Catecholamine metabolites in neuroblastoma patients

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CHAPTER 4

Plasma Free Metanephrines for diagnosis of neuroblastoma patients

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Abstract

Introduction: A substantial number of patients with neuroblastoma (NB) have increased excretion of catecholamines and metanephrines. Here, we have investigated the diagnostic role of plasma free metanephrines (PFM), metanephrine (MN), normetanephrine (NMN) and 3-methoxytyramine (3MT) for NB, the most common extra-cranial solid tumour in children.

Methods: PFM were quantified by using a commercial IVD-CE LC-MS/MS method on a TSQ Quantiva coupled to an Ultimate 3000. The method was further validated on 103 samples from paediatric subjects (54 patients with histologically confirmed NB and 49 age and sex matched controls). Correlations between PFM concentrations with clinical factors were tested. We directly compared MN, NMN, and 3MT concentrations in matched plasma and urine samples of NB patients (n=29).

Results: 3MT and NMN showed an excellent diagnostic performance with very high specificity (100% and 95.8%, respectively) and sensitivity (88.2% and 80.4%). ROC curves were obtained (AUC of 0.93 and 0.91 for 3MT and NMN, respectively) and optimal cut-offs that could discriminate between controls and NB patients were defined. A positive correlation between NMN levels in urine and plasma ($p = 0.0017$) was found.

Discussion: The determination of plasma 3MT and NMN should be taken in consideration as a new diagnostic tool for NB. Validation in prospective clinical studies in comparison to urinary catecholamines and metanephrines is warranted.

Introduction

Plasma metanephrines, which include metanephrine (MN), normetanephrine (NMN) and 3-methoxytyramine (3MT), are methylated derivatives of epinephrine (E), norepinephrine (NE) and dopamine (DA), respectively (1). Plasma metanephrines can be found in the circulation as plasma free metanephrines (PFM) or as sulfo-conjugates (2). It has been demonstrated that PFM are the most sensitive and specific biomarker for the diagnosis of pheochromocytoma (3) and their levels are not affected by renal failure (4) or by diet rich in sulphated metanephrines (5). Their quantification is challenging because of their polar nature, their low molecular weight and their very low physiological concentration in human plasma (in the order of ng/L), which requires high-end high performance liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) instruments for their detection (6).

Neuroblastoma (NB), the most common paediatric extra cranial solid tumour, arises from neural crest cells of the developing sympathetic nervous system (7). Because of their neuronal origin, patients with NB often (> 90%) showed increased excretion of catecholamine metabolites, which are commonly measured in urine as part of the diagnostic workup (8-10). However, the combination of most commonly applied metabolites, homovanillic acid (HVA) and vanillylmandelic acid (VMA), has a suboptimal diagnostic sensitivity (81.9–85%) (10-12). It has been shown that urinary normetanephrine (NMN) has a higher diagnostic sensitivity (89%) and in combination with 3MT it could be further improved to 92% (10). Furthermore, elevation of urinary 3MT was associated with poor clinical outcome independently of other known risk factors such as stage and *MYCN* amplification (13). Although analysis of PFM is the golden standard for diagnosing pheochromocytoma (3), the potential use of PFM for diagnosis of NB has only been investigated once in a small cohort (n = 10 patients) with promising results (14). This might partly be due to lack of the proper technology to measure PFM. However, with the rise of technological advances such as LC-MS/MS, exploration of PFM as potential diagnostic biomarkers became feasible (6). Taken together, there is a necessity of re-evaluation of PFM in neuroblastoma diagnostics.

In this work, we investigated the diagnostic role of PFM in patients with NB at the onset of disease and compared the PFM to urinary HVA and VMA. Possible correlations between PFM and clinical factors were assessed. In addition, we directly compared NMN and 3MT concentrations in plasma with those in its corresponding urine sample in a subset of samples.

Patients and methods

Patients and samples

The study cohort included 103 paediatric subjects of whom 54 with histologically confirmed NB patients and 49 age and sex matched controls. All NB patients were diagnosed between 2013 and 2016 in one of the centres belonging to the Associazione Italiana di Emato Oncologia Pediatrica. NB patients were staged according to the International Neuroblastoma Staging System (9) and allocated to the different risk groups according to the International Neuroblastoma Risk Group (INRG) (15). **Table 1** summarises the clinical demographics of the NB cohort. For all study subjects, peripheral blood was obtained from sodium-heparin collecting vials, centrifuged and plasma was immediately stored at −80 °C until analysed. Urine samples were obtained from 29 NB patients, acidified and stored at −80 °C until analysed. The 49 samples used as controls were obtained from leftover plasma after routine clinical analyses from outpatients. A written consent allowing the collection of samples and the use of clinical and non-genetic data for clinical research was signed by patient's guardians.

		(n = 54)	
Age	Median (range) in days	706	(1-1990)
	≤12 months	23	(43%)
	>12 months	31	(57%)
Gender	Male	33	(61%)
	Female	21	(39%)
Stage	1	4	(7%)
	3	16	(30%)
	4	24	(44%)
	4s	10	(19%)
Risk group	Low risk group	22	(41%)
	Medium risk group	6	(11%)
	High risk group	26	(48%)
MYCN	Single copy	38	(70%)
	Amplified	15	(28%)
	Unknown	1	(2%)

Table 1 - Clinical description of the NB patients.

Determination of PFM by LC-MS/MS

The analyses of PFM were performed at the Central Laboratory of Analyses of Giannina Gaslini Institute (Genoa, Italy). PFM were quantified by LC-MS/MS by applying a complete

kit (Clinmass ®, Recipe, Munich, Germany) to a TSQ Quantiva coupled to UHPLC Ultimate 3000 LC-MS/MS system (Thermofisher Scientific, Milan, Italy). The kit is based on solid-phase extraction (SPE), deuterated internal standards (NMN-D_3, MN-D_3 and 3MT-D_4), 5-level calibrators and 3 controls, analytical column and mobile phases. A 500 µL plasma aliquot was subjected to SPE following the manufacturer's instruction prior to injection into the LC-MS/MS system.

Gradient separation chromatography was carried out using the analytical column and the mobile phases (A and B) following the manufacturer's instructions. The percentage of solvent B started at 95% for 0.1 min, programmed to reach 50% in 2 min and kept for 0.75 min at flow rate of 800 µL/min, then the column was reconditioned at 95% B for 1.75 min for a total run time of 4.5 min. The column temperature was maintained at 25 °C and injection volume was 10 µL. Ionization was achieved using heated electrospray ionization source (HESI) in the positive ion mode with spray voltage set at 3500 V. Nitrogen was used as the nebulizer and auxiliary gas, set at 60 and 20 arbitrary units, respectively. Vaporizer and ion transfer tube temperature settings were 400 °C and 350 °C. For collision-induced dissociation, high purity argon was used at a pressure of 1.5 mTorr. Analytes were detected using selective reaction monitoring (SRM) of the specific transitions (for NMN: 166.1→133.9 m/z, for MN: 180.1→120.1, 148.0 m/z and for 3MT: 151.1→90.9, 118.9 m/z) and their deuterated internal standards (NMN-D_3: 169.062→137.0 m/z, MN-D_3: 183.1→123.0 m/z and 3MT-D_4: 155.1→95.0, 122.9 m/z) respectively, with a dwell time of 80 ms. In source conversion/fragmentation of the different metanephrines into each other has been verified by performing product ion scans during MN, NMN and 3MT infusion to demonstrate that the fragments used for quantitation are indeed unique to the infused substances.

Since the method used for PFM was a IVD-CE certified analysis kit applied to our LC-MS/MS system, a partial validation of the method has been performed following EMA guidelines (16) for verification of linearity, lower limit of quantification (LLOQ), intra- and inter-run accuracy and precision on our system. Accuracy and precision were determined by analysing 5 samples per level at 4 levels (3 QCs and the lowest calibrator) which are covering the calibration curve range. Following EMA guidelines, the mean concentrations found should be within 15% of the nominal values for the QC samples, except for the LLOQ which should be within 20% of the nominal value.

Determination of free urinary metanephrines by LC-MS/MS

The analyses of free urinary metanephrines (MN, NMN and 3MT) were performed at the Laboratory of Genetic Metabolic Disorders, WKZ, Utrecht the Netherlands. 3MT, NMN and MN were purchased from Sigma-Aldrich (Munich, Germany). Stable isotopes 3-Methoxytyramien-α,α,β,β-D_4·HCl (3MT-D_4), dl-Metanephrine-α-D_2,β-D_1·HCl (MN-D_3) and dl-

Normetanephrine-α-D_2,β-D_1·HCl (NMN-D_3) were purchased by Cambridge Isotope Laboratory (Massachusetts, United States). SPE procedure was adopted from Danaceu et al (17), UPLC-MS/MS conditions were based on publication by Peitzsch *et al* (6) with some modifications. Quattro Ultima Pt triple quadrupole mass spectrometer coupled to an Acquity ultra performance liquid chromatography (UPLC, Waters, Manchester, UK) were used for analyses. MRM transitions were m/z 168.2→151.2, 172.2→155.2, 198.2→180.2 and 201.2→183.2 for 3MT, 3MT-D_4, MN and MN-D_3 respectively, all [$M + H^+$]. For both NMN and NMN-D3 in source fragmentation was observed, m/z 166.1→134.1 and 169.1→137.1 were measured [$M\text{-}H_2O + H^+$].

Statistics

Robust regression and Outlier removal (ROUT) analysis was applied per metabolite to remove definitive outliers (Q = 0.1%). Descriptive statistics were reported in terms of medians and percentiles for continuous variables. Normality of variable distribution was tested using the Kolmogorov–Smirnov test. Reference values were calculated based on the 5th and 95th percentiles. Receiver Operating Characteristic (ROC) curve was applied per metabolite in order to determine the optimal cut-off to discriminate between control subjects and NB patients. Correlation between the levels of PFM and clinical parameters was tested using Mann–Whitney U test (two groups) or Kruskal–Wallis test (> 2 groups). Correlation between urine and plasma was tested using Pearson's correlation test. All statistical analyses were performed using Graphpad (GraphPad Software, version 7.03, La Jolla California USA), all tests were two-sided and a p-value < 0.05 was considered statistically significant.

Results

Performance of the PFM analytical method

The chromatographic method allowed to obtain good signal for 3MT, NMN and MN with retention time of 2.45 min, 2.48 min and 2.48 min, respectively. The specific ion transitions used allowed the correct identification of the three PFM. Linear regression fit for the calibration curves was achieved for all the three PFM with $r^2 > 0.998$. The method was linear over a wide range of concentrations (2.83–5094 ng/L for 3MT, 2.76–19860 ng/L for NMN, 2.91–20970 for MN) and has been shown to be highly accurate and reproducible (intra- and inter-assay CV% < 5.4% and RE% ranging 89.2–103.5 for all analytes (**Table 2**).The LLOQ were 2.83 (CV:12.5%, RE:97.2%), 2.76 (CV:10.7%, RE:98.4%) and 2.91 ng/L (CV:11.5%, RE:98.9%) for 3MT, NMN and MN, respectively. Plasma samples were

analysed in 4 non-consecutive runs. In each run a complete calibration curve and 3 QCs were included and results were always acceptable with back-calculated values of the calibrators < 10% of the nominal values and QCs always inside the range of acceptance.

		3MT		NMN		MN	
	QC	CV%	R.E.%	CV%	R.E.%	CV%	R.E.%
Intra-assay	I	4.1	90.1	3.2	91.1	4.6	98.7
	II	1.0	95.3	4.3	89.9	3.3	98.4
	III	3.1	92.6	3.3	92.4	3.5	103.5
Inter-assay	I	5.2	95.1	2.4	91.2	4.1	102.4
	II	2.3	96.3	5.4	89.2	2.9	97.9
	III	4.0	94.2	3.7	92.0	5.3	101.8

Table 2 - Results of the validation of the LC-MS/MS method. Precision (CV%), Accuracy (R.E.%).

Performance of the PFM analytical method

PFM were not normally distributed in control samples ($p < 0.01$) and consequently the reference ranges were defined using the 5th and 95th percentiles as follows: 2.83–9.23 ng/L for 3MT, 5.89–154.3 ng/L for NMN and 2.91–72.05 for MN. Also, in patients with NB, PFM were not normally distributed ($p < 0.0001$) and their ranges (5th and 95th percentiles) were: 1.42–255.3 ng/L, 55.3–3234 ng/L and 8.12–102.6 ng/ L for 3MT, NMN and MN, respectively. Plasma levels of 3MT (**Fig. 1A**) and NMN (**Fig. 1B**) were significantly higher in plasma of NB patients if compared to control subjects ($p < 0.001$), whereas MN levels were similar in both groups (**Fig. 1C**).

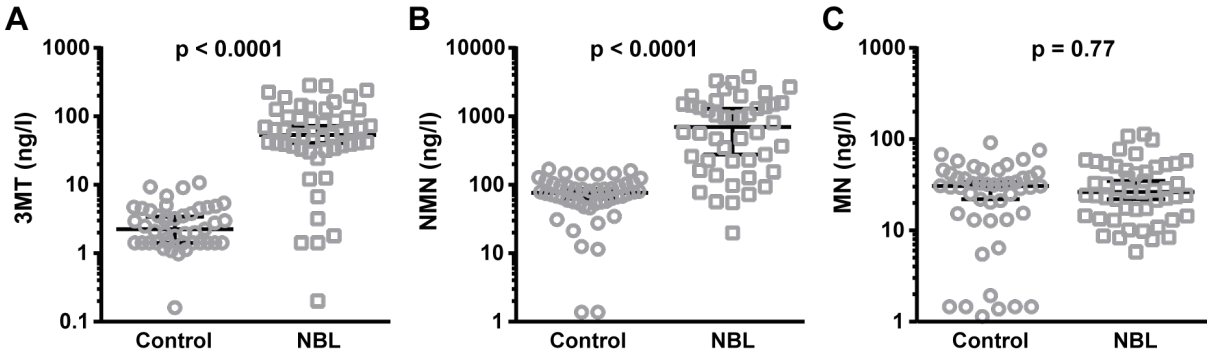

Fig. 1. Comparison of PFM levels between controls and neuroblastoma patients. A: 3-methoxytyramine (3MT), B: normetanephrine (NMN) and C: metanephrine (MN). All graphs depict the median with its 95% confidence interval. Statistical comparison was done with Mann-Whitney test.

In order to identify the optimal cut-offs that could discriminate between controls and NB patients, ROC curves were obtained for 3MT and NMN. For 3MT, the optimal cut-off was set at 11.44 ng/L (AUC = 0.93, 95% CI: 0.87–0.99, p < 0.0001), which corresponds to a sensitivity of 88.2% and specificity of 100% (**Fig. 2A**). NMN was slightly less sensitive (80.4%) and specific (95.8%) as compared to 3MT (**Fig. 2B**) and its optimal cut-off was 150.5 ng/L (AUC = 0.91, 95% CI: 0.84–0.97, p < 0.0001). The combination of NMN with 3MT showed a sensitivity and a specificity of 92% and 92%, respectively.

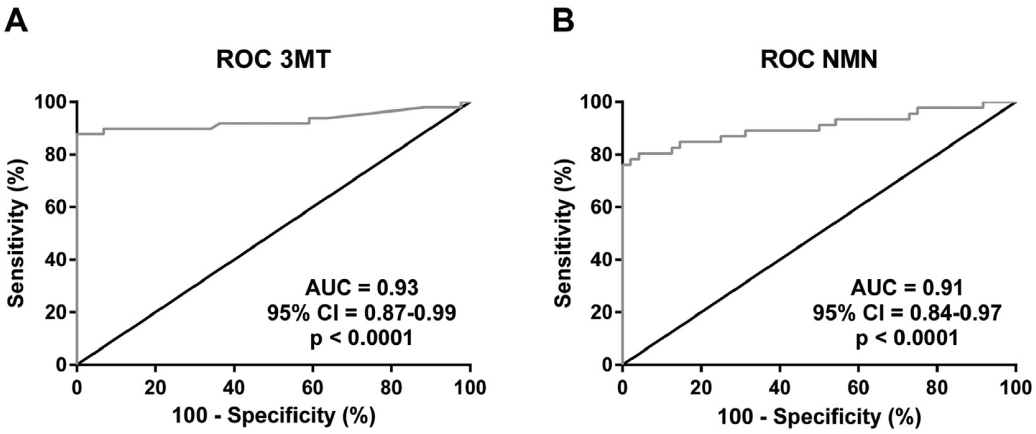

Fig. 2. ROC curve analysis for PFM. A: 3-methoxytyramine (3MT), B: normetanephrine (NMN).

Finally, correlations between different clinical factors and the levels of 3MT and NMN were tested (**Table 3**). 3MT levels were significantly higher in plasma of female patients (p = 0.034) and borderline significant in patients with stage 4 disease as compared to other stages (p = 0.074). On the other hand, NMN was higher in plasma of patients without *MYCN* amplification (p = 0.008) and borderline significant in female patients (p = 0.09) and patients younger than 18 months (p = 0.086).

Clinical factor		3MT mean	3MT p-value	NMN mean	NMN p-value
Gender	Male	59.3	0.034	762.4	0.09
	Female	107.2		1338	
Age	≤ 12 months	75.7	NS	1310	NS
	> 12 months	79.8		800.9	
Age	≤ 18 months	76.8	NS	1291	0.086
	> 18 months	79.5		755.6	
Stage	1	82.1	NS	493	NS
	3	52.6		1107	
	4	85.3		984.9	
	4s	96.4		1165	
Stage	1, 3 and 4s	71.6	0.074	1015	NS
	4	85.3		984.9	
MYCN	Non-amplified	77.4	NS	1199	0.008
	Amplified	70.7		515	
Risk-group	Low-risk	71.5	NS	1092	NS
	Medium-risk	95.3		1429	
	High-risk	80.4		820	
Risk-group	Low and Medium-risk	75.9	NS	1180	NS
	High-risk	80.4		820	

Table 3 - Analysis of the correlations between clinical factors and 3MT and NMN. NS = not significant

Comparison between PFM and urinary HVA and VMA

Since analysis of urinary HVA and VMA is still the most common way to biochemically diagnose neuroblastoma patients, the diagnostic performance of PFM 3MT and NMN was compared to retrospective urinary HVA and VMA data. Of the 38 patients with matched PFM and urinary HVA and VMA, 29 of them were positive for both plasma marker and both urinary markers, while three were negative for all four markers (**Fig. 3**). One patient was only positive for urinary VMA, while the remaining five patients had various combination of positive markers (**Fig. 3**). In this group of patients (n = 38), the sensitivity of urinary HVA and VMA was 90% and 79%, respectively. The sensitivity of PFM 3MT and PFM NMN was 90% and 79%, respectively. Taken together, these finding suggest that PFM could be just as sensitive as urinary HVA and VMA.

Comparison between PFM and urinary free metanephrines

For 29 NB patients, matched urine and plasma samples were analysed and compared for the measured metabolites (**Fig. 4A-B**). There was a clear positive correlation between NMN levels in urine and plasma ($p = 0.0017$), whereas no correlation was found between plasma and urinary 3MT ($p = 0.59$). Finally, the number of patients with elevated plasma 3MT, plasma NMN, urinary 3MT and urinary NMN was compared (**Fig. 5**). One patient was negative for

3MT and NMN in both plasma and urine, while 23 patients were positive for both makers in plasma and in urine. One patient was only positive for urinary 3MT, one for both urinary 3MT and NMN, two for both urinary markers and plasma 3MT, and one for both urinary markers and plasma NMN.

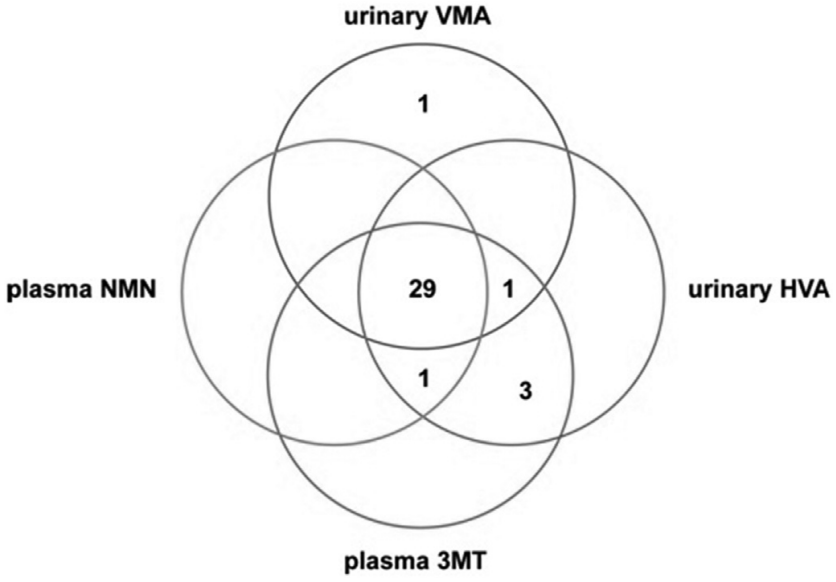

Fig. 3. PFM vs urinary HVA and VMA. Venn diagram comparing the number of patients (n = 29) with elevated urinary free 3-methoxytyramine (3MT), urinary free normetanephrine (NMN), plasma free 3MT and plasma free NMN. One patient was negative for both 3MT and NMN in both plasma and urine.

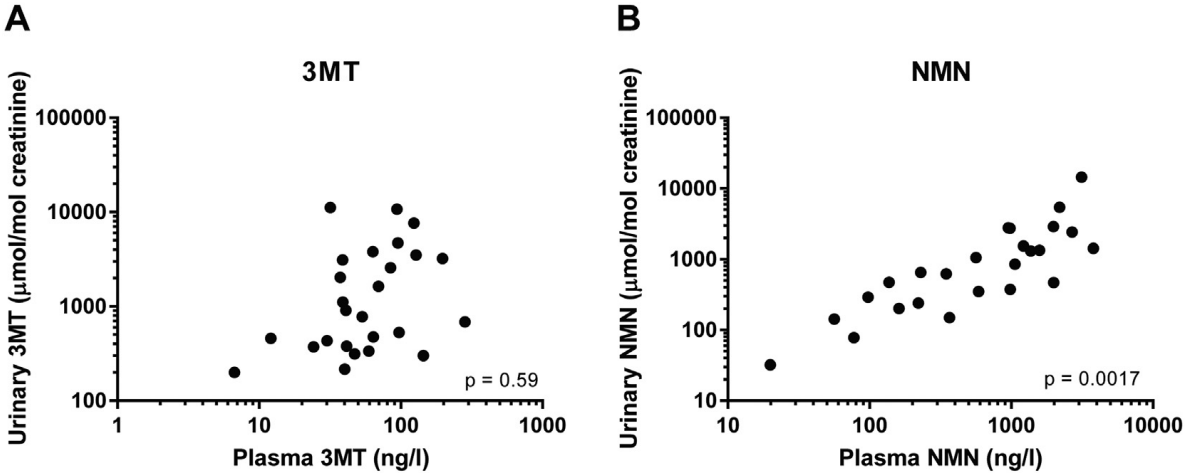

Fig. 4. Correlation between PFM and urinary free metanephrines. A: 3-methoxytyramine (3MT), B: normetanephrine (NMN).

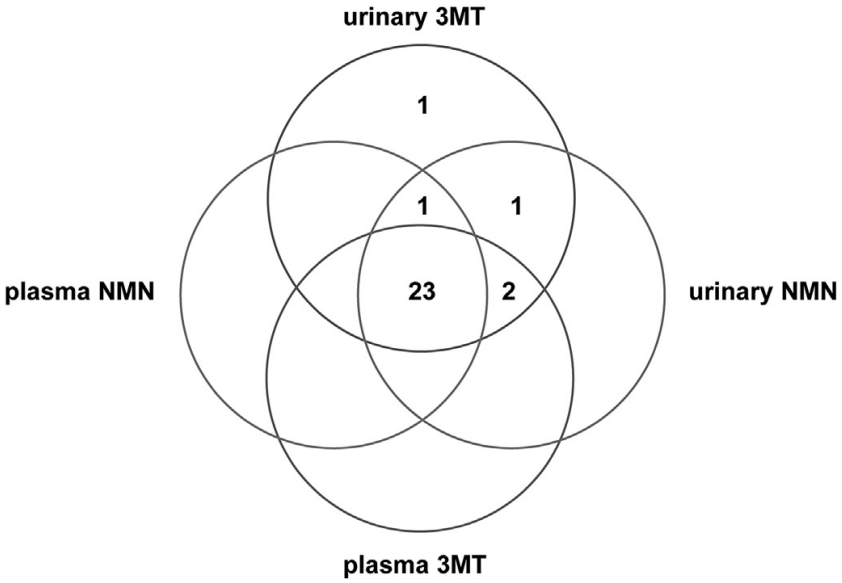

Fig. 5. PFM vs urinary free metanephrines. Venn diagram comparing the number of patients (n = 29) with elevated urinary free 3-methoxytyramine (3MT), urinary free normetanephrine (NMN), plasma free 3MT and plasma free NMN. One patient was negative for both 3MT and NMN in both plasma and urine.

Discussion

In this work we have evaluated the potential contribution of PFM for NB diagnostics and compared their values to metanephrines in matched urine samples. In line with previous work done by Franscini *et al.* we have confirmed that plasma levels of 3MT and NMN, but not of MN, are much higher in NB patients compared to control subjects (14). Although, worldwide analysis of urinary HVA and VMA is still considered the biochemical standard for diagnosis of NB (9), their diagnostic performance is suboptimal (10, 12), but could be improved by measuring urinary metanephrines (10). Furthermore, our data suggest that PFM is at least as sensitive as urinary HVA and VMA. Although this study did not analyse urinary HVA and VMA in the control group, based on a large neuroblastoma cohort, the reported specificity for both HVA and VMA was 95.8% and 95.1%, respectively (11), which is lower than the specificities we found for PFM 3MT and NMN (100% and 95.8%, respectively). In line with that conclusion and the fact that PFM became the golden standard for diagnosing pheochromocytoma and paraganglioma (3), we have shown that also NB diagnostics might also benefit from measuring PFM. The commercial IVD-CE certified analysis kit for PFM was easily applicable to our LC-MS/MS system and its analytical performance allows a routine use in the laboratory workflow. In line with the results published by Franscini *et al* (14), plasma free 3MT was the most sensitive PFM for NB diagnostics followed by plasma free

NMN (sensitivity of 88.2% and 80.4%, respectively). When the diagnostic sensitivity of 3MT and NMN in plasma is compared to their reported diagnostic sensitivity in urine (67% and 89%, respectively) (10), 3MT appeared to be more sensitive in plasma, while NMN more sensitive in urine. The combination of 3MT and NMN in plasma had a diagnostic sensitivity of 92%, which was also the case for the combination of 3MT and NMN in urine (10). Since these measurements were not performed using the same patients, further studies are warranted.

Comparison between PFM and urinary metanephrine levels showed a good correlation only for NMN, while no correlation was found for 3MT. This discrepancy might be attributed to several reasons, such as the different analytical system, the small cohort size (n = 29), more chemical instability in plasma compared to urine due to lack of acidification of the sample prior to storage at -80 °C (18). The lack of correlation between plasma and urinary 3MT might also explain the absence of the expected correlation (10) between 3MT and various adverse clinical factors such as stage 4 disease and *MYCN* amplification. For NMN, a strong inverse correlation was found between plasma free NMN and *MYCN* amplification, which is in line with the trend that was previously demonstrated for urinary NMN (10).

In conclusion, elevated plasma levels of 3MT and NMN, but not of MN, might assist in diagnosing patients with NB. A plasma-based biochemical test is relevant for the diagnosis of NB in addition or as an alternative to urine-based tests. Furthers studies are warranted.

Conflict of interest statement: none to declare.

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