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Catecholamine metabolomic and secretory phenotypes in phaeochromocytoma

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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

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-ß 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. 2004b). 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,
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 acetylsalicylic acid 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

<table>
<thead>
<tr>
<th>Established mutation or hereditary syndrome</th>
<th>VHL</th>
<th>MEN 2</th>
<th>NF1</th>
<th>SDHB</th>
<th>SDHD</th>
</tr>
</thead>
<tbody>
<tr>
<td>N</td>
<td>66</td>
<td>38</td>
<td>10</td>
<td>48</td>
<td>11</td>
</tr>
<tr>
<td>Age (mean ± s.d.)</td>
<td>31 ± 17</td>
<td>40 ± 13</td>
<td>42 ± 13</td>
<td>31 ± 15</td>
<td>31 ± 16</td>
</tr>
<tr>
<td>Gender (M/F)</td>
<td>37/29</td>
<td>17/21</td>
<td>6/4</td>
<td>32/16</td>
<td>6/5</td>
</tr>
<tr>
<td>Tumour location (A/E/B)</td>
<td>58/5/3</td>
<td>38/0/0</td>
<td>9/1/0</td>
<td>2/43/3</td>
<td>1/5/5</td>
</tr>
</tbody>
</table>

*Tumour locations indicate adrenal (A), extradrenal (E) or both adrenal and extra-adrenal (B) primary tumours.*
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. 2005b). 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, \( V = \frac{4}{3} \pi r^3 \), 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

<table>
<thead>
<tr>
<th>Analyte</th>
<th>Reference population</th>
<th>Established mutation or hereditary syndrome</th>
<th>No established mutation or hereditary syndromea</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean± S.E.M.</td>
<td>Mean± S.E.M.</td>
<td>Mean± S.E.M.</td>
</tr>
<tr>
<td></td>
<td>No tumour (n=846)</td>
<td>VHL (n=66)</td>
<td>MEN 2 (n=38)</td>
</tr>
<tr>
<td>Noradrenaline and metabolites</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Plasma NA (nmol/l)</td>
<td>1.70±0.03</td>
<td>8.64±1.50*†</td>
<td>4.19±0.64*‡</td>
</tr>
<tr>
<td>Plasma f-NMN (nmol/l)</td>
<td>0.33±0.01</td>
<td>4.38±1.09*†</td>
<td>6.34±1.58*‡</td>
</tr>
<tr>
<td>Plasma d-NMN (nmol/l)</td>
<td>11.92±0.25</td>
<td>87.57±21.61</td>
<td>132.20±45.78</td>
</tr>
<tr>
<td>Plasma DHPG (nmol/l)</td>
<td>5.12±0.06</td>
<td>6.59±0.29</td>
<td>8.53±0.94</td>
</tr>
<tr>
<td>Urine NA (μmol/day)</td>
<td>0.25±0.01</td>
<td>1.46±0.23</td>
<td>0.96±0.19</td>
</tr>
<tr>
<td>Urine d-NMN (μmol/day)</td>
<td>1.61±0.05</td>
<td>8.26±1.81</td>
<td>12.45±2.81</td>
</tr>
<tr>
<td>Urine VMA (μmol/day)</td>
<td>19.43±0.44</td>
<td>41.96±4.43</td>
<td>70.69±13.48</td>
</tr>
<tr>
<td>Adrenaline and metabolites</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Plasma A (nmol/l)</td>
<td>0.15±0.01</td>
<td>0.17±0.03†</td>
<td>0.97±0.22‡</td>
</tr>
<tr>
<td>Plasma f-MN (nmol/l)</td>
<td>0.15±0.00</td>
<td>0.16±0.01†</td>
<td>4.54±0.75‡</td>
</tr>
<tr>
<td>Plasma d-MN (nmol/l)</td>
<td>4.58±0.09</td>
<td>5.60±0.73†</td>
<td>111.50±22.37‡</td>
</tr>
<tr>
<td>Urine A (μmol/day)</td>
<td>0.02±0.00</td>
<td>0.03±0.00†</td>
<td>0.46±0.10†</td>
</tr>
<tr>
<td>Urine d-MN (μmol/day)</td>
<td>0.49±0.02</td>
<td>0.92±0.04†</td>
<td>15.29±3.45‡</td>
</tr>
<tr>
<td>Dopamine, DOPA and metabolites</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Plasma DA (nmol/l)</td>
<td>0.08±0.00</td>
<td>0.17±0.05†</td>
<td>0.15±0.02‡</td>
</tr>
<tr>
<td>Plasma f-MTY (nmol/l)</td>
<td>0.03±0.00</td>
<td>0.06±0.01†</td>
<td>0.08±0.02‡</td>
</tr>
<tr>
<td>Plasma d-MTY (nmol/l)</td>
<td>1.75±0.07</td>
<td>3.62±0.77†</td>
<td>5.95±2.12‡</td>
</tr>
<tr>
<td>Plasma DOPAC (nmol/l)</td>
<td>8.54±1.17</td>
<td>8.51±0.44</td>
<td>8.96±0.74</td>
</tr>
<tr>
<td>Plasma DOPA (nmol/l)</td>
<td>8.16±0.09</td>
<td>8.00±0.24</td>
<td>9.69±0.57</td>
</tr>
<tr>
<td>Urine DA (μmol/day)</td>
<td>1.37±0.10</td>
<td>1.55±0.08</td>
<td>2.04±0.17</td>
</tr>
</tbody>
</table>

* † Differences in patterns of relative increases above reference values for plasma NA and f-NNM. †‡ Divergent patterns of increases above reference values for adrenaline or dopamine and their metabolites among the different groups of patients. NA, noradrenaline; f-NNM, free metanephrine; d-NNM, deconjugated metanephrine; DOPA, 3,4-dihydroxyphenylacetic acid; 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-dihydroxyphenylethylamine.

 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
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

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**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.
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, 2004a, 2005b, 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. 2004b), 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 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. 2004b). 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. 2005a).

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 adrenergic-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. 2005b).

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. 2004a), 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
This work was supported by the Deutsche Forschungsgesellschaft (DFG grant no: 515/6-1), the Center for Regenerative Therapies Dresden and the Dresden Tumour Center. Support from the intramural programs of the Eunice Kennedy Shriver National Institute of Child Health and Human Development and the Center for Cancer Research, National Cancer Institute, at the National Institutes of Health, was not in the form of any specific grant.

Acknowledgements
Thanks are extended to Karen Adams, Stephanie Fliedner, Kathryn King and Tamara Prodanov for their technical help or assistance with collections of patient materials and data.

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