Biochemical testing for neuroblastoma using plasma free 3-O-methyldopa, 3-methoxytyramine, and normetanephrine

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Abstract

Background: Neuroblastoma, the most common extracranial solid tumor of childhood, produces catecholamines that are metabolized within tumor cells. Homovanillic acid (HVA) and vanillylmandelic acid (VMA), the end products of catecholamine metabolism, have limited accuracy for testing of the tumors. This study assessed whether metabolites produced in earlier steps of catecholamine metabolism might offer improved diagnostic accuracy over urinary HVA and VMA.

Procedure: Plasma concentrations of 3-methoxytyramine, normetanephrine, and metanephrine were measured in two pediatric cohorts: (i) 96 children with confirmed neuroblastoma and (ii) 41 children with signs and symptoms of a catecholamine-producing tumor or other neoplasms and in whom neuroblastoma was excluded. Additional measurements of plasma 3-O-methyldopa and relationships of metabolites to MYCN amplification were examined in patient subgroups.

Results: Overall, 94 of the 96 patients with neuroblastoma had concentrations of 3-methoxytyramine or normetanephrine above age-specific upper limits of reference intervals, providing a diagnostic sensitivity of 97.9% that was higher (P < 0.0001) than that of 82.2% for HVA and VMA. One of the two patients with normal plasma results showed an elevation of plasma 3-O-methyldopa. Diagnostic specificities were, respectively, 95.1% and 84.8%. Areas under receiver-operating characteristic curves confirmed the superior diagnostic power of the plasma than the urinary test (0.994 vs 0.945; P = 0.0095). Ratios of plasma 3-methoxytyramine to normetanephrine were 7.2-fold higher (P < 0.0001) for patients who had neuroblastomas with MYCN amplification than without MYCN amplification.

Conclusions: Measurements of plasma 3-methoxytyramine and normetanephrine provide a highly accurate diagnostic test for neuroblastoma and also offer potential for prognostic risk stratification.

KEYWORDS
3-O-methyldopa, methoxytyramine, MYCN, neuroblastoma, normetanephrine

Abbreviations: AUC, area under curve; COMT, catecholamine-O-methyltransferase; HVA, homovanillic acid; INSS, International Neuroblastoma Staging System; LC-MS/MS, Liquid chromatography-tandem mass spectrometry; L-dopa, 3,4-dihydroxyphenylanaline; ROC, receiver-operating characteristic; VMA, vanillylmandelic acid.

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1 INTRODUCTION

Neuroblastoma is an embryonal tumor of the autonomic nervous system that occurs near exclusively in children and is derived from developing and incompletely committed precursor cells of the neural crest. Representing the most common extracranial solid tumor of childhood, responsible for 8.5% of all pediatric malignancies, neuroblastomas exhibit highly heterogeneous behavior. Many run an aggressive course whereas others spontaneously and completely regress or mature into ganglioneuroblastomas or benign ganglioneuromas.

Similar to pheochromocytomas, neuroblastomas produce catecholamines but have a limited capacity for catecholamine storage and secretion so that the catecholamines produced are largely metabolized within tumor cells. Consequently, biochemical testing for neuroblastoma has relied on measurements of homovanillic acid (HVA) and vanillylmandelic acid (VMA). As the final end products of catecholamine metabolism, these metabolites are excreted in urine at micromolar concentrations, ensuring their easy measurement and continued use for biochemical testing.

Although HVA and VMA are produced in high abundance, this does not necessarily translate to high diagnostic accuracy. Diagnostic sensitivities of urinary HVA and VMA have been described at 73% to 92% at specificities of 96% to 100%. Often such studies have not outlined adequate methods for exclusion of disease by alternatives to biochemical testing so that false-negative results may be missed and diagnostic sensitivity overestimated. The largest and most reliable analysis was provided by the prospective study of Schilling et al, which included follow-up for exclusion or additional confirmation of disease. That study, involving 1.5 million infants screened for neuroblastoma at one year of age, indicated a diagnostic sensitivity of only 73%. Among the 27% of tumors that were initially missed many were aggressive, whereas among those detected many spontaneously regressed or ran a benign course so that screening did not improve outcomes and is no longer recommended.

The study of Schilling et al was, however, a screening study and as shown for other catecholamine-producing tumors, higher diagnostic sensitivities can be expected for patients in whom testing for disease is based on clinical presentation rather than screening. Clearly, however, more accurate biochemical tests for neuroblastoma would be useful, including tests that distinguish patients with aggressive versus more benign disease. To those ends, several studies have examined urinary normetanephrine and 3-methoxytyramine, the respective metabolites of norepinephrine and dopamine, as potential biomarkers for neuroblastoma. Although one of those studies indicated utility of urinary 3-methoxytyramine for prognosis, the overall diagnostic performance remained suboptimal. These findings are explained by indications that urinary 3-methoxytyramine, similar to urinary dopamine, is largely derived from renal uptake and metabolism of the amino acid precursor, 3,4-dihydroxyphenylalanine (L-dopa), which in other studies has been associated with an unfavorable prognosis. Two other studies examined use of plasma free 3-methoxytyramine and normetanephrine as tests for neuroblastoma both with promising results but limitations related to sample size, insufficient analytical sensitivity, or incompletely established age-specific reference intervals.

Using a sensitive mass spectrometric method of measurement and with recent availability of age-specific reference intervals, the present study addressed the hypothesis that measurements of plasma free 3-methoxytyramine and normetanephrine provide superior accuracy for identifying patients with neuroblastoma compared with urinary HVA and VMA. In a subgroup of patients we also assessed utility of 3-O-methyltyrosine and 3-methoxytyrosine, as an additional metabolite in the biomarker panel. Potential prognostic value was assessed according to relations of metabolites with MYCN amplification, an established prognostic biomarker.

2 METHODS

2.1 Subjects

Patients included 96 children diagnosed with neuroblastoma (40 females) at a median age of 2.7 years (range, 1 day to 15.3 years) and evaluated at the University Hospitals of Cologne and Dresden (Germany) or at St. Jude Children’s Research Hospital, Memphis, Tennessee. Neuroblastoma was confirmed by histopathology. Another 41 children (17 females) aged 5 days to 16.8 years, who were enrolled under the PRIMMS study (Pediatric Reference Intervals for Monoamine Metabolites and Steroids; Ethics committee approval EK 113042013, Technische Universität Dresden), served as a control group (Table 1). Controls were included based on suspicion of other catecholamine-producing tumors.
of a catecholamine-producing tumor or relevance to the differential diagnosis of neuroblastoma, which was excluded by an alternative diagnosis as detailed in Supporting Information Table S1. Another 533 children, described in detail elsewhere, served as a reference group from whom age-specific upper cutoffs of reference intervals for plasma metabolites were established. To assess prognostic significance, relationships of metabolite profiles to MYCN amplification were assessed in 73 children with neuroblastoma in whom tumoral MYCN copy-number data were available. MYCN amplification was defined by copy numbers higher than 10. All children were studied under approved clinical protocols with parental consent.

2.2 Preanalytics and biochemical analyses

Heparinized blood samples were drawn in the morning and then placed on ice, centrifuged for 15 minutes at 4,500 rpm and plasma aliquots stored at −80°C until analyses. Spot urine samples were collected on the same day or within a week of blood collections and aliquots stored at −80°C until analyses. When possible, blood samples were collected after 20 minutes of supine rest and an overnight fast (> 60% of patients). For children in whom fasting was not possible (i.e., neonates and younger children), a restricted diet was permitted before sampling of blood and urine according to procedures at each center.

Concentrations of plasma free 3-O-methyldopa, 3-methoxytyramine, normetanephrine, and metanephrine were analyzed by liquid chromatography-tandem mass spectrometry (LC-MS/MS) as previously described. Urinary VMA and HVA were measured at independent laboratories using LC-MS/MS, gas chromatography with mass spectrometry, or high-pressure liquid chromatography with electrochemical detection (see Supporting Information Table S2 for details) and normalized to creatinine, measured by photometry after applying the Jaffé reaction.

2.3 Data analysis

True-positive results for free 3-O-methyldopa, 3-methoxytyramine, normetanephrine, and metanephrine in the plasma panel for patients with neuroblastoma, or false-positive test results in patients without neuroblastoma were defined as values for any one of the metabolites above age-specific upper limits of reference intervals (Supporting Information Methods S1). False-negative results in patients with neuroblastoma or true-negative results in patients without neuroblastoma were defined as values for all metabolites in the plasma panel below upper limits of reference intervals. A similar procedure was used for application of age-specific upper limits of reference intervals for urinary VMA and HVA as established by the specific laboratories responsible for those analyses (Supporting Information Table S2).

Nonparametric statistical methods were used for comparisons of data between groups and within groups. Receiver-operating characteristic (ROC) curve analyses and comparisons of areas under ROC curves (AUC) for different diagnostic tests employed multivariate logistic regression models according to the JMP PRO software package 12.1.0. Discriminant analyses with 10 random permutations of training and validation sets were performed for the validation of diagnostic accuracy (Supporting Information Analyses S1).

3 RESULTS

3.1 Clinical presentation of patients with neuroblastoma

Clinical data related to MYCN amplification, INSS staging, and tumor locations were available from 73 to 74 of the 96 patients with neuroblastoma (Table 1). Patients categorized to INSS stage 3 and 4 groups were older (P < 0.0001) than patients of other groups combined, including the INSS stage 4s group (median ages, 3.2 vs 1.0 years). Tumors from INSS stage 3 and 4 patients also showed a higher (P = 0.0066) proportion of MYCN amplifications than other groups combined (means ± CI, 43% ± 11% vs 7% ± 6%). All except one of the 26 patients (96% ± 4%) with tumors characterized by MYCN amplifications were confined to the INSS stage 3 and 4 groups. Neuroblastomas at adrenal locations were also characterized by a higher (P = 0.0114) proportion of MYCN amplifications than tumors at extraadrenal locations (means ± CI, 48% ± 11% vs 19% ± 9%), but did not differ according to INSS stage.

3.2 Plasma metabolite concentrations in relationship to age

Accounting for age-specific upper cutoffs of a reference population, plasma concentrations of O-methylated metabolites in patients with neuroblastoma showed strong separation for plasma 3-methoxytyramine and good though weaker separation for 3-O-methyldopa and normetanephrine compared with the reference group (Figure 1). In contrast, there was negligible separation for metanephrine from concentrations of the reference population.

3.3 Test results for O-methylated versus deaminated acid metabolites

Plasma concentrations and urinary outputs of catecholamine metabolites showed highly variable increases in individual patients with neuroblastoma compared with the control group in whom neuroblastoma was excluded. (All data are available in Supporting Information Table S1.) Similarly, relative increases in plasma or urinary metabolites above upper limits of reference intervals varied considerably for the O-methylated metabolites and the two deaminated acid metabolites, but for all metabolites were higher (P < 0.02) among patients with neuroblastoma compared with the control group (Figure 2). Plasma free 3-methoxytyramine showed a 22.9-fold increase (P < 0.0001) above median values of the control group and with negligible overlap in values between both groups. In contrast, the 6.3-fold increase (P < 0.0001) for urinary HVA was substantially smaller (P < 0.0001) than for plasma methoxytyramine and showed considerable overlap with the control group. Similarly, plasma normetanephrine showed an
3.4 | Diagnostic test performance

True-positive results for plasma 3-methoxytyramine, normetanephrine, and metanephrine were found, respectively, in 96.8% (93/96), 72.9% (70/96), and 16.6% (16/96) of patients with neuroblastoma. For the subgroup of patients in whom plasma 3-O-methyldopa was also measured, 76.5% (39/51) demonstrated concentrations above upper cutoffs of reference intervals for this metabolite. All but two of the 96 patients with neuroblastoma showed elevations of either 3-methoxytyramine or normetanephrine above upper cutoffs demonstrating a diagnostic sensitivity of 97.9% for measurements of those two plasma metabolites (Table 2). One of the two patients without elevations of the two plasma metabolites, a 3.8-year-old female with MYCN amplification, however, showed a 30% elevation above the age-specific upper limit of reference intervals for 3-O-methyldopa.

In contrast to the plasma panel, true-positive results for HVA and VMA were found, respectively, in 75.6% (68/90) and 73.2% (60/82) of patients with neuroblastoma in whom those measurements were available. Among the 22 patients with negative results for HVA there were 20 with results available for VMA, including six patients with true-positive results. This indicated an overall diagnostic sensitivity of 82.2% (74/90) for patients with elevations of either or both urinary metabolites, which by paired comparisons for urinary and plasma data was less (P < 0.0001) than the sensitivity for the plasma test (Table 2).

Among children in whom neuroblastoma was tested for and excluded, proportions of false-positive results were minimal at less than 3% for each of the plasma O-methylated metabolites and 12.1% and 15.2% for HVA and VMA, respectively. Due to single false-positive test results for normetanephrine and 3-methoxytyramine, the diagnostic specificity for measurements of the plasma metabolites was 95.1% compared with 84.8% for urinary VMA and HVA (Table 2).

As reflected by AUCs, measurements of plasma free 3-methoxytyramine and normetanephrine showed superior performance for diagnosis of neuroblastoma than measurements of urinary HVA and VMA (Figure 3 and Table 2). Higher AUCs for plasma compared with urinary metabolites were found irrespective of whether ROC curves were constructed using absolute values.

FIGURE 1  Age-dependent distribution of plasma concentrations of 3-O-methyldopa (A), 3-methoxytyramine (B), normetanephrine (C), and metanephrine (D) in patients with neuroblastoma (▲ patients with data available for B, C and D; ■ patients with data available for all metabolites) compared with a reference population (○) as previously described elsewhere and included here to establish the importance of age-specific cutoffs for plasma metabolites. Dashed lines represent the 2.5, 25, 50, 75, and 97.5 percentiles established from data of the reference group.
FIGURE 2  Plasma concentrations of free 3-O-methyldopa (A), 3-methoxytyramine (B), normetanephrine (D) and metanephrine (E), and urinary outputs of HVA (C) and vanillylmandelic acid (F) in patients with neuroblastoma (▴) and in the control group of patients with suspected and excluded disease (●). All data are presented relative to upper cutoffs of age-specific reference intervals as shown by dashed horizontal lines. Arrows with numbers in A, B, C, D, and F indicate fold differences between median concentrations in patients with and without neuroblastoma.

TABLE 2  Characteristics of biochemical tests using combinations of either plasma free 3-methoxytyramine (MTY) and normetanephrine (NMN), or urinary HVA, and vanillylmandelic acid (VMA) in patients with neuroblastoma

|                      | Plasma MTY + NMN | Urinary HVA + VMA |
|----------------------|------------------|-------------------|
| Sensitivity          | 97.9% [95.0%–100.0%] (94/96) | 82.2% [74.3%–90.1%] (74/90) |
| Specificity          | 95.1% [88.5%–100.0%] (39/41) | 84.8% [72.6%–97.0%] (28/33) |
| AUC                  | 0.994b [0.980-0.999] | 0.945 [0.890-0.973] |
|                      | 0.996c [0.983-0.999] | 0.880 [0.800-0.931] |
| Positive predictive value | 97.9% [96.5%–99.3%] (94/96) | 93.4% [90.6%–96.2%] (71/76) |
| Negative predictive value | 95.1% [91.7%–98.5%] (39/41) | 80.0% [73.2%–86.8%] (28/35) |

aP < 0.0001.
bP = 0.0095.
cP = 0.0003. Ninety-five percent confidence intervals are provided in brackets and patient numbers in parentheses.
FIGURE 3 Receiver-operating characteristics curves for plasma free 3-methoxytyramine (MTY) and normetanephrine (NMN) (black solid lines), and urinary VMA and HVA (dashed gray lines) as biomarkers for distinguishing between patients with and without neuroblastoma. ROC curves derived from absolute concentrations measured in respective samples (A), and from normalized data (relative to upper limits of reference intervals, B). For details on patient numbers, refer to Table 2.

for plasma concentrations and urinary outputs (0.994 vs 0.945, \( P = 0.0095 \)) or values normalized to age-specific upper cutoffs of reference intervals used by the laboratories responsible for measurements (0.996 vs 0.880, \( P = 0.0003 \)). With use of reference intervals for urinary metabolites described by Pussard et al,\(^26\) the AUC for the urinary test (0.928) was higher (\( P = 0.0016 \)) than for laboratory specific upper cutoffs, but nevertheless remained lower (\( P = 0.0028 \)) than for plasma metabolites (Supporting Information Figure S1). Higher AUCs for plasma than urinary metabolites were similarly found after data were restricted to complete sets of paired results for both plasma and urinary tests according to either absolute values (0.994 vs 0.946, \( P = 0.0105 \)) or normalized to reference intervals (0.996 vs 0.880, \( P = 0.0003 \)).

In addition to the above logistic regression analysis, discriminant analysis using random permutations of training and validation data sets confirmed the higher performance of the plasma than the urinary test (Supporting Information Analysis S1).

3.5 Correlations with clinical features

Among the 73 patients with available information on MYCN amplification, ratios of plasma 3-methoxytyramine to normetanephrine and urinary HVA to VMA were, respectively, 7.2- and 4.0-fold higher (\( P < 0.0001 \)) in patients with than in patients without MYCN amplification (Figure 4). ROC curve analyses revealed similar AUCs for plasma (0.898, 95% CI 0.782-0.955) and urinary metabolite ratios (0.887, 95% CI 0.763-0.950), with optimal cutoffs of 0.37 for the 3-methoxytyramine:normetanephrine ratio and 2.75 for the HVA:VMA ratio as determined by Youden’s index (Figure 4). At those cutoffs, diagnostic sensitivities and specificities for assessing MYCN amplification were, respectively, 76.9% and 93.6% for the plasma test and 80.0% and 90.2% for the urinary test.

4 DISCUSSION

This study establishes that measurements of plasma free 3-methoxytyramine and normetanephrine provide a highly accurate diagnostic test for neuroblastoma. Preliminary evidence is also provided that addition of 3-O-methydopa to the panel might be useful for detecting occasional tumors characterized by limited production of downstream catecholamine metabolites, as previously indicated for L-dopa.\(^{27,28}\) The panel also appears to offer opportunities for establishing prognostic risk.

Reasons for the improved diagnostic accuracy of plasma free 3-methoxytyramine and normetanephrine compared with the downstream metabolites, HVA and VMA, can be appreciated from an understanding of the different sources of catecholamines and how they are sequentially metabolized within different compartments of synthesis and after leaving those compartments (Supporting Information Figure S2).\(^{29,30}\) Most metabolism of norepinephrine occurs initially by deamination within sympathetic nerves with subsequent extra-neuronal conversion by catechol-O-methyltransferase (COMT) to 3-methoxy-4-hydroxyphenylglycol before further metabolism to VMA in the liver.\(^{31}\) The final end-product of epinephrine metabolism is also VMA. As such, VMA reflects the combined synthesis and metabolism of norepinephrine and epinephrine within numerous cellular compartments of the body. Similarly, HVA reflects the combined synthesis and metabolism of all dopamine produced within the body.

In contrast to VMA and HVA, the small amounts of O-methylated catecholamine metabolites that escape deamination to enter the bloodstream have restricted sources that more specifically reflect their formation within catecholamine-synthesizing cells that express
FIGURE 4  Differentiation of MYCN amplification status according to metabolite ratios of plasma 3-methoxytyramine to normetanephrine (A, C) and urinary HVA to VMA (B, D). Data are restricted to the 73 patients with neuroblastoma in whom results for MYCN amplification were available.

COMT (Supporting Information Figure S2). Such cells include neural crest-derived chromaffin cells and related pheochromocytoma and neuroblastoma tumor cells.32,33 Thus, although produced in small quantities, the O-methylated metabolites provide a more specific signal than the final metabolic end products for indicating the presence of catecholamine-producing and metabolizing tumors that express COMT. According to mathematical modeling (Supporting Information Analyses S2), the 3.6-fold larger diagnostic signal for 3-methoxytyramine than for HVA can be accounted for by a 6.3-fold larger proportion of 3-methoxytyramine relative to HVA produced by neuroblastoma tumor cells compared with all other sources in the body.

Reflecting their suboptimal utility as biomarkers, measurements of urinary VMA and HVA have now been omitted from international neuroblastoma response criteria.34 Furthermore, because use of the metabolites in screening is no longer recommended, diagnosis has come to depend on histopathology and imaging.35 This in part reflects a common mode of discovery where a mass may be indicated by palpation or ultrasound; in such cases, a biopsy and radiographic and then nuclear medicine-based imaging represent the immediate options for diagnosis and staging.36,37 Nevertheless, there are clinical presentations where neuroblastoma may be suspected without presence of an obvious mass and where tests that are less invasive or do not involve radiation exposure remain useful. Ideally, such tests might offer additional utility for prognosis and disease monitoring, including response to treatment or likelihood of relapse.

Although differences in catecholamine metabolite profiles have been linked previously to high-risk versus low-risk disease and associated clinical outcomes,15–17,38 the evidence of any relationships to MYCN amplification has until now been obscure.39,40 Because catecholamine synthesis involves sequential conversion of L-dopa to dopamine and of dopamine to norepinephrine, the present findings of higher ratios of 3-methoxytyramine to normetanephrine or of HVA to VMA in neuroblastomas with than without MYCN amplification are
in accordance with concepts that more aggressive disease is associated with more poorly differentiated catecholamine biosynthetic pathways. This is also in agreement with suggestions that measurements of L-dopa can provide additional utility for identifying high-risk cases of neuroblastoma in whom the usually measured catecholamine metabolites are not increased. This in turn provides a rationale for inclusion of 3-O-methyltyrosine in the plasma panel.

Other efforts directed to the development of circulating molecular biomarkers for diagnosis, disease stratification, and management of patients with neuroblastoma, while promising, will take time for validation and translation to the clinic. In the meantime, measurements of plasma-free O-methylated metabolites are already available at many centers as recommended tests for diagnosis of pheochromocytoma and paraganglioma. Extending this application to neuroblastoma is therefore relatively straightforward. Nevertheless, even for pheochromocytoma and paraganglioma, there has been more than a 25-year delay from the initial introduction of plasma-free metanephrines to final prospective validation for diagnosis. The present report, along with others, therefore only represents first steps in the diagnostic test development pipeline.

There are several limitations of the present study that must be addressed to bring the plasma test forward. Dependence on retrospectively collected patient samples mandates further prospective confirmatory studies. Another weakness involves the incomplete clinical and biochemical data that limited test interpretation. Thus, before the plasma test can be fully implemented into routine practice, there must be justification for inclusion of 3-O-methyltyrosine in the panel as well as validation of potential for disease monitoring and prognosis. It is, however, first crucial to prospectively establish utility for diagnosis. For that, there must be inclusion of an appropriate comparison group of children in whom neuroblastoma is suspected and then appropriately excluded. In addition to urinary VMA and HVA measured by a single LC-MS/MS method, comparisons need to also include measurements of urinary free 3-methoxytyramine and normetanephrine. The considerable age-related falls in 3-O-methyltyrosine, 3-methoxytyramine, and normetanephrine mandate the use of age-specific reference intervals. Considerations of preanalytical precautions and age- and sex-specific reference intervals should be considered similarly for comparisons of both plasma and urinary tests.

In summary, measurements of plasma-free 3-methoxytyramine and normetanephrine provide a highly accurate test for neuroblastoma, with preliminary evidence indicating superior diagnostic performance compared with commonly used measurements of urinary VMA and HVA. With the availability of age-specific continuous reference intervals, a prerequisite for test interpretation, it is time to start the process of bringing laboratory testing for neuroblastoma in pediatric populations up to the same level already established for the diagnosis of chromaffin cell tumors in adults.

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CONFLICTS OF INTEREST

The authors declare no conflicts of interest.

DATA AVAILABILITY STATEMENT

All data are available in Supporting Information Table S1.

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SUPPORTING INFORMATION
Additional supporting information may be found online in the Supporting Information section at the end of the article.

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