CLINICAL STUDY

Plasma levels of free metanephrines and 3-methoxytyramine indicate a higher number of biochemically active HNPGL than 24-h urinary excretion rates of catecholamines and metabolites

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Abstract

Context: A substantial number of patients with head and neck paragangliomas (HNPGLs) have biochemically active tumors, evidenced by increased urinary excretion of catecholamines and metabolites, including 3-methoxytyramine (3MT). It is unclear whether plasma levels of these parameters are more sensitive to detect biochemical activity in HNPGL patients than urinary excretion rates.

Objective: To compare plasma free levels vs urinary excretion rates of deconjugated 3MT and combined metanephrines (MNs) in patients with HNPGL.

Patients and methods: We included 124 consecutive patients with HNPGL for screening of catecholamine excess by measurement of 24-h urinary excretion rates of deconjugated (nor)metanephrine, (nor)epinephrine, dopamine, vanillylmandelic acid, 3MT, and plasma free levels of (nor)metanephrine and 3MT.

Results: Plasma free 3MT levels were increased in 35 of the 124 patients (28%), whereas 24-h urinary excretion of deconjugated 3MT was increased in 30 patients (24%) ($P=0.13$). Plasma free MN levels were increased in seven patients (6%) and urinary deconjugated MN levels in six patients (5%) ($P=1.00$). Plasma free normetanephrine (NMN) levels were increased in seven patients (6%), and five patients had increased urinary excretion of deconjugated NMN (4%) ($P=0.69$). Plasma free combined MN levels (NMN, MN, and 3MT) were increased in 41 patients (33%), whereas 24-h urinary excretion rates of deconjugated combined MNs were increased in 33 patients (27%, $P<0.05$).

Conclusions: The combined levels of free MNs and free 3MT in plasma indicate a higher number of biochemically active HNPGLs than the 24-h urinary excretion rates of these markers.

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Introduction

Head and neck paragangliomas (HNPGLs) are rare neuroendocrine tumors derived from parasympathetic ganglia (1). Some patients with HNPGLs have biochemically active tumors, evidenced by increased urinary excretion rates of catecholamines and their metabolites (2, 3, 4). The majority of those patients have increased urinary excretion rates of 3-methoxytyramine (3MT), the 3-O-methylated metabolite of dopamine (3).

It is presently unknown whether plasma free concentrations of 3-O-methylated metabolites of catecholamines, including 3MT, are more sensitive parameters of biochemical activity of HNPGL than urinary excretion rates of catecholamines or deconjugated 3-O-methylated metabolites. For the diagnosis of pheochromocytoma, the measurement of plasma free metanephrine (MN) concentrations is the optimal biochemical test with the highest sensitivity and specificity (5, 6, 7, 8). Therefore, the aim of this study

was to assess whether plasma levels of free MNs and 3MT are more sensitive parameters of biochemical activity of HNPGL than urinary excretion rates of free catecholamines and their deconjugated metabolites.

Subjects and methods

We performed a cross-sectional study of 130 consecutive patients with HNPGL who were followed at the out-patient clinic of the Leiden University Medical Center, a tertiary referral center for patients with paragangliomas. For this purpose, clinical, biochemical, and radiological data of all consecutive patients with HNPGL were evaluated. All patients were investigated at the out-patient clinic according to structured standard clinical protocols. These included questions focused on tumor- and catecholamine-related signs and symptoms, measurement of blood pressure in the supine position, and after 5 min in the upright position, in order to screen

for orthostatic hypotension. In all patients, head and neck magnetic resonance imaging (MRI) was performed or had been performed within the previous 2 years.

Urine was collected during 24 h in duplicate under strict dietary regulations (patients abstained from pineapple, avocado, bananas, kiwi, nuts, plums, coffee, tea, and other caffeine-containing beverages) and after withdrawal of medication for at least 1 week or after changing antihypertensive medication to doxazosin for several weeks. In order to ascertain adequacy of urinary collection, 24-h urinary creatinine excretion rates were also measured. Blood samples were drawn after the second day of urine collection in the post-absorptive state. Blood samples were drawn from an i.v. catheter inserted into a forearm vein after 30 min of rest in the supine position and collected in cold, glutathione-containing vacutainers. All blood samples were centrifuged immediately at 3000 rpm for 10 min at 4 °C. Plasma samples were stored at –80 °C until analyses.

Biochemical screening included the measurement of urinary excretion rates of deconjugated (nor)metanephrine, free (nor)epinephrine, dopamine, and deconjugated 3MT excretion in two 24-h urinary samples and the measurement of plasma free (nor)metanephrines and 3MT concentrations.

In case of excessive catecholamine or metabolite excretion (i.e. any value above the upper reference limit in two urine samples or in the plasma sample), radiological assessment by 123I-metaiodobenzylguanidine (MIBG) scans and MRI and/or CT scans of thorax and abdomen were performed to identify the source of excessive catecholamine production. All patients with documented paragangliomas in the abdomen or thorax were excluded in this study because the interpretation of the biochemical results in relation to HNPGL could be confounded by the presence of these other paragangliomas.

We performed screening for succinate dehydrogenase (SDH) mutations in those HNPGL patients, who agreed upon genetic testing. Hereditary disease was diagnosed if mutations in the *SDHB*, *SDHC*, or *SDHD* genes were documented in the HNPGL patient and/or a family member.

We screened 130 consecutive HNPGL patients for catecholamine excess. Four patients with catecholamine excess were excluded from the current analyses because no additional radiological assessment was performed to exclude the presence of other paragangliomas not related to the head and neck regions. Two patients were excluded because they were diagnosed with a pheochromocytoma. Therefore, the study group comprised 124 patients with HNPGL.

Plasma MN levels in HNPGL patients were compared with those of 26 healthy subjects. The subjects who participated in the 'Dietary influences on plasma and urinary MNs' served as a healthy control group. The healthy controls were studied using the same methods as described in our study (e.g. blood was drawn from a forearm vein after an overnight fast; the sample was drawn after 30 min of rest in the supine position,

and furthermore, subjects avoided catecholamine-containing products). The control group comprised 26 healthy subjects (13 men and 13 women) with a median age of 38 years (9).

The study was an evaluation of routine patient care. According to the requirements of Dutch law, it was not necessary to obtain permission from the Institutional Ethical Commission. Prior to germ line mutation testing, informed consent was obtained from each patient.

Assays

Free epinephrine, norepinephrine, and dopamine excretion rates in 24-h urine collections were quantified by reversed phase HPLC with electrochemical detection. Inter- and intra-assay coefficients of variation (CV) for epinephrine were 4.3–9.0% ranging from low to high concentrations. For norepinephrine, these data are 2.7–3.6% and for dopamine 3.1–4.8%. Vanillylmandelic acid (VMA) excretion in urine was measured using HPLC with fluorometric detection with inter- and intra-assay CV of 2.4–9.1%. Urinary deconjugated (nor)metanephrine and 3MT were determined by isotope dilution gas chromatography with mass spectrometric detection. The CV of the 3-O-methylated catecholamine metabolites (MN, normetanephrine (NMN), and 3MT) ranged from 1.7 to 4.2% (10). Plasma free MNs were determined by automated in-line solid-phase extraction and isotope dilution liquid chromatography with mass spectrometric detection (11).

Reference ranges were obtained in healthy volunteers. These values were for urinary excretion: free norepinephrine 0.06–0.47 µmol/24 h, epinephrine <0.16 µmol/24 h, dopamine 0.46–3.40 µmol/24 h, VMA <30 µmol/24 h (deconjugated), MN (deconjugated) 33–99 µmol/mol creatinine, NMN (deconjugated) 64–260 µmol/mol creatinine, and 3MT (deconjugated) 45–197 µmol/mol creatinine (12).

The reference intervals for plasma free MNs were determined using blood samples collected in the supine position from 115 volunteers (57 males and 58 females; age range, 36–81 years; median age, 55 years) (13). The reference ranges for plasma free MNs were MN 0.07–0.33 nmol/l, NMN 0.23–1.07 nmol/l, and 3MT <0.17 nmol/l. SDH mutation analysis was performed by restriction digestion as described by Taschner *et al.* (14, 15).

Statistical analysis

SPSS for Windows version 16.0 (SPSS, Inc.) was used for data analysis. Results are expressed as means $\pm$ S.E.M., unless otherwise specified. Test sensitivity was calculated from the patients with a positive test result divided by the total number of patients tested. Test sensitivities were compared using the McNemar test. The average value of catecholamine and metabolite excretion rates of two urine samples was used for calculation of *P* values. A *P* value <0.05 was considered to represent a significant difference.

Table 1 Clinical characteristics of 124 patients with head and neck paragangliomas (HNPGs). Data are shown as mean $\pm$ S.E.M., unless otherwise indicated.

	All patients with HNPG (n=124)	Hereditary HNPG (n=95)	Sporadic HNPG (n=16)
Patient characteristics			
Age (years)	49 $\pm$ 1	47 $\pm$ 1	58 $\pm$ 3
Gender (n (%))			
Men	64 (52%)	51 (54%)	4 (25%)
Women	60 (48%)	44 (46%)	12 (75%)
Type of glomus tumor (n (%))			
Caroticum	87 (70%)	73 (77%)	4 (25%)
Vagale	51 (41%)	42 (44%)	6 (38%)
Jugulare	13 (11%)	11 (12%)	1 (6%)
Jugulotympanicum	21 (17%)	13 (14%)	5 (31%)
Tympanicum	12 (10%)	6 (6%)	5 (31%)
No. of HNPG (n (%))			
1	55 (44%)	37 (39%)	12 (75%)
>1	69 (56%)	58 (61%)	4 (25%)
No. of patients with genetic analysis			
SDHD	86 (69%)		
SDHB	8 (7%)		
SDHC	1 (1%)		
Sporadic	16 (13%)		

Results

Clinical characteristics

The study group comprised 124 patients with HNPG. Mean age of the patients was 49 years (range 13–77 years; Table 1). Fifty-five patients (44%) had a single HNPG, whereas 69 patients (56%) had multiple HNPGs. Paragangliomas occurred most frequently in the glomus caroticum (70%). Fifty-one patients (41%) had a glomus vagale tumor, 21 (17%) a glomus jugulotympanicum tumor, 13 (11%) a glomus jugulare tumor, and 12 (10%) a glomus tympanicum tumor.

Genetic testing for SDHx mutations was performed in 111 of the 124 patients (90%) with HNPG. In 95 patients, mutations were found (86 SDHD, eight SDHB, and one SDHC), whereas in 16 patients, no mutation was found. In four patients with multiple HNPGs, no mutation in the SDHB, SDHC, or SDHD gene was found. We screened these four patients for a mutation in the SDAF2 gene, but in none of these four patients was a mutation found.

Biochemical characteristics

Forty-six patients (37%) had biochemically active HNPG, evidenced by increased plasma concentrations of free (nor)metanephrine, 3MT and/or increased 24-h urinary concentrations of free catecholamines, and deconjugated metabolites, including 3MT (Table 2). There was no difference between the number of subjects with increased plasma free 3MT concentrations compared with the number of subjects with increased

urinary excretion rates of deconjugated 3MT ($n=35$ vs 30, 28 vs 24%, $P=0.13$). Urinary deconjugated MN excretion rates (MN and NMN) were increased in 8% (10 of 124 patients), which in combination with the patients with increased urinary deconjugated 3MT concentration increased the number of patients with biochemically active HNPG to 27% (33 of 124 patients). Eleven patients (9%) had increased urinary excretion rates of VMA. Increased urinary excretion rates of free catecholamines (adrenaline, noradrenaline, or dopamine) were present in 10% of the patients (12 of 124 patients). Increased urinary excretion rates of combined MNs in combination with catecholamines were present in 28% of the patients (35 of 124 patients).

Plasma free (nor)metanephrine concentrations were increased in 11% of the patients (14 of 124 patients). The addition of plasma free 3MT levels to these measurements increased the proportion of patients with evidence of biochemically active HNPG to 33% (41 of 124 patients). The number of HNPG patients with increased plasma free MN levels was not significantly different from that of patients with increased 24-h urinary excretion rates of deconjugated MNs (11 vs 8%, $P=0.34$). Increased plasma free MN (MN and NMN) concentrations in combination with increased plasma free 3MT levels were present in significantly more HNPG subjects than increased urinary excretion rates of combined MNs (MN and NMN) including 3MT (33 vs 27%, $P<0.05$).

In total, ten patients had either increased urinary or increased plasma MN levels. Of these ten patients, four had increased plasma 3MT levels, one had an increased

Table 2 Plasma metanephrine levels and mean urinary excretion rates of catecholamines, VMA, and metanephrines in 124 HNPG patients.

	Mean (range)	N tested positive
Plasma		
NMN (nmol/l)	0.6 (0.16–6.64)	7 (6%)
MN (nmol/l)	0.18 (0.04–0.48)	7 (6%)
3MT (nmol/l)	0.46 (0.03–12.8)	35 (28%)
MN + NMN (nmol/l)	0.78 (0.28–6.78)	14 (11%)
MN + NMN + 3MT (nmol/l)	1.24 (0.32–13.9)	41 (33%)
Urine		
NMN (μ mol/mol creatinine)	176 (55–1720)	5 (4%)
MN (μ mol/mol creatinine)	55 (16–162)	6 (5%)
3MT (μ mol/mol creatinine)	312 (43–6391)	30 (24%)
MN + NMN (μ mol/mol creatinine)	232 (95.5–1783)	10 (8%)
MN + NMN + 3MT (μ mol/mol creatinine)	544 (154–6598)	33 (27%) ^a
Adrenaline (μ mol/24 h)	0.02 (0.0–0.11)	0
Noradrenaline (μ mol/24 h)	0.40 (0.11–5.2)	6 (5%)
Dopamine (μ mol/24 h)	1.92 (0.77–6.43)	8 (7%)
Catecholamines (μ mol/24 h)	2.34 (0.91–9.78)	12 (10%)
VMA (μ mol/24 h)	22.5 (0–62)	11 (9%)
Catecholamines + combined MNs		35 (28%)

VMA, vanillylmandelic acid; MN, metanephrine; NMN, normetanephrine; 3MT, 3-methoxytyramine; combined MNs, MN + NMN + 3MT.

^aA significant difference in test sensitivity between urine and plasma.

plasma NMN level, and one had increased NMN and 3MT levels. Overall, six patients had additionally increased noradrenergic or dopaminergic activity. Four patients showed no increased noradrenergic or dopaminergic activity.

Eight patients had increased urinary excretion rates of dopamine. Seven of these eight patients had increased plasma 3MT levels and the other patient had an increased plasma NMN level.

Nine patients tested negative with urinary screening of catecholamine excretion rates but positive with screening of plasma MN levels. Of these patients, six had increased plasma free 3MT levels, two had increased plasma free MN levels, and one had an increased plasma free NMN level.

In the healthy control group, two persons had slightly elevated MN levels, none had increased plasma free NMN levels, and none had increased plasma 3MT levels. We have presented the results of the plasma free MN measurements graphically in Fig. 1.

Catecholamine and metabolite excretion, tumor load, and genotype

We identified 55 patients with only a single HNPGL with a mean tumor diameter of 3.3 cm (range 0.3–9.7 cm). In those patients, there was a weak positive correlation between tumor diameters and plasma concentrations of free 3MT ($r=0.30$, $P=0.04$), and the combined plasma concentrations of free MN, NMN, and 3MT ($r=0.35$, $P=0.02$). There were also weak correlations between tumor diameters and mean urinary excretion rates of deconjugated NMN ($r=0.40$, $P<0.01$), VMA ($r=0.32$, $P=0.03$), dopamine ($r=0.40$, $P<0.01$), the combined excretion rates of deconjugated MN and NMN ($r=0.40$, $P<0.01$), the combined excretion rates of deconjugated (nor)metanephrine and 3MT ($r=0.43$, $P<0.01$), and the mean excretion rate of catecholamines ($r=0.37$,

$P=0.01$). There was no correlation between the number of HNPGL and plasma free MN levels and mean urinary excretion rates of deconjugated MNs and catecholamines.

We found no specific biochemical phenotype for patients with either a *SDHB* or a *SDHD* genotype. The number of patients with a biochemically active tumor was equal between patients with a *SDHB*, a *SDHD*, or a sporadic genotype.

Catecholamine and metabolite excretion, age, BMI, and gender

Stepwise linear regression analysis was performed in a model including age, gender, and BMI as independent variables and plasma free MN, NMN, and 3MT levels as dependant variables. We identified age ($\beta=0.35$, $P<0.001$), BMI ($\beta=-0.29$, $P<0.001$), and gender ($\beta=-0.35$, $P<0.001$) as independent predictors of plasma free MN levels. The patients with increased plasma free MN levels were significantly older compared with patients with normal levels (48.0 ± 1.2 vs 61.5 ± 3.7 years, $P<0.01$). Men had significantly higher plasma free MN levels compared with women (0.21 ± 0.0 vs 0.15 ± 0.0 , $P<0.001$).

There was no relationship of plasma levels of free NMN with age ($\beta=0.09$, $P=0.35$), BMI ($\beta=-0.11$, $P=0.26$), and gender ($\beta=0.09$, $P=0.35$). There was a relationship of age ($\beta=0.09$, $P=0.38$), BMI ($\beta=0.02$, $P=0.41$), and gender ($\beta=-0.08$, $P=0.41$) with plasma free 3MT levels.

Long-term follow-up of patients with biochemically active tumor

Forty-six patients (37%) had biochemically active HNPGL, evidenced by increased plasma concentrations of free (nor)metanephrine, 3MT and/or increased 24-h urinary concentrations of free catecholamines, and deconjugated metabolites, including 3MT. In this cohort of 46 patients, mean follow-up time was 2.8 ± 1.1 years, with a total follow-up time of 129 patient years. Forty patients had persistent catecholamine excess during follow-up. Five patients had normalization of catecholamine levels, of whom two patients underwent surgery. Three patients showed normalization of catecholamine excretion levels without intervention (surgery) and one patient was lost to follow-up.

Thirty-eight patients had persistent catecholamine excess during follow-up. Three patients had normalization of catecholamine levels, of whom two underwent surgery. In four patients, either one of the two urine samples showed increased MN levels. As we consider a patient positive when two urine samples contain excess catecholamines, these patients were considered to have normalization of catecholamine excretion. One patient was lost to follow-up.

Figure 1 Plasma concentrations of free methoxytyramine, normetanephrine, and metanephrine in HNPGL patients (left) and controls (right). The dashed line represents the upper limit of the reference interval.

Discussion

The results of this study show that one-third of HNPGL patients have biochemically active tumors. The combined plasma concentrations of free MNs and 3MT indicate a higher proportion of patients with biochemically active HNPGL than 24-h urinary excretion rates of deconjugated MNs and 3MT. In addition, the current data indicate that in HNPGL patients, plasma free 3MT concentrations and urinary excretion rates of deconjugated 3MT do not identify significantly different numbers of subjects with biochemically active HNPGL. Plasma MN levels were additionally measured in a healthy control group. In these controls, two persons had slightly elevated MN levels, none had increased plasma free NMN levels, and none had increased plasma 3MT levels.

HNPGLs have the ability to produce and secrete catecholamines (2, 3). Biochemically active paragangliomas are identified by the measurement of plasma (nor)metanephrine and 3MT concentrations and 24-h urinary excretion rates of free catecholamines and their deconjugated metabolites (6, 16). The measurements of urinary excretion rates of catecholamine metabolites (i.e. MN and NMN) and especially plasma levels of catecholamines and their metabolites are recommended for the biochemical screening of pheochromocytoma because of their high diagnostic sensitivity and specificity (7). However, the majority of patients with biochemically active HNPGL secrete 3MT, which is a metabolite of dopamine. Therefore, the measurement of this metabolite should be included if biochemical activity of HNPGL in general is assessed. 3MT can be measured in urine and plasma by HPLC–tandem mass spectrometric detection (XLC–MS/MS) (13). This study indicates that the assessment of plasma free 3MT levels does not add to the measurement of urinary excretion rates of deconjugated 3MT.

As patients with HNPGL have the ability to (co)secrete noradrenaline (11, 17, 18), this catecholamine and its metabolite NMN should be measured as well. Combining the results of free MNs and 3MT in plasma resulted in a slightly, but significantly, higher number of patients with biochemically active HNPGL compared with the combined results of urinary excretion rates.

Consumption of catecholamine-rich food products can result in substantial increases in urinary excretion rates of deconjugated NMN and 3MT and to a lesser extent in plasma free 3MT levels. Therefore, dietary restrictions are indicated prior to collection of blood for measurements of plasma free 3MT levels, and urinary excretion rates of deconjugated NMN and 3MT (9). Although patients in our study collected urine during 48 h under strict dietary regulations, we cannot exclude potential confounding effects of the diet. In contrast, the measurements of plasma free MN concentrations are not influenced by the confounding effects of dietary components; this contributes to the highest sensitivity

of plasma levels compared with the measurement of urinary excretion rates of MN (9, 19). In accordance, plasma free MNs (MN, NMN, and 3MT) levels were increased in a higher percentage of HNPGL patients compared with urinary excretion rates of MNs.

Our findings are in agreement with the observations in patients with pheochromocytomas (6, 7, 8). Lenders *et al.* reported a test sensitivity of 97% of plasma free MNs vs only 60% of urinary combined MNs in patients with hereditary pheochromocytomas and 99% of plasma MN levels vs 88% in urinary excretion rates in patients with sporadic pheochromocytomas.

HNPGLs are devoid of phenylethanolamine N-methyltransferase enzymatic activity. The increase in urinary MN levels observed in patients with HNPGL is therefore somewhat peculiar. We identified age, BMI, and gender as independent predictors of plasma MN levels. Patients with increased plasma free MN levels were significantly older compared with those with normal free plasma MN levels. For the levels of plasma free NMN and 3MT, we did not find a relationship between plasma and/or urine levels and age, gender, and BMI. Deutschbein *et al.* found that MN levels were significantly influenced by sex and age. Our data are therefore in correspondence with the data of Deutschbein *et al.* (20). They also found a relationship between plasma NMN levels and BMI and sex. We did not find these relationships. As HNPGLs have the ability to secrete noradrenaline, but not adrenaline, the presence of HNPGLs could influence the correlation between plasma NMN levels, age, sex, and BMI. Because in all patients with increased catecholamine/MN levels additional radiological assessment excluded the presence of a pheochromocytoma, the increased levels of MN may be regarded as physiological. It could indicate that men and older people have increased adrenergic activity and/or the removal of MN spillover from the adrenals may be less adequate compared with younger people. The results of the (nor)metanephrine measurements in healthy subjects strengthen our assumption. As none of the healthy subjects had increased NMN and 3MT levels, but two had increased MN levels, the excretion of MN could be considered as physiological.

Medication (tricyclic antidepressants, β -blockers, calcium antagonists, and acetaminophen) can either cause direct analytical interference in measurements of catecholamines and metabolite levels or may influence the physiological processes that determine these levels. Therefore, urine and plasma samples are collected after withdrawal of medication for several weeks or after changing antihypertensive medication to doxazosin. In total, 16 patients used doxazosin because of hypertension during the study period. Eisenhofer *et al.* (21) studied the effect of medication-associated false-positive test results in patients screened for pheochromocytoma. They found that selective α 1-blockers were not associated with an increased frequency of false-positive results for plasma norepinephrine or NMN but were associated with false-positive elevations of

urinary norepinephrine. Because of these findings, we do not expect that doxazosin caused an increase in either plasma or urinary NMN levels.

In conclusion, one-third of HNPGL patients have biochemically active tumors. The combined assessment of plasma concentrations of free MNs and 3MT detects a higher number of biochemically active HNPGLs than the measurement of 24-h urinary excretion rates of combined MNs and 3MT. In addition, the current data indicate that in HNPGL patients, urinary excretion rates of deconjugated 3MT and plasma free 3MT levels do not identify significantly different numbers of subjects with biochemically active HNPGL.

Declaration of interest

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

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