

Deconjugated Urinary Metanephrine, Normetanephrine and 3-Methoxytyramine in Laboratory Diagnosis of Pheochromocytoma and Paraganglioma

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Summary

This work discusses the clinical performance of deconjugated metanephrine (MN), normetanephrine (NMN) and 3-methoxytyramine (3MT) determined in the basal first morning urine using a chromatographic method with electrochemical detection for the clinical diagnosis of pheochromocytoma (PHEO) and paraganglioma (PGL). Urine samples were collected from 44 patients (36 with PHEO, 8 with PGL) aged 54+/-17 (20-78) years (22 females, 22 males). A sampling of biological materials was performed preoperatively and about one week, six months and one year after adrenal gland surgery. The control group consisted of 34 PHEO/PGL patients more than 4 months after adrenal gland surgery. All subjects in the control group were without a diagnosis of PHEO or PGL. Clinical sensitivity was 55 % for MN, 64 % for NMN, 80 % for combination of both MN and NMN, and only 23 % for 3MT. Clinical specificity calculated from the control group was 93 % for MN, 95 % for NMN, 95 % for the combination MN and NMN, and 97 % for 3MT. Cut-off values for deconjugated metanephrines in the basal urine were 310 (MN), 690 (NMN) and 250 µg/l (3MT). Chromatographic determination of deconjugated urinary metanephrines, which is simple without the necessity of special laboratory material, can serve for the screening of PHEO or PGL patients. Urine NMN and 3MT exerts

an association to malignancy, and all markers are associated with tumor mass. However, the principal laboratory diagnosis of PHEO or PGL must be based on plasma-free metanephrines and plasma chromogranin A with better performance in the laboratory diagnosis of PHEO or PGL.

Key words

Urine • Deconjugated metanephrine • Deconjugated normetanephrine • Deconjugated 3-methoxytyramine • Chromatography • Electrochemical detection • Pheochromocytoma • Paraganglioma

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Introduction

Pheochromocytoma (PHEO) or paraganglioma (PGL) are rare tumors associated with neuroendocrine chromaffin cells usually found in the adrenal medulla and other ganglia of the nervous system (Tischler 2006). PHEO is a tumor arising from adrenomedullary

chromaffin cells that commonly produces catecholamines and it is associated with adrenal localization in 80-85 % of patients. PGL is a neuroendocrine tumor derived from extra-adrenal chromaffin cells of the sympathetic paravertebral ganglia (thorax, abdomen, pelvis) producing catecholamines, or parasympathetic ganglia located in the neck and at the base of the skull, which do not produce catecholamines (Chen *et al.* 2010, Lenders *et al.* 2014). Extra-adrenal occurrence of sympathetic and parasympathetic PGL is observed in 10-20 % patients (Chen *et al.* 2010, van Berkel *et al.* 2014). In this study we have used PPGL as a general term for both PHEO and PGL. Approximately 10 % of the PHEOs and 20 % of the PGLs are malignant with poor survival (Andersen *et al.* 2011). Up to 30 % of PPGL patients have recognizable germline mutations including multiple endocrine neoplasia type 2A (MEN2A) or 2B (MEN2B), von Recklinghausen neurofibromatosis type 1 (NF1), von Hippel-Lindau syndrome (VHL) and succinate dehydrogenase complex – subunits A, B, C, D (SDHx) syndrome (Mazzaglia 2012). Since 1990, 14 different PPGL susceptibility genes have been reported (Lenders *et al.* 2014). Mainly mutations in the tumour suppressor gene von Hippel-Lindau (*VHL*) and the mitochondrial succinate dehydrogenase enzyme complex subunit B (*SDHB*) are associated with malignancy (Andersen *et al.* 2011).

The genetic diversity commonly associated with PPGLs is based on the noradrenergic phenotype of VHL and SDHx or on the adrenergic phenotype characteristic for MEN2A, MEN2B and for NF1 (van Berkel *et al.* 2014). In noradrenergic phenotype the biosynthesis of the enzyme phenylethanolamine-N-methyltransferase converting norepinephrine to epinephrine is downregulated at both the mRNA and protein level in sympathetic or parasympathetic paragangliomas (Gouzmann *et al.* 2015). Increased production of norepinephrine results in an elevated concentration of normetanephrine in circulation after the conversion of norepinephrine to normetanephrine by the enzyme catechol O-methyltransferase. The epinephrine and metanephrine are usually within the normal reference range in noradrenergic PGLs. In the adrenergic phenotype, the phenylethanolamine-N-methyltransferase presented in the adrenal gland converts norepinephrine to epinephrine, which is further converted by the catechol O-methyltransferase to metanephrine. The adrenergic phenotype is typical with an elevated concentration of

epinephrine and secondary metanephrine with varying amounts of norepinephrine and normetanephrine in the circulation (van Berkel *et al.* 2014). A lack of the catechol O-methyltransferase in sympathetic nerves, the major site of initial norepinephrine metabolism, means that the O-methylated metabolites are relatively specific markers of chromaffin tumors. These metabolites are produced continuously within tumors by a process that is independent of exocytotic catecholamine release, which for some tumors occurs at low rates or is episodic in nature (Lenders *et al.* 2014). In addition, 70 % of patients with SDHB and SDHD mutations increase 3-methoxytyramine (dopamine) production (Eisenhofer *et al.* 2011) due to the absence of tyrosine hydroxylase, the enzyme that catalyses the initial and rate-limiting step in catecholamine biosynthesis (van Berkel *et al.* 2014). Dopaminergic phenotype can be a good indicator of metastatic disease (Eisenhofer *et al.* 2012, Fliedner *et al.* 2010). Sympathetic PPGL usually produce significant amounts of catecholamines, whereas parasympathetic PGL can be endocrinologically inactive (Lenders *et al.* 2005). It was shown (Eisenhofer *et al.* 2011) that patients with NF1 and MEN2 can be discriminated from those with VHL, SDHB and SDHD mutations in 100 % cases by the combined analysis of MN, NMN and 3MT. Within this subgroup of VHL, SDHB or SDHD patients, measurements of plasma 3MT discriminated patients with SDHB and SDHD mutations from those with VHL mutations in 78 % of cases. The histological evaluation of primary PPGL tumors is based on PASS scoring scale. A value less than 4 out of 20 points supports the benign character of the tumor (Thomson 2002).

High-performance liquid chromatography (HPLC) continues to be the dominant analytical method for biogenic amine quantitation. The most widely used method for the determination of catecholamines and metanephrines that have been extracted from urine and separated by HPLC is electrochemical detection (ECD). However, the use of more sensitive liquid chromatography with tandem mass spectrometry (LC-MS/MS) is becoming more popular (Whiting and Doogue 2009). Simultaneous measurements of urinary free O-methylated metabolites of catecholamines by the liquid chromatographic tandem-mass spectrometric method provide potential advantages over urinary deconjugated metanephrines for the diagnosis of PPGLs with analytical sensitivity 1.2 nmol/l (Peitsch *et al.* 2013). The Endocrine Society (Lenders *et al.* 2014) recommends that the initial screening for PPGLs include

measurements of plasma-free metanephrines or urine fractionated metanephrines using liquid chromatography with mass spectrometric or electrochemical detection methods. LC-MS/MS offers numerous advantages over other analytical methods and is the method of choice when measurements include methoxytyramine (Eisenhofer and Peitzsch 2014). LC-MS/MS limit of quantification under 75 pmol/l was achieved for MN, NMN and 3-methoxytyramine using only 150 µl of plasma samples (Adaway *et al.* 2015).

Prior to chromatography, a pre-analytical clean-up step is required to extract and sometimes to concentrate the metanephrines from urine. Metanephrines are metabolised by sulfatation and excreted in the urine predominantly as sulphated metabolites (Eisenhofer *et al.* 2004). Urinary metanephrines are usually measured after deconjugation, where the results reflect the total concentration of metanephrines in urine. An acid hydrolysis of urinary metanephrines, where the biogenic amines are protonated as cations, and can be isolated using solid-phase extraction (SPE) based on both cation-exchange and anion-exchange chromatography to separate metanephrines from catecholamines and other interfering compounds, is nearly always included to release the conjugated forms, which are the major molecular species present (Whiting and Doogue 2009). New column technology has been developed with smaller particle sizes and with increased resolving power based on reverse-phase chromatography with modifications to allow retention and separation of polar biogenic amines without using ion-pairing reagents.

The plasma test offers advantages over the urine test, although it is rarely implemented correctly, rendering the urine test preferable for mainstream use (Eisenhofer and Peitzsch 2014). Urinary collection provides an integral measurement over 24 h, but is potentially incomplete and is inconvenient for patients (van Berkel *et al.* 2014). Several pre-analytical factors, such as exercise, posture, food with substantial quantities of biogenic amines, stress, hypoglycemia and medications may affect test results (van Berkel *et al.* 2014).

In this study we presented our experiences with high performance liquid chromatography with electrochemical detection (HPLC-ECD) for determination of urine deconjugated MN, NMN and 3MT in groups of patients who suffered of PPGLs.

Materials and Methods

The group of patients

Included in the study were the group of PPGL patients and the control group of the same patients more than 4 months after adrenalectomy without diagnosis of PPGL in this time. Patients with pheochromocytoma or paraganglioma consisted of 44 patients aged 54+/-17 (20-78) years, 22 females aged 60+/-16 (28-78) years, 22 males aged 49+/-17 (20-76) years. Pheochromocytoma occurred in 36 patients, paraganglioma in 8 patients. Adrenergic phenotype of disease was observed in 23 patients (1 MEN2A+MTC, 2 NF1, 20 PHEO), noradrenergic phenotype was found in 21 patients (11 PHEO, 7 PGL, 1 SDHD, 2 VHL).

A sampling of biological materials (first morning urine) was performed preoperatively and about one week, six months and one year after surgery. All four collections were conducted in 24 patients (17 PHEO, 2 PGL, 2 NF1, 1 VHL, 1 SDHD, 1 MEN2a+MTC), 3 collections in 8 patients (5 PHEO, 2 PGL, 1 VHL), 2 collections in 8 patients (5 PHEO, 2 PGL, 1 VHL), and 1 collection taking before surgery was conducted in 4 patients (1 PHEO, 3 PGL).

The control group consisted of 34 PHEO or PGL patients more than 4 months after adrenalectomy (24 PHEO, 3 PGL, 2 NF1, 3 VHL, 1 SDHD, 1 MEN2a+MTC).

Laboratory examination

MN, NMN and 3MT were determined in the basal first morning urine using a solid-phase extraction (SPE) after deconjugation of metanephrines by acid hydrolysis of metanephrine-sulphates. The deconjugated MN, NMN and 3MT were determined by HPLC-ECD.

Acid hydrolysis: 100 µl of urine (or calibrators) together with 100 µl of internal standard consisting of 2 µmol/l 4-hydroxy-3-methoxybenzylamine hydrochloride (HMBA) in 100 µl of 95 µmol/l CH₃COOH: CH₃CN=200 : 0.01 and 50 µl 1 mol/l HCl were heated for 20 min at 90 °C.

SPE with Separon HEMA 1000 CM (Tessek, Prague, Czech Republic, d_p=60 µm, weakly acidic cation exchanger): 50 µl (=20 µl of urine with 40 pmol of HMBA) of hydrolyzate and 200 µl of borate buffer (pH 6.5; 3 g of (NH₄)B₅O₈·8H₂O and 2 g of boric acid in 100 ml of water) were applied to filter tip with sorbent, washed with 500 µl of water and eluted with 500 µl of 3 mol/l NH₄OH.

SPE with Dowex 1x2 chloride form (Sigma-Aldrich s.r.o., Prague, Czech Republic, 200-400 mesh, strong anion exchanger): 500 µl of eluate from Separon HEMA 1000 CM (=20 µl of urine and 40 pmol of HMBA) were applied to filter tip with sorbent, washed with 500 µl of water and eluted with 250 µl of 50 mmol/l citrate buffer (pH 2.95).

HPLC (Double piston pump Knauer 1050, Wissenschaftliche Gerätebau Dr. Ing. Herbert Knauer GmbH, Berlin, Germany): 10 µl of eluate (= 0.4 µl of urine and 0.8 pmol of HMBA) was injected to the stationary phase Synergi 4 µm Polar-RP 80A (Phenomenex, Torrance, CA, USA) in 150 x 3 mm stainless steel column. Mobile phase consisted of acetonitrile: water containing 0.17 mmol/l EDTA, 2 mmol/l KCl, 40 mmol/l NaH₂PO₄·2H₂O, 10 mmol/l H₃PO₄, 1 mmol/l 1-octanesulfonic acid sodium salt and 0.06 mmol/l 1,4,7,10,13,16-hexaoxacyclooctadecane =80:920. The isocratic elution was used with the flow rate 0.6 ml/min. Separated metanephrines were detected using amperometric detector Decade II (Antec, Leyden, The Netherlands) with working voltage +0.65 V, current range 2 nA.

Statistics and calculations

All statistical calculations were made using the computer program NCSS 2004 (Number Cruncher Statistical Systems, Kayville, Utah, USA). The individual points in the time series of the CGA, MN, and NMN examination were interpolated after an inverse or logarithmic transformation by the Newton polynomial programmed up to the 12th degree using our own program working in Lotus 1-2-3 of the IBM Corporation, USA. Individual curves were counted for all patients with 3 and 4 determinations of CGA, MN, and NMN during more than the annual monitoring of their condition.

Results

The curves modeling the course of changes in MN, NMN and 3MT concentrations during the 75 weeks of monitoring PPGL patients following adrenal gland operations are based on 3 to 4 determinations of MN, NMN and 3MT. These curves along with specific values of MN, NMN and 3MT are shown in Figures 1, 2 and 3. For all patients diagnosed with PPGL, the nature of the illness was differentiated in the figures by solid symbols given in the legend of the Figures. These

were patients suffering from PHEO, PGL, SDHD, NF1, VHL and MEN2A with MTC syndromes. The curves were modelled so that the concentrations of monitored analytes before the operation were transferred to the Y axis to the 0 time corresponding to the operation on the adrenal gland. The reference range is defined by a transparent rectangle on the curves. The course of the curves in a given reference range was recorded in the part of the Figures labelled with the letter B. The clinical sensitivity of the individual tests for determining MN, NMN and 3MT is well evident in this part. The mean curve of the individual analytes consists of patient values for whom concentrations at time 0 were greater than the cut-off value for MN, NMN or 3MT is depicted on the Figures in part C. A dashed curve shown here consists of the mean curve to which twice the value of the standard deviation was added. The maximum of this dashed curve in time more than 10 weeks after surgery of adrenal gland corresponds with cut-off value which is upper part of reference range. This curve tells us with 95 % probability when the concentration of the individual analytes will return to the normal reference range following the operation.

Figure 1 describes the changes in MN concentration in a given time period and it is evident (Fig. 1A, 1B) that the clinical sensitivity is relatively low for patients with PPGL (55 %), the clinical specificity is 93 % and Figure 1C shows the reference range with the cut-off value 310 µg/l. MN returns to a normal reference range in an average of 2.5 weeks (Fig. 1C).

Figure 2 describes changes in NMN concentration in a given time period and it is evident (Fig. 2A, 2B) that clinical sensitivity reaches only 64 % in PPGL patients. The clinical sensitivity is higher than the clinical sensitivity for MN, but it is insufficient for laboratory diagnostics of PPGL patients. The combined sensitivity of MN and NMN corresponds to a value of 80 %. As with MN, clinical specificity reached 95 % (Fig. 2A) and NMN returned to a reference interval with a cut-off value of 690 µg/l in 2.4 weeks on average.

Figure 3 describes changes in 3MT concentration in a given time period and it is evident (Fig. 3A, 3B) that clinical sensitivity reaches only 23 % and 3MT is completely inappropriate for general laboratory diagnostics of PPGL patients. Clinical specificity was in the case of 3MT equal to 97 %. 3MT returned to the normal reference range with a cut-off value of 250 µg/l in 15 weeks on average.

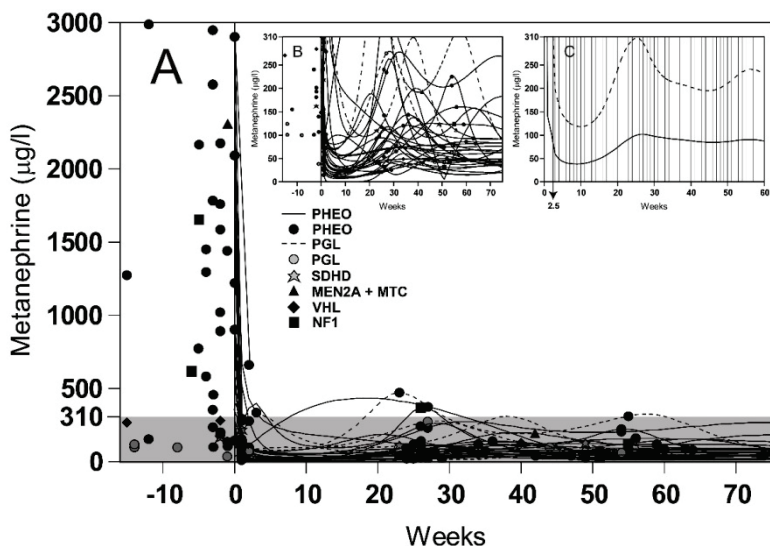


Fig. 1. Shown in **part A** are the results of determining metanephrine (MN) with specific values determined before the operation and after the operation using symbols describing the type of syndrome of pheochromocytoma (PHEO) or paraganglioma (PGL) patients. The values are linked by the curves calculated using a Newton polynomial of the 12th degree and a transparent rectangle indicates the reference range. The values and curves in **part B** correspond to the given reference range. The mean MN curve taken from patient values for whom concentrations at time 0 were greater than the cut-off value for MN is depicted on the figures in **part C**. A dashed curve shown here consists of the mean curve to which twice the value of the standard deviation (SD) was added. PHEO, pheochromocytoma; PGL, paraganglioma; MEN, multiple endocrine neoplasia; MTC, medullary thyroid cancer; NF1, von Recklinghausen neurofibromatosis type 1; VHL, von Hippel-Lindau syndrome; SDHD, succinate dehydrogenase complex - subunit D syndrome.

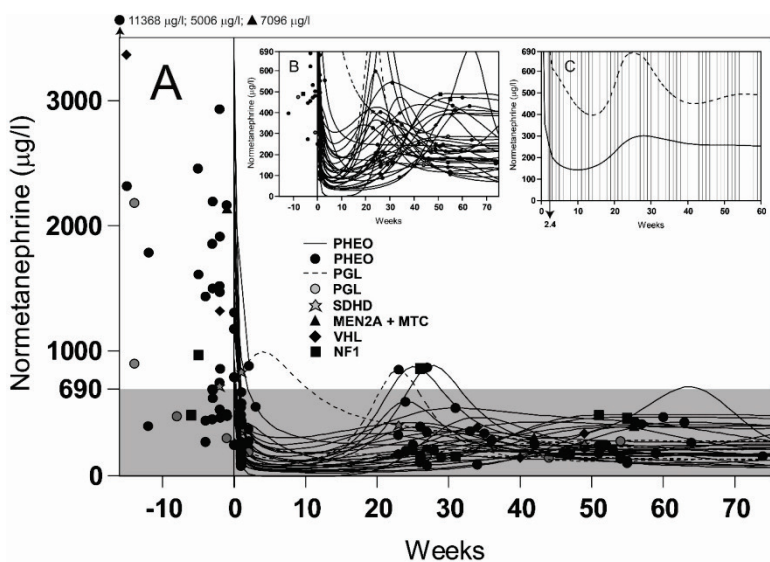


Fig. 2. Shown in **part A** are the results of determining normetanephrine (NMN) with specific values determined before the operation and after the operation using symbols describing the type of syndrome of pheochromocytoma (PHEO) or paraganglioma (PGL) patients. The values are linked by the curves calculated using a Newton polynomial of the 12th degree and a transparent rectangle indicates the reference range. The values and curves in **part B** correspond to the given reference range. The mean NMN curve taken from patient values for whom concentrations at time 0 were greater than the cut-off value for NMN is depicted on the figures in **part C**. A dashed curve shown here consists of the mean curve to which twice the value of the standard deviation (SD) was added. PHEO, pheochromocytoma; PGL, paraganglioma; MEN, multiple endocrine neoplasia; MTC, medullary thyroid cancer; NF1, von Recklinghausen neurofibromatosis type 1; VHL, von Hippel-Lindau syndrome; SDHD, succinate dehydrogenase complex - subunit D syndrome.

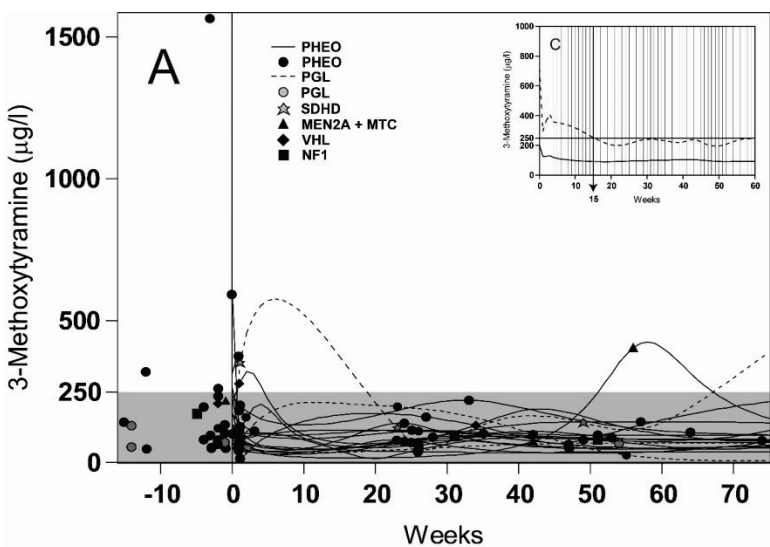


Fig. 3. Shown in **part A** are the results of determining 3-methoxytyramine (3MT) with specific values determined before the operation and after the operation using symbols describing the type of syndrome of PGL patients. The values are linked by the curves calculated using a Newton polynomial of the 12th degree and a transparent rectangle indicates the reference range. The mean 3MT curve taken from patient values for whom concentrations at time 0 were greater than the cut-off value for 3MT is depicted on the figures in **part C**. A dashed curve shown here consists of the mean curve to which twice the value of the standard deviation (SD) was added. PHEO, pheochromocytoma; PGL, paraganglioma; MEN, multiple endocrine neoplasia; MTC, medullary thyroid cancer; NF1, von Recklinghausen neurofibromatosis type 1; VHL, von Hippel-Lindau syndrome; SDHD, succinate dehydrogenase complex - subunit D syndrome.

All results concerning clinical sensitivity in patients with a PPGL diagnosis, measured before the adrenal gland operation, are shown in Table 1. Patients are divided into adrenergic and noradrenergic phenotypes and both cut-off values for MN, NMN, 3MT and their mean values, standard deviations, ranges of measured concentration in first morning urine and the number of PPGL patients diagnosed with a specific disease syndrome are given here. In summary, it can be stated that clinical sensitivity had a value of 55 % for MN, 64 % for NMN, 80 % for a combination of MN + NMN and 23 % for 3MT.

Table 2 gives all results concerning clinical specificity for both PPGL patients with MN, NMN and 3MT values that were included in calculations only in the case that they were determined at least 4 months following the adrenal gland operation. This concerned a total of 34 patients. The Table 2 gives both cut-off values for MN, NMN, 3MT and their mean values, standard deviations, ranges of measured concentration in first morning urine and the number of PPGL patients diagnose. Clinical specificity has a value of 93 % for MN, 95 % for NMN, 95 % for a combination of MN/NMN and 97 % for 3MT. The mass of the operated tumor correlated to the MN, NMN and 3MT concentration according to the equations:

$$\log(\text{mass (g)}) = 0.1406 + 0.5845 * \log(\text{MN}(\mu\text{g/l}))$$

$$p < 0.0001, r = 0.6870$$

$$\log(\text{mass (g)}) = -0.4888 + 0.7498 * \log(\text{NMN}(\mu\text{g/l}))$$

$$p = 0.0022, r = 0.5079$$

$$\log(\text{mass (g)}) = 0.0012 + 0.7923 * \log(\text{3MT}(\mu\text{g/l}))$$

$$p = 0.0002, r = 0.6023$$

PASS scores correlated only with concentrations NMN and 3MT according to equations:

$$\log(\text{PASS}) = -0.2393 + 0.3032 * \log(\text{NMN}(\mu\text{g/l}))$$

$$p = 0.0333, r = 0.3417$$

$$\log(\text{PASS}) = -0.0512 + 0.3264 * \log(\text{3MT}(\mu\text{g/l}))$$

$$p = 0.0096, r = 0.4097$$

A statistically important correlations between the ascertained AUC (area under curve) logarithmic values of MN, NMN, 3MT and logarithmic values of the tumor mass or PASS score were not found.

Discussion

Chromatographic determination of MN, NMN, 3MT employs a simple and feasible technology for detection of PPGL diseases. The problem is that during initial diagnostic workup in patients with suspected PPGL tumors a single test with very high negative

predictive value is preferred. The increase of clinical sensitivity can lower the clinical specificity (van Berkel *et al.* 2014). In the case of MN, the predictive value of the test failed from a clinical sensitivity standpoint for patients with noradrenergic phenotype. By contrast, there can be a problem with NMN for patients with adrenergic phenotype. In the case of PPGL patients, it is therefore very important to conduct a parallel determination of both MN and NMN. In our group of patients, determination of clinical sensitivity *via* 3MT was the least successful. Increased concentrations of 3MT can be helpful in the laboratory diagnosis of patients with an endocrinologically inactive PGL nature or in those cases in which dedifferentiation of chromaffin cells no longer allows for the biosynthesis of catecholamines. This type of patient did not occur in the group we observed.

The modeling of the individual curves from 3 to 4 of the values determined over the course of over a year monitoring PPGL patients was complicated. The values had to be inversely or logarithmically transformed so that a Newton polynomial of the 12th degree could be applied to modeling the course *via* a program developed in our laboratory. The result of the modeling enabled the course of change to be predicted, provided information on the used reference ranges and enabled a determination of the mean period of the return of MN, NMN and 3MT to the normal reference range following the adrenal gland operation. This period was less than 3 weeks for MN or NMN, but nearly 15 weeks for 3MT. The AUC (area under) given in Table 2 provides information on the overall biosynthesis of MN, NMN and 3MT during the monitored period. We did not found the statistically significant relationship between mass of removed tumor or PASS score and values of MN, NMN or 3MT. The AUC values should be further examined from a clinical standpoint. Statistically important associations were found for the logarithmic transformation between the mass of the operated tumor and MN ($p < 0.0001$), NMN ($p = 0.0022$) or 3MT ($p = 0.0002$). The PASS scores only correlated with NMN ($p = 0.0333$) and 3MT ($p = 0.0096$) after the logarithmic transformation of values.

Our cut-off values for urinary MN, NMN and 3MT were based on basal values measured in the first morning urine. This is a more practical method than measuring the values related to a 24-h urine sample, which for many cases, especially those patients immediately following surgery, is feasible only with difficulties.

Table 1. The clinical sensitivity of PPGL (PHEO or PGL) patients with concentrations of deconjugated urinary metanephrine (MN), normetanephrine (NMN) and 3-methoxytyramine (3MT) determined before the adrenal operation. PHEO, pheochromocytoma; PGL, paraganglioma; MEN, multiple endocrine neoplasia; MEN, multiple endocrine neoplasia; MTC, medullary thyroid cancer; NF1, von Recklinghausen neurofibromatosis type 1; VHL, von Hippel-Lindau syndrome; SDHD, succinate dehydrogenase complex – subunit D syndrome.

Patients with pheochromocytoma or paraganglioma before surgery												
Phenotype	Syndrome	N	MN (µg/l)			NMN (µg/l)			3MT (µg/l)			
			mean	SD	range	mean	SD	range	mean	SD	range	
Adrenergic	PHEO	20	1664	798	156-2989	1183	684	251-2324	261	338	47-1573	
	PHEO	1	2316	-	-	2137	-	-	219	-	-	
	MEN2A+MTC	2	1141	734	622-1660	730	339	490-970	156	23	140-172	
	PHEO - NF1	11	177	75	101-358	1212	870	471-2933	140	83	49-288	
Noradrenergic	PHEO - VHL	2	277	10	270-284	2347	1439	1329-3364	393	259	210-576	
	PGL	7	170	147	38-458	727	692	167-2194	101	75	50-258	
	PGL - SDHD	1	163	-	-	714	-	-	61	-	-	
CLINICAL SENSITIVITY		44	MN = 55 %	NMN = 64 %	MN + NMN = 80 %				3MT = 23 %			

Table 2. The clinical specificity determined from the values of PPGL (PHEO or PGL) patients taken at least 4 months after the adrenal operation. MN, deconjugated urinary metanephrine; NMN, normetanephrine; 3MT, 3-methoxytyramine; PHEO, pheochromocytoma; PGL, paraganglioma; MEN, multiple endocrine neoplasia; MTC, medullary thyroid cancer; NF1, von Recklinghausen neurofibromatosis type 1; VHL, von Hippel-Lindau syndrome; SDHD, succinate dehydrogenase complex - subunit D syndrome; AUC, area under curve.

Syndrome	N	MN (µg/l)			NMN (µg/l)			3MT (µg/l)					
		cut-off value = 310 µg/l			cut-off value = 690 µg/l			cut-off value = 250 µg/l					
		mean	SD	range	mean	SD	range	mean	SD	range			
				mean AUC (0-60 weeks)			mean AUC (0-60 weeks)			mean AUC (0-60 weeks)			
<i>MEN2A+MTC</i>	2	152	58	111-193	9797 (n=1)	353	62	309-396	14895 (n=1)	241	235	74-407	6621 (n=1)
<i>NF1</i>	4	151	152	32-372	6953 (n=2)	494	289	159-864	20226 (n=2)	173	104	82-299	9070 (n=2)
<i>PGL</i>	3	284	207	64-474	9159 (n=3)	418	382	119-849	13703 (n=3)	98	88	27-197	6948 (n=3)
<i>Adrenergic PHEO</i>	31	91	64	22-241	6725 (n=15)	253	126	89-596	14532 (n=15)	92	48	31-232	5245 (n=15)
<i>Noradrenergic PHEO</i>	14	97	90	21-377	5529 (n=8)	295	196	92-871	15933 (n=8)	110	53	57-220	6258 (n=8)
<i>SDHD</i>	2	117	10	110-124	10686 (n=1)	328	108	251-404	27373 (n=1)	131	14	121-141	14471 (n=1)
<i>VHL</i>	5	60	47	15-133	3728 (n=2)	257	110	149-398	18103 (n=2)	98	35	54-136	6026 (n=2)
CLINICAL SPECIFICITY	61	MN = 93 %	NMN = 95 %	MN + NMN = 95 %				3MT = 97 %					

Assuming normal fluid intake, this method does not have to overly increase the uncertainty of the measurement with regard to the clinical interpretation of the results. The cut-off values were set so that maximum specificity is achieved, which, on the other hand, reduces the predictive value for clinical sensitivity. This is another reason why the laboratory diagnosis of PPGL patients must be augmented by measuring free metanephrines and chromogranin A in plasma.

Conclusion

We can conclude that the determination of deconjugated urine MN, NMN and 3MT using HPLC-ECD, which is relatively simple without the necessity of special laboratory material, can be an effective markers for screening of PPGLs. Urinary metanephrines exerts association to malignity and tumor mass. One limitation is that their clinical sensitivity and clinical specificity were worse than those parameters determined using radioimmunoassay or chromatographic processes in the

plasma. Urinary metanephrines are appropriate after screening PPGL patients following adrenal gland surgery, but their predictive values is not sufficient with enough certainty to draw definitive conclusions regarding clinical sensitivity and specificity. The laboratory diagnosis of PPGL patients must be augmented by measuring plasma-free MN and NMN. It is also certainly useful in this respect to augment and confirm the laboratory diagnosis *via* the values of chromogranin A determined by radioimmunoassay in plasma or serum. The advantage of the immunoassay together with HPLC-ECD approach is that these methods are common in a clinical-biochemical laboratory and do not require additional expensive instrumentation.

Conflict of Interest

There is no conflict of interest.

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