Clinica Chimica Acta 490 (2019) 46–54

Contents lists available at ScienceDirect

Clinica Chimica Acta

journal homepage: www.elsevier.com/locate/cca

Reference intervals for LC-MS/MS measurements of plasma free, urinary free and urinary acid-hydrolyzed deconjugated normetanephrine, metanephrine and methoxytyramine

Graeme Eisenhofer[a,b,*], Mirko Peitzsch[a], Denise Kaden[a], Katharina Langton[a], Anastasios Mangelis[a], Christina Pamporaki[b], Jimmy Masjkur[b], Aikaterini Geroula[b], Max Kurlbaum[c], Timo Deutschbein[c], Felix Beuschlein[d,e], Aleksander Prejbisz[f], Stefan R. Bornstein[b], Jacques W.M. Lenders[b,g]

[a] Institute of Clinical Chemistry and Laboratory Medicine, University Hospital Carl Gustav Carus, Technische Universität Dresden, Fetscherstrasse 74, 01307 Dresden, Germany
[b] Department of Medicine III, University Hospital Carl Gustav Carus, Technische Universität Dresden, Fetscherstrasse 74, 01307 Dresden, Germany
[c] Department of Internal Medicine, Division of Endocrinology, University Hospital, University of Würzburg, Würzburg, Germany.
[d] Medizinische Klinik und Poliklinik IV, Klinikum der Ludwig-Maximilians-Universität München, Munich, Germany
[e] Klinik für Endokrinologie, Diabetologie und Klinische Ernährung, Universitätsspital Zürich, Zürich, Switzerland
[f] Department of Hypertension, Institute of Cardiology, Warsaw, Poland
[g] Department of Internal Medicine, Radboud University Medical Centre, Nijmegen, The Netherlands

ARTICLE INFO

Keywords:
Liquid chromatography tandem mass spectrometry (LC-MS/MS)
Normetanephrine
Metanephrine
Methoxytyramine
Reference intervals
Age

ABSTRACT

Background: Plasma or urinary metanephrines are recommended for screening of pheochromocytomas and paragangliomas (PPGLs). Measurements of urinary free rather than deconjugated metanephrines and additional measurements of methoxytyramine represent other developments. For all measurements there is need for reference intervals.

Methods: Plasma free, urinary free and urinary deconjugated O-methylated catecholamine metabolites were measured by LC-MS/MS in specimens from 590 hypertensives and normotensives. Reference intervals were optimized using data from 2,056 patients tested for PPGLs.

Results: Multivariate analyses, correcting for age and body surface area, indicated higher plasma and urinary metanephrine in males than females and sex differences in urinary normetanephrine and free methoxytyramine that largely reflected body size variation. There were positive associations of age with plasma metabolites, but negative relationships with urinary free metanephrine and methoxytyramine. Plasma and urinary normetanephrine were higher in hypertensives than normotensives, but differences were small. Optimization of reference intervals using the data from patients tested for PPGLs indicated that age was the most important consideration for plasma normetanephrine and sex most practical for urinary metabolites.

Conclusion: This study clarifies impacts of demographic and anthropometric variables on catecholamine metabolites, verifies use of age-specific reference intervals for plasma normetanephrine and establishes sex-specific reference intervals for urinary metabolites.

1. Introduction

Current guidelines for the biochemical diagnosis of pheochromocytomas and paragangliomas (PPGLs) stipulate measurements of plasma or urinary normetanephrine and metanephrine (together termed metanephrines) as first-line screening tests [1]. At lower levels of evidence, liquid chromatographic methods are recommended over other methods of measurement and for the plasma test blood should be drawn in the supine position [1]. There has been some debate concerning the latter recommendation, this based largely on inconvenience to clinical staff and added costs of supine sampling [2–4]. Nevertheless, accumulating evidence supports both the advantages of liquid

* **Corresponding author at:** Institute for Clinical Chemistry and Laboratory Medicine, Technische Universität Dresden, Fetscherstraße 74, 01307 Dresden, Germany.
E-mail address: Graeme.Eisenhofer@uniklinikum-dresden.de (G. Eisenhofer).

https://doi.org/10.1016/j.cca.2018.12.019
Received 24 August 2018; Received in revised form 9 November 2018; Accepted 16 December 2018
Available online 17 December 2018

chromatography with tandem mass spectrometry (LC-MS/MS) over immunoassay measurements and the importance of supine sampling for optimal diagnostic accuracy of the plasma test [5–8].

Although supine blood sampling is clearly important for ensuring high diagnostic accuracy of the plasma test, many laboratories use reference intervals determined from blood samples drawn in the seated position [9–12]. Reported upper cut-offs from seated measurements of plasma normetanephrine, up to two times above those for supine sampling, are too high to reliably exclude PPGLs. There is thus need for reliable reference intervals for the plasma test determined for blood collected in the recommended supine position. Since with seated sampling the plasma test offers no diagnostic advantage over urinary fractionated metanephrines [6,13], the urine test may be preferable for those centers where sampling blood in the fully recumbent supine position is not possible [14]. For this and other reasons measurements of urinary fractionated metanephrines will likely remain important.

Metanephrines in urine are commonly measured after an acid hydrolysis step that converts the large proportion of sulfate-conjugated metabolites to free metabolites [15,16]. The sulfate-conjugates are different metabolites from the free metanephrines and are produced by a sulfotransferase enzyme located primarily in the gastrointestinal tract where most of the sulfate-conjugated metabolites are formed [17,18]. In contrast, the single largest tissue source of the free metabolites is the adrenal medulla or in patients with PPGLs, the tumor cells themselves [19,20]. This offers diagnostic advantages for measurements of free versus deconjugated metanephrines in both plasma and urine [21,22]. Sample preparation is also simpler and there are less problems with the validity of quality assurance procedures for measurements of urinary free than deconjugated metabolites [14,16,23]. Consequently some laboratories have moved to measurements of the free metabolites in urine [23–25]. This is now made relatively simple by availability of highly sensitive LC-MS/MS instruments, but is hindered by lack of established reference intervals. Apart from the need for reference intervals for urinary free metanephrines there is also increasing need for reliable reference intervals for both plasma and urinary methoxytyramine, which at least for plasma measurements shows utility for identification of dopamine-producing PPGLs [26,27].

Recognising the aforementioned needs, the present analysis outlines results for plasma free, urinary free and urinary deconjugated O-methylated catecholamine metabolites determined by LC-MS/MS in specimens collected from 590 volunteers. In addition to impact of age, gender and anthropometric variables we also assessed the importance of considering the presence or absence of hypertension. To assess potential impact of different cut-offs on diagnostic performance we examined their utility using publically available data for a population of 2,056 patients tested for PPGLs [22]. This population also served to further validate and refine reference intervals.

2. Materials and methods

2.1. Subjects

Subjects for the reference interval population included 590 volunteers, aged 18 to 82 years (median 42), including 329 females and 261 males among whom 110 (33%) of the females and 129 (49%) of the males had hypertension (Table 1). Enrolment of subjects was facilitated by advertisement with a small financial renumeration for time. All subjects underwent a standard medical history and physical examination that included recordings of prescribed and non-prescribed medications and dietary supplements, body weight, height, heart rate and office blood pressure, the latter recorded in triplicate. Presence of hypertension was established by systolic blood pressure above or equal to 140 mmHg or diastolic blood pressure above or equal to 90 mmHg and further confirmed or excluded in 437 subjects by 24 hour ambulatory blood pressure monitoring (systolic blood pressure ≥ 130 and diastolic blood pressure ≥80). Hypertension was also defined in patients with a stated history of high blood pressure controlled by antihypertensive medications. To facilitate comparisons for other laboratories the data from the reference population are made available in the supplement to this report.

Data and details about the 2056 patients tested for PPGLs under a multicenter prospective clinical protocol are provided elsewhere [22]. In brief, tumors were confirmed in 236 patients and excluded in the other 1,860 patients. All volunteers and patients provided written informed consent under protocols approved by Ethics committees at participating centers.

2.2. Blood and urine samples

All blood sampling in volunteers was carried out between 8:00-10:00 AM after an overnight fast and refraining from caffeinated beverages in the morning before sampling, which was carried out after a minimum of 20 minutes of supine rest. Heparinized blood samples were kept chilled and centrifuged within 2 hours of collection to separate plasma, which was stored at -80°C until assayed. Collections of 24-hr urine specimens were initiated after discarding the first void upon awakening in the morning and continuing until collection of the first void upon awakening the next morning. Collections were returned to the laboratory on the final day of the collection. As outlined previously [28], metanephrines in urine are relatively stable and samples were not acidified in order to avoid deconjugation at low pH. Completeness of collections was assessed by volume and 24-hr creatinine outputs, with subjects asked to perform repeat collections in cases of questionable results. Blood sampling and urine collections in patients tested for PPGLs were carried out according to similar standard-operating procedures used for volunteers.

2.3. LC-MS/MS

LC-MS/MS was performed using a QTRAP 5500 triple quadrupole mass spectrometer (AB Sciex, Darmstadt, Germany) coupled to an Acquity® ultra performance liquid chromatography system (Waters Corporation, Milford, MA, USA). The methods for analysis of plasma and urinary metabolites are detailed elsewhere [28,29], including modifications for measuring plasma methoxytyramine [30]. To facilitate comparisons of data from this report with measurements by other laboratories, data are provided from the results of an international inter-laboratory proficiency program [31]; for this comparison, measurements of those metabolites have been compared to medians of other participating laboratories (Supplemental figures 1 & 2)

2.4. Data analyses

Statistical analyses utilized the JMP statistics software package (SAS Institute Inc, Cary, NC). Differences between men and women or between hypertensives and normotensives were initially assessed using the Wilcoxon test. Associations of body surface area were assessed since larger body size in males than females (Table 1) was hypothesized to contribute to higher urinary outputs of catecholamine metabolites in males than females. Similarly, differences between normotensives and hypertensives were hypothesized to be influenced by the predominance of male sex and advanced age in hypertensives than normotensives. Therefore, impacts of sex, blood pressure status, age and body surface area on plasma and urinary catecholamine metabolites were subsequently assessed by least squares multivariate analyses.

Further analyses to validate or refine reference intervals utilized publically available data for a population of 2,056 patients tested for PPGLs [22]. Influences of sex on proportions of false-positive versus true-negative results were assessed by application of both sex-specific and non-specific upper cut-offs of 97.5 and 99.5 percentiles derived from the reference population. Impact of body size on proportions of false-positive versus true-negative results were similarly assessed using

Table 1
Demographic, anthropometric and urinary volume and creatinine data for the subjects of the reference population

	Normotensives (NT)		Hypertensives (HT)		NT vs HT
Gender	**Males**	**Females**	**Males**	**Females**	**P-value**
N	132	219	129	110	< 0.0001
Age (yr)	31	34	51	52	< 0.0001
	(18-81)	(18-71)	(18-81)	(23-82)	HT > NT
Height (m)	1.80**	1.68	1.79**	1.67	0.6116
	(1.62-1.97)	(1.50-1.84)	(1.65-2.01)	(1.49-1.80)	
Weight (kg)	81**	66	90**	70	< 0.0001
	(59-133)	(46-112)	(61-151)	(50-139)	HT > NT
BSA (m^2)	1.95**	1.69	1.98**	1.75	< 0.0001
	(1.61-2.46)	(1.34-2.25)	(1.62-2.55)	(1.36-2.40)	HT > NT
BMI (kg/m^2)	24.5*	23.3	27.6*	25.6	< 0.0001
	(18.2-42.0)	(17.0-43.6)	(19.6-50.4)	(17.6-50.4)	HT > NT
24 hr Urine Volume (L)	2.00	1.90	2.04	1.84	0.3989
	(0.54-4.05)	(0.46-4.80)	(0.80-4.68)	(0.50-3.86)	
Urine Creatinine (mmol/day)	15.6**	10.3	15.2**	9.4	0.4522
	(4.1-32.2)	(3.6-21.1)	(5.3-32.9)	(2.3-18.3)	

Data are shown as medians and ranges. * P < 0.01, **P < 0.0001 higher in males than females for either normotensive or hypertensive groups. Abbreviations: BSA, body surface area; BMI, body mass index.

height and body mass data available in 1,248 patients without PPGLs who were divided into three equally sized groups according to distributions of body surface area. Similarly, to assess need for age-specific upper cut-offs, patients in whom PPGLs were excluded were divided into three equal age-groups and differences in proportions of false-positive results examined between groups; assessments included examination of age-specific upper cut-offs according to a previously established equation [32]. The various combinations of upper cut-offs were also applied to the group of 236 patients with PPGLs to establish those that provided optimal diagnostic performance. Results of diagnostic performance according to cut-offs derived from the reference population and optimized using the patient population were compared to results using cut-offs derived from the Youden index (maximal value of sensitivity + specificity − 1) for ROC curves of each of the three metabolites in the three test panels [33].

3. Results

3.1. Sex

Plasma concentrations of normetanephrine did not differ between males and females, whereas plasma concentrations of metanephrine and methoxytyramine were respectively 29% and 14% higher (P < 0.0001) in males than females (Figure 1 A-C). Urinary outputs of free and deconjugated metabolites were all consistently higher (P < 0.0001) in males than females (Figure 1 D-I). Largest differences were observed for urinary free and deconjugated metanephrine; these were respectively 39% and 42% higher in males than females compared to 21% and 27% differences for free and deconjugated normetanephrine and 22% and 37% differences for methoxytyramine.

Higher plasma concentrations in males than females for plasma metanephrine and methoxytyramine remained significant (P < 0.0001) after multivariate correction for age, body surface area and hypertension (Table 2); however, leverage plots indicated larger differences for metanephrine with than without correction (46% versus 29%). Similarly, an influence of sex on urinary outputs of metanephrine remained significant (P < 0.0001) after multivariate analysis with leverage plots indicating 45-46% higher mean outputs of free and deconjugated metanephrine in males than females.

In contrast to metanephrine, differences between males and females for urinary outputs of free normetanephrine and methoxytyramine disappeared after multivariate analysis (Table 2), this reflecting significant (P < 0.0001) positive relationships of urinary outputs with body surface area that was larger in males than females (Table 1). Similarly, although still higher (P < 0.002) in males than females after correction for body surface area (Table 2), magnitudes of differences were diminished from 27% to 14% for urinary deconjugated normetanephrine and from 37% to 28% for methoxytyramine.

3.2. Age

Age showed a positive relationship with plasma concentrations of normetanephrine ($r_s = 0.3260$, P < 0.0001) and weaker positive relationships with plasma metanephrine ($r_s = 0.1418$, P = 0.0006) and methoxytyramine ($r_s = 0.1991$, P < 0.0001). Positive relationships of the three O-methylated metabolites with age remained significant after multivariate analysis (Table 2). There were no relationships of age with either urinary outputs of free or deconjugated normetanephrine or with deconjugated metanephrine and methoxytyramine. However, age showed significant weak negative relationships with urinary free metanephrine ($r_s = -0.2058$, P < 0.0001) and methoxytyramine ($r_s = -0.1996$, P < 0.0001). Similar results were observed after multivariate analyses (Table 2).

3.3. Hypertension

Hypertensives had 17% higher (P < 0.0001) plasma concentrations of normetanephrine and 14% and 22% higher (P < 0.0005) respective urinary outputs of free and deconjugated normetanephrine than normotensives (Figure 2 A,D,G). Among other O-methylated metabolites, hypertensives showed slightly higher plasma concentrations of plasma metanephrine (P < 0.05) and methoxytyramine (P < 0.005) than normotensives (Figure 2, B,C), but no differences for urinary free or deconjugated metanephrine and methoxytyramine (Figure 2, E,F,H,I).

With multivariate analysis the differences for plasma metanephrine and methoxytyramine between hypertensives and normotensives disappeared, whereas those for plasma as well as urinary free and deconjugated normetanephrine remained significant (Table 2). Nevertheless, leverage plots indicated that differences between hypertensives and normotensives were reduced to only 4%, 7% and 13% higher values for plasma, urinary free and urinary deconjugated normetanephrine respectively.

3.4. Impact of sex-specific reference intervals on false-positive results

As expected the 97.5 and 99.5 percentiles for plasma concentrations of free normetanephrine differed little between the two sexes (Table 3). Nevertheless, among the 1820 patients tested for PPGLs in whom

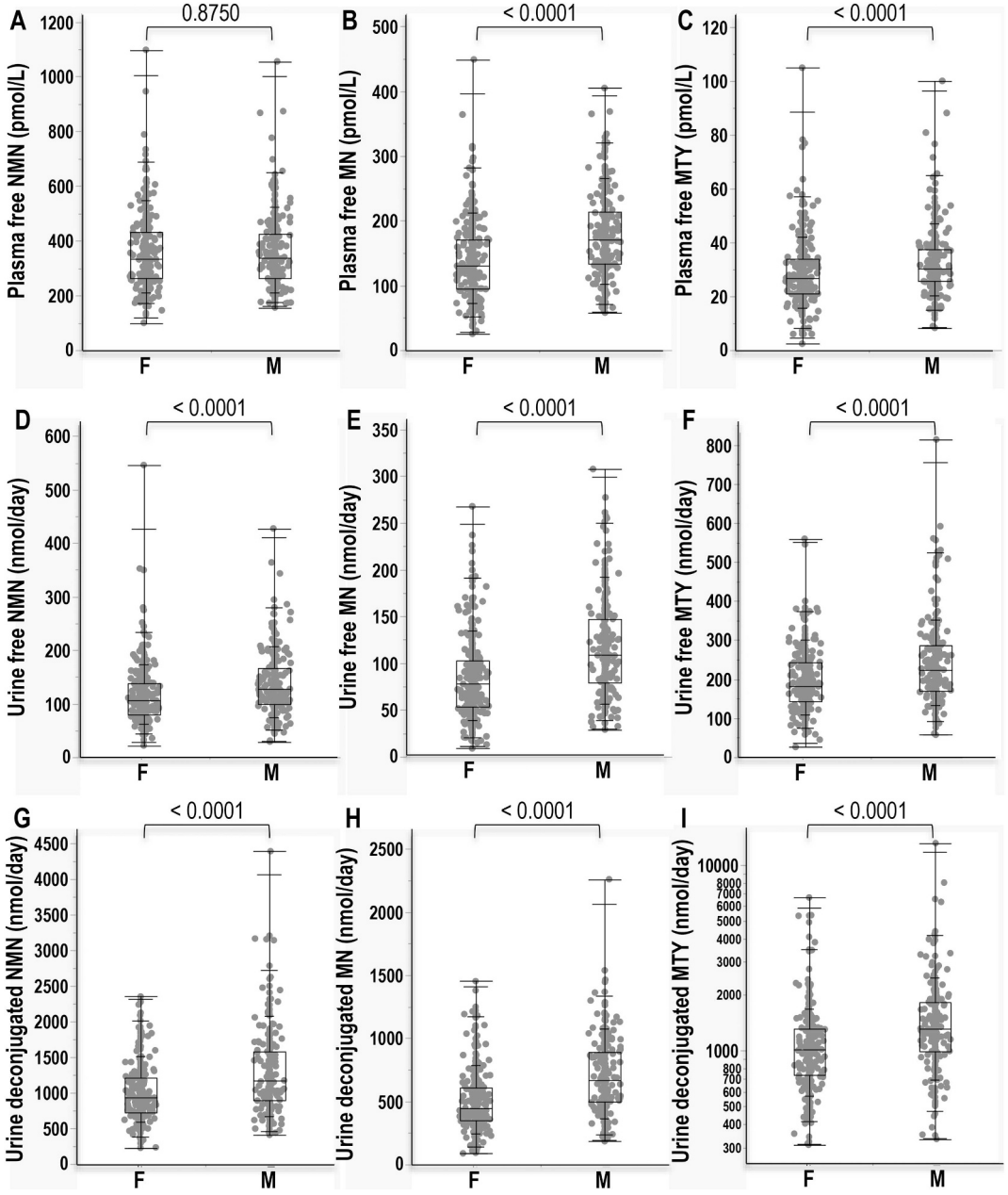

Fig. 1. Plasma free (A,B,C), urinary free (D,E,F) and urinary deconjugated (G,H,I) normetanephrine (NMN, A,D,G), metanephrine (MN, B,E,H) and methoxytyramine (MTY, C,F,I) in females (F) versus males (M) of the reference population. Data are shown as dot, box and whisker plots with whiskers showing the 90, 97.5 and 99.5 percentiles with ranges at limits.

disease was excluded, females showed a slightly higher (P = 0.0368) proportion of false-positive results than males for plasma normetanephrine at the 97.5 percentile-derived cut-offs and a nearly 2-fold higher (P = 0.0099) proportion at the 99.5 percentile-derived cut-offs (Table 4); differences lost significance with application of sex-specific 97.5 percentile-derived cut-offs but remained significant (P = 0.0103) with sex-specific 99.5 percentile-derived cut-offs.

In contrast to normetanephrine, although plasma concentrations of metanephrine and methoxytyramine were higher (P < 0.0001) in males than females (Figure 1 & Table 2), there were no significant differences in proportions of false-positive results for either metabolite between males and females with and without application of sex-specific upper cut-offs (Table 4).

Without sex-specific upper cut-offs, measurements of urinary free metabolites showed substantially higher proportions of false-positive results in males than females compared to plasma measurements (Table 4), this reflecting the large impacts of sex on urinary free metabolites (Table 3). False-positive results for all three urinary free metabolites were 3.3- to 7.0-fold higher (P < 0.03) with use of both 97.5 and 99.5 percentile-derived cut-offs in males than females. With sex-specific 97.5 percentiles there were no differences between males and females in proportions of false-positive results for urinary free metabolites; however, with application of sex-specific 99.5 percentiles there remained a slightly higher (P = 0.0220) proportion of false-positive results for urinary free normetanephrine.

There was a smaller impact of sex on proportions of false-positive results for urinary deconjugated than for the free metabolites (Table 4). Without adjustment for sex, proportions of false-positive results using the 97.5 percentiles were 1.9- to 2.3-fold higher (P < 0.0001) in males than females for urinary deconjugated normetanephrine and metanephrine and 2.1-fold higher (P = 0.0036) for methoxytyramine. After application of sex-specific upper cut-offs there were no differences in

Table 2
Multivariate analysis of influences of sex, age, body surface area (BSA) and presence of hypertension on plasma concentrations of free metabolites and urinary outputs of free and deconjugated metabolites

Amine	Sex		Age		BSA		Hypertension	
Plasma free metabolites								
Normetanephrine	0.2089		< 0.0001	+ve	0.0022	-ve	0.0174	HT > NT
Metanephrine	< 0.0001	M > F	0.0045	+ve	< 0.0001	-ve	0.4522	
Methoxytyramine	< 0.0001	M > F	< 0.0001	+ve	0.5896		0.7002	
Urinary free metabolites								
Normetanephrine	0.0971		0.7038		< 0.0001	+ve	0.0093	HT > NT
Metanephrine	< 0.0001	M > F	< 0.0001	-ve	0.1185		0.9605	
Methoxytyramine	0.1107		< 0.0001	-ve	< 0.0001	+ve	0.4681	
Urinary deconjugated metabolites								
Normetanephrine	0.0019	M > F	0.8750		0.0020	+ve	< 0.0001	HT > NT
Metanephrine	< 0.0001	M > F	0.4559		0.0270	-ve	0.5056	
Methoxytyramine	< 0.0001	M > F	0.0768		0.0195	+ve	0.9982	

Data are shown as P-values. Where significant (i.e., $P < 0.05$) differences are shown for sex as higher in females than males (F > M) or higher in males than females (M > F), for relationships with age or BSA according to the negative (-ve) or positive (+ve) nature of relationships and for any differences between normotensives (NT) and hypertensives (HT). Data were normalized by logarithmic transformation before statistical analyses.

false-positive results for metanephrine and methoxytyramine, whereas the difference for normetanephrine was reversed to a 1.6-fold higher ($P = 0.0047$) proportion of false-positives in females than males. Reversals in proportions of false-positive results between males and females with and without sex-specific cut-offs were further exaggerated with application of the 99.5 percentiles for both urinary deconjugated normetanephrine and methoxytyramine; however, for urinary deconjugated metanephrine the 2.7-fold higher ($P = 0.0017$) proportion of false-positives without sex-specific cut-offs disappeared after application of sex-specific cut-offs.

3.5. Association of body surface area with false-positive results

Larger body surface area was associated with lower ($P < 0.01$) proportions of false-positive results for plasma normetanephrine using both 97.5 and 99.5 percentile-derived upper cut-offs (Supplemental Table 1). This impact of body surface area on false-positives for plasma normetanephrine remained significant ($P < 0.05$) with use of sex-specific cut-offs and gained significance ($P = 0.0002$) for metanephrine with use of the 97.5 but not 99.5 percentile-derived cut-offs.

In contrast to plasma normetanephrine, larger body surface area was associated with increased ($P < 0.0001$) proportions of false-positive results for urinary free and deconjugated normetanephrine with use of 97.5 percentile-derived upper cut-offs (Supplemental Table 1). Increased body surface area was also associated with increased ($P < 0.005$) proportions of false-positive results for urinary free and deconjugated metanephrine and urinary free methoxytyramine. With sex-specific upper cut-offs, the association of body surface area with false-positive results for urinary metabolites was diminished, but remained significant ($P < 0.05$) for urinary free and deconjugated normetanephrine.

3.6. Age-specific reference intervals

False-positive results for plasma normetanephrine increased ($P < 0.0001$) with advancing age such that patients over 60 years of age had 3.5- and 4.3-fold higher proportions of false-positive results than patients under 45 years at respective 97.5 and 99.5 percentile-derived upper cut-offs (Supplemental Table 2). With use of age-specific cut-offs, calculated according to a previously described equation [32], the impact of advancing age on proportions of false-positive results was reversed. With these age-specific cut-offs the overall proportion of false-positive results for plasma normetanephrine was 4.7% compared to 11.0% and 2.8% for respective use of 97.5 and 99.5 percentile-derived cut-offs.

Advancing age was associated with an increased rate of false-positive results for plasma metanephrine ($P = 0.0028$) with use of 97.5 but not 99.5 percentile-derived upper cut-offs and had no impact on rates of false positives for methoxytyramine (Supplemental Table 2). Apart from a small reduction in false-positive results for urinary free normetanephrine in patients over 60 years of age, there were no other differences in false-positive results for urinary metabolites among age groups with use of either 97.5 or 99.5 percentile-derived upper cut-offs.

3.7. Reference versus patient groups

Patients tested for PPGLs in whom tumors were excluded were older ($P < 0.0001$), more often female ($P = 0.0156$), had a higher body mass index ($P < 0.0001$) and a higher prevalence of hypertension ($P < 0.0001$) than subjects of the reference group (Supplemental table 3). Accordingly they also had higher ($P < 0.05$) plasma concentrations and urinary outputs of nometanephrine than subjects of the reference group, with most pronounced differences ($P < 0.0001$) observed for plasma free and urinary deconjugated normetanephrine. Urinary excretion of deconjugated methoxytyramine was also slightly but significantly higher ($P = 0.0030$) in the patient than the reference group. Excretion of urinary free metanephrine on the other hand was higher in the reference than the patient group.

Likelihood of false-positive results according to the 97.5 percentile-derived cut-offs of the reference population and without adjustment for sex was at least 2-fold higher than expected for panels of plasma free and urinary deconjugated metabolites, but only marginally higher than expected for the urinary free metabolites (Table 4). Multivariate analyses indicated that the presence of hypertension had negligible impact on false-positive results for each of the three test panels (Supplemental figure 3). The high proportion of false-positive results for the plasma panel was also largely independent of body surface area, body mass index and gender, but was clearly impacted by age to a similar level as unidentified group differences in patient and reference populations. Variations in body surface area, age and gender contributed to the high proportion of false-positive results for urinary deconjugated metabolites, but not one of these factors contributed to the likelihood of false-positive results beyond that of unidentified group differences between patient and reference populations. In contrast, sex was the only factor identified to contribute to the relatively minimal increase in likelihood of false-positive results for urinary free metabolites in the patient compared to the reference population.

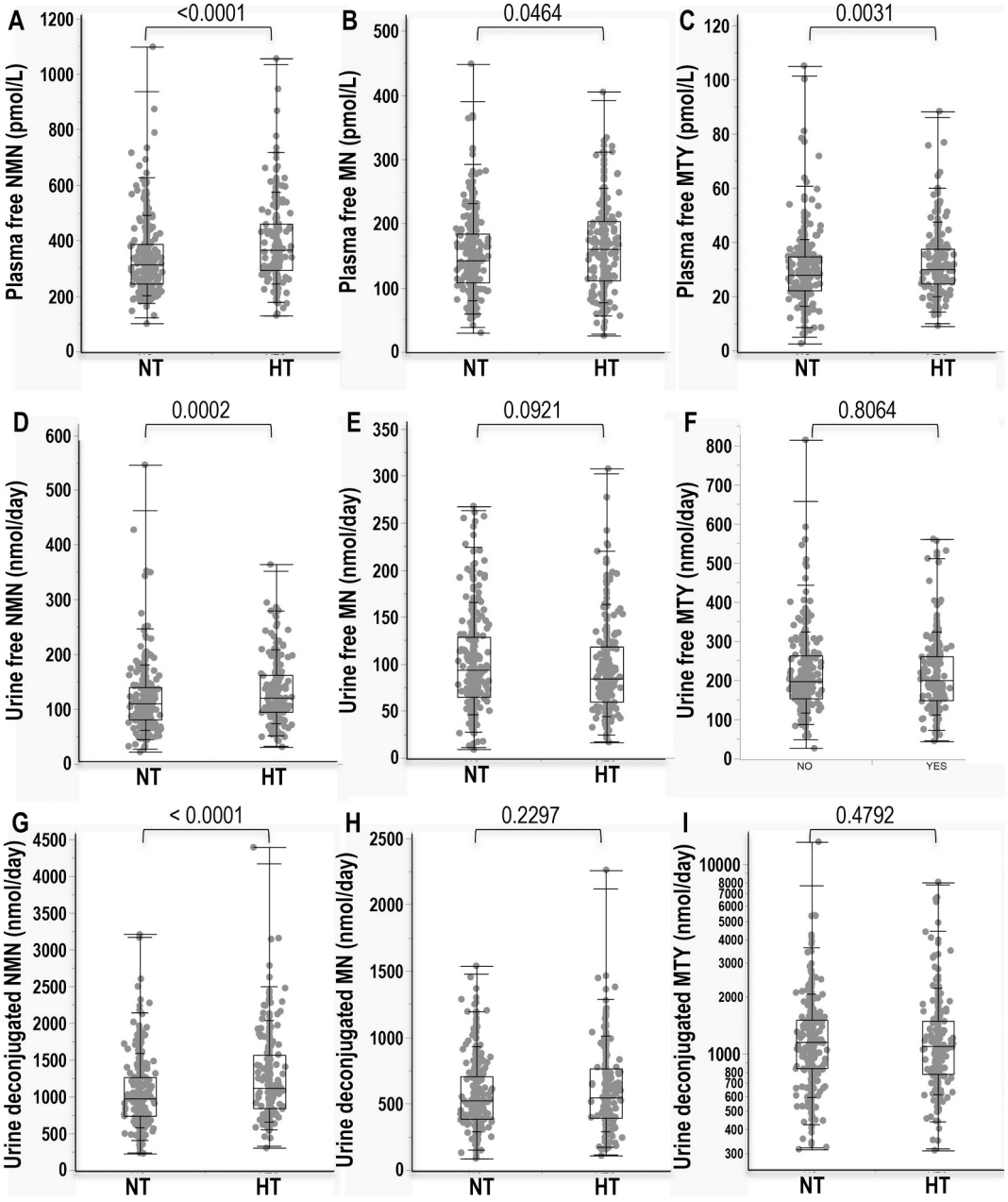

Fig. 2. Plasma free (A,B,C), urinary free (D,E,F) and urinary deconjugated (G,H,I) normetanephrine (NMN, A,D,G), metanephrine (MN, B,E,H) and methoxytyramine (MTY, C,F,I) in normotensives (NT) versus hypertensives (HT) of the reference population. Data are shown as dot, box and whisker plots with whiskers showing the 90, 97.5 and 99.5 percentiles with ranges at limits.

3.8. Optimized cut-offs

With use of 97.5 percentiles as upper cut-offs for all metabolites, the plasma test delivered high diagnostic sensitivity (99.2%), but sub-optimal specificity with a false-positive rate of over 20% (Table 5). With use of 99.5 percentile-derived upper-cut-offs, specificity was increased to 95.1% but at a loss in diagnostic sensitivity to 94.9%. Further improvement in overall diagnostic performance of the plasma test was achieved using age-specific upper cut-offs for normetanephrine and optimized cut-offs for metanephrine and methoxytyramine, this manifest mainly by increased diagnostic sensitivity (97.9%) with minimal loss of specificity (94.2%) compared to use of 99.5 percentiles.

Considerably improved diagnostic specificity of urinary tests was achieved using sex-specific 99.5 compared to the 97.5 percentiles, though this was associated with unacceptable loss in diagnostic sensitivity to below 90% (Table 5). For urinary free metabolites, acceptable diagnostic sensitivity of 93.4% at a specificity of 94.2% was achieved using sex-specific 97.5 percentiles for normetanephrine and sex-specific 99.5 percentiles for methoxytyramine and metanephrine. For urinary deconjugated metabolites diagnostic sensitivity was increased to 92.9% at a specificity of 92.1% using adjustments to sex-specific 97.5 percentiles for normetanephrine, sex-specific 99.5 percentiles for metanephrine and adjusted sex-specific 99.5 percentiles for methoxytyramine according to the data of tables 3 and 4.

Use of cut-offs derived from ROC curves for each metabolite (i.e., Youden's index), although associated with slight gains in diagnostic sensitivity, was associated with unacceptable loss of diagnostic specificity and considerably reduced overall diagnostic performance (Table 5).

Table 3
Medians and 97.5 and 99.5 percentiles for plasma concentrations and urinary outputs of O-methylated catecholamine metabolites.

	All	Males	Females
Plasma NMN (pmol/L)			
Median	337	340	334
97.5 percentiles	(174-676)	(175-674)	(173-688)
99.5 percentiles	(129-953)	(161-999)	(118-1002)
Plasma MN (pmol/L)			
Median	149	172	130
97.5 percentiles	(59-312)	(71-321)	(52-281)
99.5 percentiles	(36-371)	(59-394)	(28-396)
Plasma MTY (pmol/L)			
Median	29	30	27
97.5 percentiles	(12-60)	(15-65)	(8-57)
99.5 percentiles	(6-90)	(8-96)	(5-88)
Urinary free NMN (nmol/24hr)			
Median	114	127	106
97.5 percentiles	(49-268)	(52-280)	(45-242)
99.5 percentiles	(31-370)	(30-410)	(28-424)
Urinary free MN (nmol/24hr)			
Median	89	109	78
97.5 percentiles	(28-220)	(39-249)	(21-191)
99.5 percentiles	(15-269)	(30-299)	(11-248)
Urinary free MTY (nmol/24hr)			
Median	197	224	181
97.5 percentiles	(82-475)	(93-523)	(75-374)
99.5 percentiles	(56-563)	(58-751)	(38-550)
Urinary deconjugated NMN (nmol/24hr)			
Median	1031	1157	929
97.5 percentiles	(448-2313)	(466-2708)	(380-2004)
99.5 percentiles	(285-3164)	(412-4041)	(230-2311)
Urinary deconjugated MN (nmol/24hr)			
Median	531	669	447
97.5 percentiles	(172-1217)	(241-1333)	(140-1175)
99.5 percentiles	(91-1471)	(191-2051)	(90-1408)
Urinary deconjugated MTY (nmol/24hr)			
Median	1125	1316	1004
97.5 percentiles	(441-3946)	(477-4179)	(418-3487)
99.5 percentiles	(322-6793)	(338-11600)	(313-5853)

Abbreviations: NMN, normetanephrine; MN, metanephrine; MTY, methoxytyramine

4. Discussion

Apart from confirming the already established importance of sex-specific reference intervals for urinary metanephrines and age-specific reference intervals for plasma normetanephrine, the present study provides new data for LC-MS/MS measurements of urinary free metabolites and clarifies impacts of body size and blood pressure status on all measured variables in relation to sex and age. Presumably reflecting population differences it is further illustrated that care should be exercised in application of 97.5 percentiles in reference populations as diagnostic cut-offs in patient populations. During testing for PPGLs, such differences between reference and patient populations are commonly ascribed to presence of hypertension in the latter population. However, as shown here presence of hypertension has relatively minimal impacts on plasma and urinary metabolites and likelihood of false-positive results.

As expected, false-positive results for panels of tests increased according to numbers of tests in each panel. However, use of 97.5 percentile-derived cut-offs resulted in more than 2-fold higher proportions of false-positive results for plasma free and urinary deconjugated metabolites than expected if reference and patient populations had been matched. In contrast, proportions of false-positive results for the panel of urinary free metabolites were close to the maximum expected with matched populations (i.e., 7.5%), supporting use of reference population-derived cut-offs for this panel. Age was the most important single factor identified contributing to the higher than expected proportion of false-positive results for plasma metabolites, with additional factors identified to contribute to higher than expected proportions of false-positive results for urinary deconjugated metabolites. Nevertheless, other differences in patient and reference populations clearly contributed to the higher than expected proportions of false-positive results in patient populations tested for PPGLs. Acute anxiety, panic attacks and comorbidities in tested patient populations such as heart failure, chronic kidney disease and obstructive sleep apnea are all known to activate sympathetic nervous or adrenal medullary systems [34–39] and thereby may contribute to the relatively high prevalence of false-positive results in patient populations. Medications that alter the disposition of catecholamines are other factors that can contribute to false-positive results for LC-MS/MS based measurements [14]. However, patients on such medications were excluded from the patient study population.

With the above considerations in mind it becomes apparent that use of reference-population derived 97.5 percentiles do not always provide an optimal method for selecting upper cut-offs. The Youden index provides an alternative method [33], but as illustrated here a major limitation to this approach is that such cut-offs are inappropriate for tests involving panels of multiple analytes some of which carry more power for disease discrimination than others. As a result, derived cut-offs for some analytes, although optimal when used alone, are not optimal when used in combination. The result, as shown here with use of Youden index-derived cut-offs, is unacceptably low diagnostic specificity.

Until new mathematical algorithms are established for optimizing cut-offs of multiple analytes in test panels it seems that the most appropriate approach for reference interval optimization is to place most weight in the analyte of a panel that provides the highest diagnostic performance when considered alone. For the O-methylated catecholamine metabolites, normetanephrine provides the single most important analyte for discrimination of patients with and without PPGLs. Thus, use of sex-specific 97.5 percentiles for normetanephrine appeared most appropriate for urinary panels, with a further adjustment for urinary deconjugated normetanephrine to correct a reversal in false-positive results. Accompanying use of the 99.5 percentiles for metanephrine and methoxytyramine improved diagnostic specificity with minimal loss in sensitivity and overall improved diagnostic performance. For plasma metabolites optimal performance was obtained using age-specific reference intervals for normetanephrine as established previously [32]; for metanephrine and methoxytyramine single cut-offs for males and females that were slightly above 99.5 percentiles contributed to optimized performance. Together the above considerations support use of the cut-offs outlined here and applied in a recent study [22] as well as part of our routine diagnostic service.

Whether the aforementioned age- and gender-specific cut-offs may be applicable to other laboratories or whether further adjustments are required depends on any bias of the currently described methods compared to others. Today such bias can be assessed by participation in inter-laboratory quality assurance programs [31], facilitated in the present report by provision of results from one program (Supplemental figures 1 & 2). Participation in such programs thereby provides a vehicle for harmonization of reference intervals [40], which can further benefit from availability and common use of certified reference material.

Independent of whether the optimized cut-offs outlined here are applicable for other laboratories, further validation is recommended. Nevertheless, the present data do provide useful background information for any laboratories seeking to establish or update their LC-MS/MS measurements of plasma or urinary free metanephrines, the methods of choice for diagnosis of PPGLs [22]. Age-specific reference intervals for plasma normetanephrine are particularly useful for optimized diagnostic performance of the plasma test. Although sex is one of the more

Table 4
Percent false-positive test results according to use of the 97.5 and 99.5 percentiles with and without adjustment for sex in patients tested for PPGLs

	Without adjustment for sex			With adjustment for sex		
	F	M	F&M	F	M	F&M
Plasma free metabolites (n = 1820)						
NMN (97.5)	12.7*	9.5	11.1	11.8	9.5	10.7
MN (97.5)	5.2	6.7	6.0	7.6	5.8	6.6
MTY (97.5)	7.2	9.8	8.5	8.4	7.9	8.1
ALL (97.5)	19.9	21.5	20.8	22.4	19.4	20.9
NMN (99.5)	3.7*	1.8	2.5	3.4*	1.5	2.5
MN (99.5)	2.3	2.6	2.5	1.5	1.9	1.7
MTY (99.5)	1.3	2.0	1.7	1.3	1.4	1.4
ALL (99.5)	6.6	5.8	6.0	5.7	4.2	4.9
Urine free metabolites (n = 1756)						
NMN (97.5)	2.3	7.1***	4.7	4.1	6.0	5.1
MN (97.5)	0.7	4.4***	2.5	1.8	2.4	2.1
MTY (97.5)	0.7	3.3***	2.0	3.1	1.7	2.4
ALL (97.5)	3.5	11.8***	7.7	8.0	8.2	8.1
NMN (99.5)	0.3	1.9**	1.1	0.2	1.3*	0.7
MN (99.5)	0.3	1.7**	1.1	0.5	1.1	0.8
MTY (99.5)	0.2	1.3*	0.7	0.3	0.6	0.5
ALL (99.5)	0.9	3.7**	2.2	1.0	2.4*	1.7
Urine deconjugated metabolites (n=1757)						
NMN (97.5)	6.3	11.7***	9.0	10.4**	6.6	8.5
MN (97.5)	3.3	7.5***	5.4	3.6	5.0	4.3
MTY (97.5)	2.4	5.1**	3.7	3.2	4.8	4.0
ALL (97.5)	10.2	20.3***	15.3	15.1	14.1	14.6
NMN (99.5)	2.4	1.8	2.1	6.3***	0.9	3.5
MN (99.5)	1.3	3.5**	2.4	1.7	0.9	1.3
MTY (99.5)	0.5	0.9	0.7	0.8*	0.1	0.5
ALL (99.5)	3.8	5.7	4.7	7.9***	1.6	4.7

* $P < 0.05$; ** $P < 0.005$; *** $P < 0.0001$ higher than other gender. Abbreviations: F, females; M, males; NMN, normetanephrine; MN, metanephrine; MTY. methoxytyramine.

important variables to consider for measurements of urinary metabolites, the present data indicate that at least for urinary free normetanephrine and methoxytyramine the higher outputs in males than females appear to largely reflect differences in body size. Higher urinary outputs of deconjugated normetanephrine and methoxytyramine in males than females were also impacted by body size, but for these metabolites gender appears to have an independent influence. In contrast, consistently higher urinary outputs of both urinary free and deconjugated metanephrine, as well as plasma metanephrine in males than females appears to be completely independent of body size. These sex-dependent differences likely reflect the negative relationships of epinephrine and metanephrine with both body fat and body mass index and the higher body fat content of females than males [41].

From the above considerations it might be concluded that at least for urinary free normetanephrine and methoxytyramine, reference intervals should be tailored to body size. To make this possible, however, height and body weight information from patients would need to be recorded and ideally made available with laboratory requests to generate personalized reference intervals. Since the impact of body size on false-positive urinary test results is largely negated using sex-specific reference intervals, such reference intervals provide a more practical and unified approach to correct for both body size and sex. Nevertheless, body size likely remains an important variable to consider for interpreting urinary results in specific patients who are particularly large or small for their sex. Normalization to body size may be particularly useful for children.

In summary, this study provides information for implementation of sex- and age-specific reference intervals for plasma and urinary O-methylated metabolites particularly important for laboratories modernizing to LC-MS/MS measurements that for urinary tests should now include a shift from deconjugated to the free metabolites [22]. The data are additionally useful for clarifying underlying factors than can impact likelihood of false-positive results.

Table 5
Diagnosis test performance according to different selections of upper cut-offs

Cut-off selection	Plasma free	Urinary free	Urinary deconjugated
1. 97.5 percentile - sex specific			
NMN UC	M, 674; F, 688	M, 280; F, 242	M, 2708; F, 2004
MN UC	M, 321; F, 281	M, 249; F, 191	M, 1333; F, 1175
MTY UC	M, 65; F, 57	M, 523; F, 374	M, 4179; F, 3487
Sensitivity	99.2%	94.2%	93.8%
Specificity	79.1%	92.3%	85.4%
2. 99.5 percentile - sex specific			
NMN UC	M, 999; F, 1002	M, 410 F, 424	M, 4041; F, 2311
MN UC	M, 394; F, 396	M, 299; F, 248	M, 2051; F, 1408
MTY UC	M, 96; F, 88	M, 751; F, 550	M, 11600; F, 5853
Sensitivity	94.9%	88.9%	89.4%
Specificity	95.1%	98.3%	95.3%
3. Age-specific plasma NMN*, optimized 97.5 percentiles for urinary NMN & optimized MN & MTY			
NMN UC	542-1092*	M, 280; F, 242	M, 2708; F, 2313
MN UC	446	M, 299; F, 248	M, 2051; F, 1408
MTY UC	107	M, 751; F, 550	M, 6793; F, 5853
Sensitivity	97.9%	93.4%	92.9%
Specificity	94.2%	94.2%	92.1%
4. Youden's-derived cut-offs - sex specific			
NMN UC	M, 761; F, 873	M, 346; F, 259	M, 2768; F, 2776
MN UC	M, 349; F, 301	M, 264; F, 200	M, 1872; F, 997
MTY UC	M, 47; F, 42	M, 318; F, 235	M, 2632; F, 1370
Sensitivity	98.7%	93.8%	95.1%
Specificity	74.0%	77.1%	75.0%

Upper cut-offs (UC) for normetanephrine (NMN), metanephrine (MN) and methoxytyramine (MTY) are shown in units of pmol/L for plasma free metabolites and nmol/24 hr for urinary metabolites. Where specified sex-specific cut-offs are shown separately for males (M) and females (F). *Diagnostic performance of the plasma test using age-specific upper cut-offs for NMN (UC_{NMN}) was calculated according to the equation ($UC_{NMN} = 2.07x10^{-6} . age^3 + 0.545$) from a minimum of 542 pmol/L at age 5 to a maximum of 1092 pmol/L at age 65 and beyond.

Acknowledgements

This study was supported by grants from the Deutsche Forschungsgemeinschaft (EI855/1/2, KFO252 and CRC/Transregio 205/1) and the European Union Horizon 2020 Program (ENS@T-HT - #633983-2).

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.cca.2018.12.019.

References

[1] J.W.M. Lenders, Q.Y. Duh, G. Eisenhofer, A.P. Gimenez-Roqueplo, S.K. Grebe, M.H. Murad, M. Naruse, K. Pacak, W.F. Young, Pheochromocytoma and paraganglioma: An Endocrine Society Clinical Practice Guideline, J. Clin. Endocrinol. Metab. 99 (2014) 1915–1942.

[2] V. Chortis, I. Bancos, R.K. Crowley, W. Arlt, Supine or sitting? Economic considerations regarding patient position during plasma metanephrine analysis for the exclusion of chromaffin tumours, Clin. Endocrinol. (Oxf) 82 (2015) 462–463.

[3] G. Eisenhofer, R. Darr, C. Pamporaki, M. Peitzsch, S. Bornstein, J.W. Lenders, Supine or Sitting? Economic and other considerations for use of plasma metanephrines for diagnosis of phaeochromocytoma, Clin. Endocrinol. (Oxf) 82 (2015) 463–464.

[4] C. Boot, B. Toole, S.J. Johnson, S. Ball, D. Neely, Single-centre study of the diagnostic performance of plasma metanephrines with seated sampling for the diagnosis of phaeochromocytoma/paraganglioma, Ann. Clin Biochem. 54 (2017) 143–148.

[5] D. Weismann, M. Peitzsch, A. Raida, A. Prejbisz, M. Gosk, A. Riester, H.S. Willenberg, R. Klemm, G. Manz, T. Deutschbein, M. Kroiss, R. Darr, M. Bidlingmaier, A. Januszewicz, G. Eisenhofer, M. Fassnacht, Measurements of plasma metanephrines by immunoassay vs liquid chromatography with tandem mass spectrometry for diagnosis of pheochromocytoma, Eur. J. Endocrinol. 172 (2015) 251–260.

[6] R. Darr, C. Pamporaki, M. Peitzsch, K. Miehle, A. Prejbisz, M. Peczkowska, D. Weismann, F. Beuschlein, R. Sinnott, S.R. Bornstein, H.P. Neumann, A. Januszewicz, J. Lenders, G. Eisenhofer, Biochemical diagnosis of phaeochromocytoma using plasma-free normetanephrine, metanephrine and methoxytyramine: importance of supine sampling under fasting conditions, Clin. Endocrinol. (Oxf) 80 (2014) 478–486.

[7] R. Casey, T.P. Griffin, D. Wall, M.C. Dennedy, M. Bell, P.M. O'Shea, Screening for phaeochromocytoma and paraganglioma: impact of using supine reference intervals for plasma metanephrines with samples collected from fasted/seated patients, Ann Clin Biochem 54 (1) (2017) 170–173.

[8] R. Darr, M. Kuhn, C. Bode, S.R. Bornstein, K. Pacak, J.W.M. Lenders, G. Eisenhofer, Accuracy of recommended sampling and assay methods for the determination of plasma-free and urinary fractionated metanephrines in the diagnosis of pheochromocytoma and paraganglioma: a systematic review, Endocrine 56 (2017) 495–503.

[9] S.A. Lagerstedt, D.J. O'Kane, R.J. Singh, Measurement of plasma free metanephrine and normetanephrine by liquid chromatography-tandem mass spectrometry for diagnosis of pheochromocytoma, Clin. Chem. 50 (2004) 603–611.

[10] W.H. de Jong, K.S. Graham, J.C. van der Molen, T.P. Links, M.R. Morris, H.A. Ross, E.G. de Vries, I.P. Kema, Plasma free metanephrine measurement using automated online solid-phase extraction HPLC tandem mass spectrometry, Clin. Chem. 53 (2007) 1684–1693.

[11] B.J. Petteys, K.S. Graham, M.L. Parnas, C. Holt, E.L. Frank, Performance characteristics of an LC-MS/MS method for the determination of plasma metanephrines, Clin. Chim. Acta. 413 (2012) 1459–1465.

[12] R.T. Peaston, K.S. Graham, E. Chambers, J.C. van der Molen, S. Ball, Performance of plasma free metanephrines measured by liquid chromatography-tandem mass spectrometry in the diagnosis of pheochromocytoma, Clin. Chim. Acta. 411 (2010) 546–552.

[13] H.J. Kim, J.I. Lee, Y.Y. Cho, S.Y. Lee, J.H. Kim, B.C. Jung, S.W. Kim, J.H. Chung, Y.K. Min, M.S. Lee, M.K. Lee, J.H. Kim, Diagnostic accuracy of plasma free metanephrines in a seated position compared with 24-hour urinary metanephrines in the investigation of pheochromocytoma, Endocr. J. 62 (2015) 243–250.

[14] G. Eisenhofer, M. Peitzsch, Laboratory evaluation of pheochromocytoma and paraganglioma, Clin. Chem. 60 (2014) 1486–1499.

[15] G. Eisenhofer, Free or total metanephrines for diagnosis of pheochromocytoma: what is the difference? Clin. Chem. 47 (2001) 988–989.

[16] J. Simonin, S. Gerber-Lemaire, C. Centeno, C. Seghezzi, K. Iglesias, K. Abid, E. Grouzmann, Synthetic calibrators for the analysis of total metanephrines in urine: revisiting the conditions of hydrolysis, Clin. Chim. Acta 413 (2012) 998–1003.

[17] G. Eisenhofer, A. Aneman, D. Hooper, B. Rundqvist, P. Friberg, Mesenteric organ production, hepatic metabolism, and renal elimination of norepinephrine and its metabolites in humans, J. Neurochem. 66 (1996) 1565-73.

[18] G. Eisenhofer, M.W. Coughtrie, D.S. Goldstein, Dopamine sulphate: an enigma resolved, Clin. Exp. Pharmacol. Physiol. Suppl 26 (1999) S41–S53.

[19] G. Eisenhofer, B. Rundquist, A. Aneman, P. Friberg, N. Dakak, I.J. Kopin, M.C. Jacobs, J.W. Lenders, Regional release and removal of catecholamines and extraneuronal metabolism to metanephrines, J. Clin. Endocrinol. Metab. 80 (1995) 3009–3017.

[20] G. Eisenhofer, H. Keiser, P. Friberg, E. Mezey, T.T. Huynh, B. Hiremagalur, T. Ellingson, S. Duddempudi, A. Eijsbouts, J.W. Lenders, Plasma metanephrines are markers of pheochromocytoma produced by catechol-O-methyltransferase within tumors, J. Clin. Endocrinol. Metab. 83 (1998) 2175–2185.

[21] C. Pamporaki, R. Darr, M. Bursztyn, S. Glockner, S.R. Bornstein, J.W. Lenders, K. Pacak, A. Krinner, G. Eisenhofer, Plasma-free vs deconjugated metanephrines for diagnosis of phaeochromocytoma, Clin. Endocrinol. (Oxf) 79 (2013) 476–483.

[22] G. Eisenhofer, A. Prejbisz, M. Peitzsch, C. Pamporaki, J. Masjkur, N. Rogowski-Lehmann, K. Langton, E. Tsourdi, M. Peczkowska, S. Fliedner, T. Deutschbein, F. Megerle, H. Timmers, R. Sinnott, F. Beuschlein, M. Fassnacht, A. Januszewicz, J.W.M. Lenders, Biochemical Diagnosis of Chromaffin Cell Tumors in Patients at High and Low Risk of Disease: Plasma versus Urinary Free or Deconjugated O-Methylated Catecholamine Metabolites, Clin. Chem. 64 (2018) 1646–1656.

[23] A.S. Davison, Urinary free metanephrines and suitability of available quality control material, Clin. Chim. Acta. 424 (2013) 83–84.

[24] J.G. Boyle, D.F. Davidson, C.G. Perry, J.M. Connell, Comparison of diagnostic accuracy of urinary free metanephrines VMA, and catecholamines and plasma catecholamines for diagnosis of pheochromocytoma, J. Clin. Endocrinol. Metab. 92 (2007) 4602–4608.

[25] M.J. Whiting, Simultaneous measurement of urinary metanephrines and catecholamines by liquid chromatography with tandem mass spectrometric detection, Ann Clin Biochem 46 (Pt 2) (2009) 129–136.

[26] G. Eisenhofer, J.W. Lenders, G. Siegert, S.R. Bornstein, P. Friberg, D. Milosevic, M. Mannelli, W.M. Linehan, K. Adams, H. Timmers, K. Pacak, Plasma methoxytyramine: A novel biomarker of metastatic pheochromocytoma and paraganglioma in relation to established risk factors of tumour size, location and SDHB mutation status, Eur. J. Cancer 48 (2012) 1739–1749.

[27] N. van Duinen, E.P. Corssmit, W.H. de Jong, D. Brookman, I.P. Kema, J.A. Romijn, 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, Eur. J. Endocrinol. 169 (2013) 377–382.

[28] M. Peitzsch, D. Pelzel, S. Glockner, A. Prejbisz, M. Fassnacht, F. Beuschlein, A. Januszewicz, G. Siegert, G. Eisenhofer, Simultaneous liquid chromatography tandem mass spectrometric determination of urinary free metanephrines and catecholamines, with comparisons of free and deconjugated metabolites, Clin. Chim. Acta. 418 (2013) 50–58.

[29] M. Peitzsch, A. Prejbisz, M. Kroiss, F. Beuschlein, W. Arlt, A. Januszewicz, G. Siegert, G. Eisenhofer, Analysis of plasma 3-methoxytyramine, normetanephrine and metanephrine by ultraperformance liquid chromatography-tandem mass spectrometry: utility for diagnosis of dopamine-producing metastatic phaeochromocytoma, Ann Clin Biochem 50 (Pt 2) (2013) 147–155.

[30] M. Peitzsch, J.E. Adaway, G. Eisenhofer, Interference from 3-O-methyldopa with Ultra-High Performance LC-MS/MS Measurements of Plasma Metanephrines: Chromatographic Separation Remains Important, Clin. Chem. 6 (2015) 993–996.

[31] D. Pillai, S. Callen, Pilot quality assurance programme for plasma metanephrines, Ann. Clin. Biochem. 47 (Pt 2) (2010) 137–142.

[32] G. Eisenhofer, P. Lattke, M. Herberg, G. Siegert, N. Qin, R. Darr, J. Hoyer, A. Villringer, A. Prejbisz, A. Januszewicz, A. Remaley, V. Martucci, K. Pacak, H.A. Ross, F.C. Sweep, J.W. Lenders, Reference intervals for plasma free metanephrines with an age adjustment for normetanephrine for optimized laboratory testing of phaeochromocytoma, Ann. Clin. Biochem. 50 (2013) 62–69 Pt 1.

[33] R. Fluss, D. Faraggi, B. Reiser, Estimation of the Youden Index and its associated cutoff point, Biom. J. 47 (4) (2005) 458–472.

[34] E.C. Villacres, M. Hollifield, W.J. Katon, C.W. Wilkinson, R.C. Veith, Sympathetic nervous system activity in panic disorder, Psychiatry Res. 21 (1987) 313–321.

[35] B. Rundqvist, M. Elam, Y. Bergmann-Sverrisdottir, G. Eisenhofer, P. Friberg, Increased cardiac adrenergic drive precedes generalized sympathetic activation in human heart failure, Circulation 95 (1997) 169–175.

[36] M.P. Schlaich, F. Socratous, S. Hennebry, N. Eikelis, E.A. Lambert, N. Straznicky, M.D. Esler, G.W. Lambert, Sympathetic activation in chronic renal failure, J. Am. Soc. Nephrol. 20 (2009) 933–939.

[37] S.W. Holwerda, R.E. Luehrs, A.L. Gremaud, N.A. Wooldridge, A.K. Stroud, J.G. Fiedorowicz, F.M. Abboud, G.L. Pierce, Relative burst amplitude of muscle sympathetic nerve activity is an indicator of altered sympathetic outflow in chronic anxiety, J. Neurophysiol. 120 (2018) 11–22.

[38] Y. Sharabi, D.S. Goldstein, O. Bentho, A. Saleem, S. Pechnik, M.F. Geraci, C. Holmes, K. Pacak, G. Eisenhofer, Sympathoadrenal function in patients with paroxysmal hypertension: pseudopheochromocytoma, J. Hypertens. 25 (2007) 2286–2295.

[39] V.K. Somers, M.E. Dyken, M.P. Clary, F.M. Abboud, Sympathetic neural mechanisms in obstructive sleep apnea, J. Clin. Invest. 96 (1995) 1897–1904.

[40] G. Koerbin, K. Sikaris, G.R.D. Jones, R. Flatman, J.R. Tate, A.H.C.f.C.R. Intervals, An update report on the harmonization of adult reference intervals in Australasia, Clin. Chem. Lab. Med. "in press" PMID: 29305566 (2018).

[41] M. Reimann, N. Qin, M. Gruber, S.R. Bornstein, C. Kirschbaum, T. Ziemssen, G. Eisenhofer, Adrenal medullary dysfunction as a feature of obesity, Int. J. Obes. (Lond) 41 (2017) 714–721.