Brouwers FM & Pacak K 2005*b* Pheochromocytoma catecholamine phenotypes and prediction of tumor size and location by use of plasma free metanephrines. *Clinical Chemistry* 51 735–744. (doi:10.1373/clinchem.2004.045484)

Eisenhofer G, Huynh TT, Elkahloun A, Morris JC, Bratslavsky G, Linehan WM, Zhuang Z, Balgley BM, Lee CS, Mannelli M *et al.* 2008 Differential expression of the regulated catecholamine secretory pathway in different hereditary forms of pheochromocytoma. *American Journal of Physiology. Endocrinology and Metabolism* 295 E1223–E1233. (doi:10.1152/ajpendo.90591.2008)

Eisenhofer G, Timmers HJ, Lenders JW, Bornstein SR, Tiebel O, Mannelli M, King KS, Vocke C, Linehan WM, Bratslavsky G *et al.* 2011 Age at diagnosis of pheochromocytoma differs according to catecholamine phenotype and tumor location. *Journal of Clinical Endocrinology and Metabolism* [in press]. (doi:10.1210/jc.2010-1588)

Favier J, Kempf H, Corvol P & Gasc JM 1999 Cloning and expression pattern of EPAS1 in the chicken embryo. Colocalization with tyrosine hydroxylase. *FEBS Letters* 462 19–24. (doi:10.1016/S0014-5793(99)01476-3)

Favier J, Briere JJ, Burnichon N, Riviere J, Vescovo L, Benit P, Giscos-Douriez I, De Reynies A, Bertherat J, Badoual C *et al.* 2009 The Warburg effect is genetically determined in inherited pheochromocytomas. *PLoS ONE* 4 e7094. (doi:10.1371/journal.pone.0007094)

Feldman JM 1981 Phenylethanolamine-*N*-methyltransferase activity determines the epinephrine concentration of pheochromocytomas. *Research Communications in Chemical Pathology and Pharmacology* 34 389–398.

Feldman JM, Blalock JA, Zern RT, Shelburne JD, Gaede JT, Farrell RE & Wells SA Jr 1979 Deficiency of dopamine-beta-hydroxylase. A new mechanism for normotensive pheochromocytomas. *American Journal of Clinical Pathology* 72 175–185.

Grossman A, Pacak K, Sawka A, Lenders JW, Harlander D, Peaston RT, Reznek R, Sisson J & Eisenhofer G 2006 Biochemical diagnosis and localization of pheochromocytoma: can we reach a consensus? *Annals of the New York Academy of Sciences* 1073 332–347. (doi:10.1196/annals.1353.038)

Huynh TT, Pacak K, Brouwers FM, Abu-Asab MS, Worrell RA, Walther MM, Elkahloun AG, Goldstein DS, Cleary S & Eisenhofer G 2005 Different expression of catecholamine transporters in phaeochromocytomas from patients with von Hippel–Lindau syndrome and multiple endocrine neoplasia type 2. *European Journal of Endocrinology* 153 551–563. (doi:10.1530/eje.1.01987)

Isobe K, Nakai T, Yashiro T, Nanmoku T, Yukimasa N, Ikezawa T, Suzuki E, Takekoshi K & Nomura F 2000 Enhanced expression of mRNA coding for the adrenaline-synthesizing enzyme phenylethanolamine-*N*-methyl transferase in adrenaline-secreting pheochromocytomas. *Journal of Urology* 163 357–362. (doi:10.1016/S0022-5347(05)68051-2)

Ito Y, Fujimoto Y & Obara T 1992 The role of epinephrine, norepinephrine, and dopamine in blood pressure disturbances in patients with pheochromocytoma. *World Journal of Surgery* 16 759–763 (discussion 763–764). (doi:10.1007/BF02067379)

de Jong WH, Eisenhofer G, Post WJ, Muskiet FA, de Vries EG & Kema IP 2009 Dietary influences on plasma and urinary metanephrines: implications for diagnosis of catecholamine-producing tumors. *Journal of Clinical Endocrinology and Metabolism* 94 2841–2849. (doi:10.1210/jc.2009-0303)

Krause W, Michael N, Lubke C, Livett BG & Oehme P 1996 Catecholamine release from fractionated chromaffin cells. *European Journal of Pharmacology* 302 223–228. (doi:10.1016/0014-2999(96)00103-3)

Langley K & Grant NJ 1995 Do adrenergic chromaffin cells exocytose like noradrenergic cells? *Trends in Neurosciences* **18** 440–441. (doi:10.1016/0166-2236(95)94492-N)

Lee S, Nakamura E, Yang H, Wei W, Linggi MS, Sajan MP, Farese RV, Freeman RS, Carter BD, Kaelin WG Jr *et al.* 2005 Neuronal apoptosis linked to EglN3 prolyl hydroxylase and familial pheochromocytoma genes: developmental culling and cancer. *Cancer Cell* **8** 155–167. (doi:10.1016/j.ccr.2005.06.015)

Lenders JW, Eisenhofer G, Armando I, Keiser HR, Goldstein DS & Kopin IJ 1993 Determination of metanephrines in plasma by liquid chromatography with electrochemical detection. *Clinical Chemistry* **39** 97–103.

Lenders JW, Pacak K, Walther MM, Linehan WM, Mannelli M, Friberg P, Keiser HR, Goldstein DS & Eisenhofer G 2002 Biochemical diagnosis of pheochromocytoma: which test is best? *Journal of the American Medical Association* **287** 1427–1434. (doi:10.1001/jama.287.11.1427)

Manger WM 2009 The protean manifestations of pheochromocytoma. *Hormone and Metabolic Research* **41** 658–663. (doi:10.1055/s-0028-1128139)

Marley PD & Livett BG 1987 Differences between the mechanisms of adrenaline and noradrenaline secretion from isolated, bovine, adrenal chromaffin cells. *Neuroscience Letters* **77** 81–86. (doi:10.1016/0304-3940(87)90611-2)

Nagatsu T & Stjarne L 1998 Catecholamine synthesis and release. Overview. *Advances in Pharmacology* **42** 1–14. (doi:10.1016/S1054-3589(08)60682-1)

Pollard PJ, El-Bahrawy M, Poulsom R, Elia G, Killick P, Kelly G, Hunt T, Jeffery R, Seedhar P, Barwell J *et al.* 2006 Expression of HIF-1alpha, HIF-2alpha (EPAS1), and their target genes in paraganglioma and pheochromocytoma with VHL and SDH mutations. *Journal of Clinical Endocrinology and Metabolism* **91** 4593–4598. (doi:10.1210/jc.2006-0920)

Srirangalingam U, Khoo B, Walker L, MacDonald F, Skelly RH, George E, Spooner D, Johnston LB, Monson JP, Grossman AB *et al.* 2009 Contrasting clinical manifestations of SDHB and VHL associated chromaffin tumours. *Endocrine-Related Cancer* **16** 515–525. (doi:10.1677/ERC-08-0239)

Teraoka H, Sugawara T & Nakazato Y 1993 Difference in the effectiveness of Ca^{2+} to evoke catecholamine secretion between adrenaline- and noradrenaline-containing cells of bovine adrenal medulla. *Journal of Neurochemistry* **60** 1936–1940. (doi:10.1111/j.1471-4159.1993.tb13423.x)

Tian H, Hammer RE, Matsumoto AM, Russell DW & McKnight SL 1998 The hypoxia-responsive transcription factor EPAS1 is essential for catecholamine homeostasis and protection against heart failure during embryonic development. *Genes and Development* **12** 3320–3324. (doi:10.1101/gad.12.21.3320)

Timmers HJ, Kozupa A, Eisenhofer G, Raygada M, Adams KT, Solis D, Lenders JW & Pacak K 2007 Clinical presentations, biochemical phenotypes, and genotype–phenotype correlations in patients with succinate dehydrogenase subunit B-associated pheochromocytomas and paragangliomas. *Journal of Clinical Endocrinology and Metabolism* **92** 779–786. (doi:10.1210/jc.2006-2315)

Timmers HJ, Pacak K, Huynh TT, Abu-Asab M, Tsokos M, Merino MJ, Baysal BE, Adams KT & Eisenhofer G 2008 Biochemically silent abdominal paragangliomas in patients with mutations in the Sdhb gene. *Journal of Clinical Endocrinology and Metabolism* **93** 4826–4832. (doi:10.1210/jc.2008-1093)

Tischler AS 2006 Molecular and cellular biology of pheochromocytomas and extra-adrenal paragangliomas. *Endocrine Pathology* **17** 321–328. (doi:10.1007/s12022-006-0003-3)

Unger N, Deutschbein T, Walz MK, Mann K & Petersenn S 2009 The value of immunoassays for metanephrines in the biochemical diagnosis of pheochromocytomas. *Hormone and Metabolic Research* **41** 676–679. (doi:10.1055/s-0029-1224133)

Woodward ER & Maher ER 2006 Von Hippel–Lindau disease and endocrine tumour susceptibility. *Endocrine-Related Cancer* **13** 415–425. (doi:10.1677/erc.1.00683)