Urinary 3-Methoxytyramine Is a Biomarker for MYC Activity in Patients With Neuroblastoma

Iedan R. N. Verly, MD, PhD[1,2]; Yvette A. H. Matser, MD[1,2]; René Leen, MSc[2]; Rutger Meinsma, MSc[2]; Marta Fiocco, PhD[1,3,4]; Jan Koster, PhD[5]; Richard Volckmann, MSc[5]; Dilara Savci-Heijink, MD, PhD[6]; Giuliana Cangemi, PhD[7]; Sebastiano Barco, MSc[7]; Linda J. Valentijn, PhD[5]; Godelieve A. M. Tytgat, MD, PhD[1]; and André B. P. van Kuilenburg, PhD[2]

PURPOSE Elevated urinary 3-methoxytyramine (3MT) level at diagnosis was recently put forward as independent risk factor for poor prognosis in neuroblastoma. Here, we investigated the biologic basis underlying the putative association between elevated 3MT levels and poor prognosis.

METHODS Urinary 3MT levels and prognosis were investigated in both retrospective Italian (N = 90) and prospective Dutch (N = 95) cohorts. From the Dutch Cancer Oncology Group cohort (N = 122), patients with available urinary 3MT and gene expression data (n = 90) were used to generate a 3MT gene signature. The 3MT gene signature score was then used to predict survival outcome in the Children's Oncology Group (N = 247) and German Pediatric Oncology Group (N = 498) cohorts and compared with other known gene signatures. Immunohistochemistry of MYCN and dopamine β-hydroxylase proteins was performed on primary tumors.

RESULTS Elevated urinary 3MT levels were associated with poor prognosis in a retrospective cohort and a prospective cohort. Moreover, elevated urinary 3MT levels were associated with eight differentially expressed genes, providing a 3MT gene signature that successfully predicted poor clinical outcome. Even among low-risk patients, high 3MT signature score was associated with poor 5-year overall survival (72% *v* 99% among low-risk patients with a low 3MT signature score), and the 3MT signature score was correlated with *MYC* activity in the tumor (R = 82%, *P* < .0001). Finally, a strong MYCN and weak dopamine β-hydroxylase staining of tumors derived from patients with elevated urinary 3MT levels was observed, linking MYC activity in the tumor to both catecholamine biosynthesis and elevated urinary 3MT levels.

CONCLUSION Elevated urinary 3MT is a promising biomarker for poor prognosis and reflects increased MYC activity in the tumor. Therefore, urinary 3MT levels should be measured at diagnosis and may assist in assessing risk.

INTRODUCTION

Neuroblastoma is the most common extracranial pediatric solid tumor, accounting for approximately 15% of pediatric cancer–related deaths.[1] Patients with neuroblastoma are typically allocated into specific risk groups on the basis of a variety of factors such as the disease stage, the patient's age at diagnosis, and amplification of the *MYCN* gene (MNA).[2,3] These risk groups were originally developed to optimize the match between expected disease severity—defined as event-free survival (EFS) and overall survival (OS)—and treatment intensity.[2,3] Nevertheless, new risk factors that might be correlated more accurately with disease severity are continually being investigated to improve upon the existing models used to assess risk.[2-6] Currently, tumor biopsy is routinely performed to confirm the diagnosis and to obtain material for molecular diagnostics (eg, *MYCN* status).[7,8] However,

because a biopsy typically represents only a portion of the entire tumor, it may not necessarily reflect the biologic state of the entire tumor.[9] By contrast, tumor biomarkers such as metabolites and cell-free nucleic acids can be detected in blood and/or urine samples, thereby providing a more complete clinical picture with respect to the entire tumor.[10-12]

We previously reported that elevated levels of urinary 3-methoxytyramine (3MT) at time of diagnosis are correlated with poor outcome in patients with neuroblastoma, independent of other risk factors such as disease stage, the patient's age, and MNA status when tested in multivariate analyses.[11] Furthermore, even among high-risk patients and patients with MNA, elevated urinary 3MT levels were able to accurately identify patients with significantly worse prognosis.[11] The biologic basis underlying the association between elevated 3MT levels and poor prognosis is currently

CONTEXT

Key Objective

Urinary 3-methoxytyramine (3MT) has been put forward as a novel independent biomarker for poor prognosis in patients with neuroblastoma. In this study, we investigated the biologic rationale of the association of elevated urinary 3MT levels with poor outcome.

Knowledge Generated

We demonstrated that elevated urinary 3MT levels were associated with eight differentially expressed genes, providing a 3MT gene signature, which successfully predicted poor clinical outcome. The 3MT signature score proved to be correlated with MYC activity in the tumor and MYC activity affected the catecholamine metabolism resulting in elevated urinary 3MT levels.

Relevance

3MT is a promising biomarker for poor prognosis and reflects increased MYC activity in the tumor. Given the increasing availability of therapies that target MYC/MYCN signaling, analyzing urinary 3MT levels may help identify patients who would likely benefit from these therapies.

unknown, given that 3MT levels were not correlated with tumor burden[11] and given that 3MT is a degradation metabolite of dopamine with no known biologic activity.[13] Here, we analyzed matched tumor gene expression profiles and urinary 3MT levels and demonstrated that urinary 3MT levels strongly reflect MYC activity in the tumor.

METHODS

Patient Cohorts

Urinary 3MT levels were analyzed in a retrospective Italian cohort (N = 90) and a prospective Dutch cohort (N = 95, Fig 1A; for additional information, see the Data Supplement). All urine samples were collected at diagnosis. Urinary 3MT was extracted and analyzed as described previously,[14] and elevated 3MT was defined as exceeding the published prognostic cutoff value of a > 2.9-fold change,[11] which is calculated as 3MT concentrations per mmol creatinine divided by the upper limit of the age-related reference range. The Dutch Cancer Oncology Group (DCOG) cohort (N = 122; GSE16476 and GSE73537, Fig 1B) was used to establish a correlation between urinary 3MT levels and gene expression data. In 32 patients of this DCOG cohort, urinary 3MT levels were not available. Urinary and outcome data of these 90 patients were included in a previous publication.[11] Patients from whom both urine and gene expression data were available were allocated into a DCOG test cohort (n = 64, GEO accession number GSE16476) and a DCOG validation cohort (n = 26, GSE73537) on the basis of the expression data set that contained the profiling of their primary tumor. To investigate the correlation between the 3MT gene signature and outcome, we used gene expression data (Fig 1C) from the previously published Children's Oncology Group (COG, N = 247[15]) and German Pediatric Oncology Group (GPOH, N = 498, GSE62564).[16] The clinical characteristics of all cohorts are described in the Data Supplement. This study was approved by the local medical ethics committee.

Statistics and Bioinformatics

Median follow-up was calculated using the reverse Kaplan-Meier method. EFS and OS were calculated using the Kaplan-Meier method in combination with the log-rank test (SPSS version 25.0; IBM Corp, Armonk, NY). Multivariate analyses were performed using Cox proportional regression (SPSS). All gene expression data sets used in this study are available in the R2 bioinformatics platform,[17] and all analyses were performed using R2 (see the Data Supplement for further information). Using the DCOG test cohort (n = 64), gene expression was compared between patients with elevated and nonelevated urinary 3MT. Differentially expressed genes with $P < .001$, after correction for multiple testing (false discovery rate), were selected to form the 3MT gene signature. To test the accuracy of the 3MT gene signature, the DCOG validation cohort (n = 26) was divided into a high and low 3MT signature score using k-means analysis, which was subsequently compared with the actual urinary 3MT status. Finally, the 3MT gene signature scores were compared with the actual urinary 3MT status using Pearson's correlation.

Immunohistochemistry

Sections of primary tumor samples were stained for MYCN and dopamine β-hydroxylase (DBH) using standard immunohistochemistry methods as previously described.[12,18]

RESULTS

Validation of Urinary 3MT As a Prognostic Biomarker

First, we confirmed the association between elevated urinary 3MT levels at diagnosis and poor clinical outcome in the retrospective Italian and the prospective Dutch cohorts, which had a median follow-up of 4.9 years (interquartile range: 1.7-9.0 years) and 2.2 years (interquartile range: 1.2-3.1 years), respectively. In the retrospective Italian cohort, patients with elevated urinary 3MT levels had significantly worse EFS (Fig 2A) and OS (Fig 2C) compared with patients without elevated 3MT levels. Similarly, in the
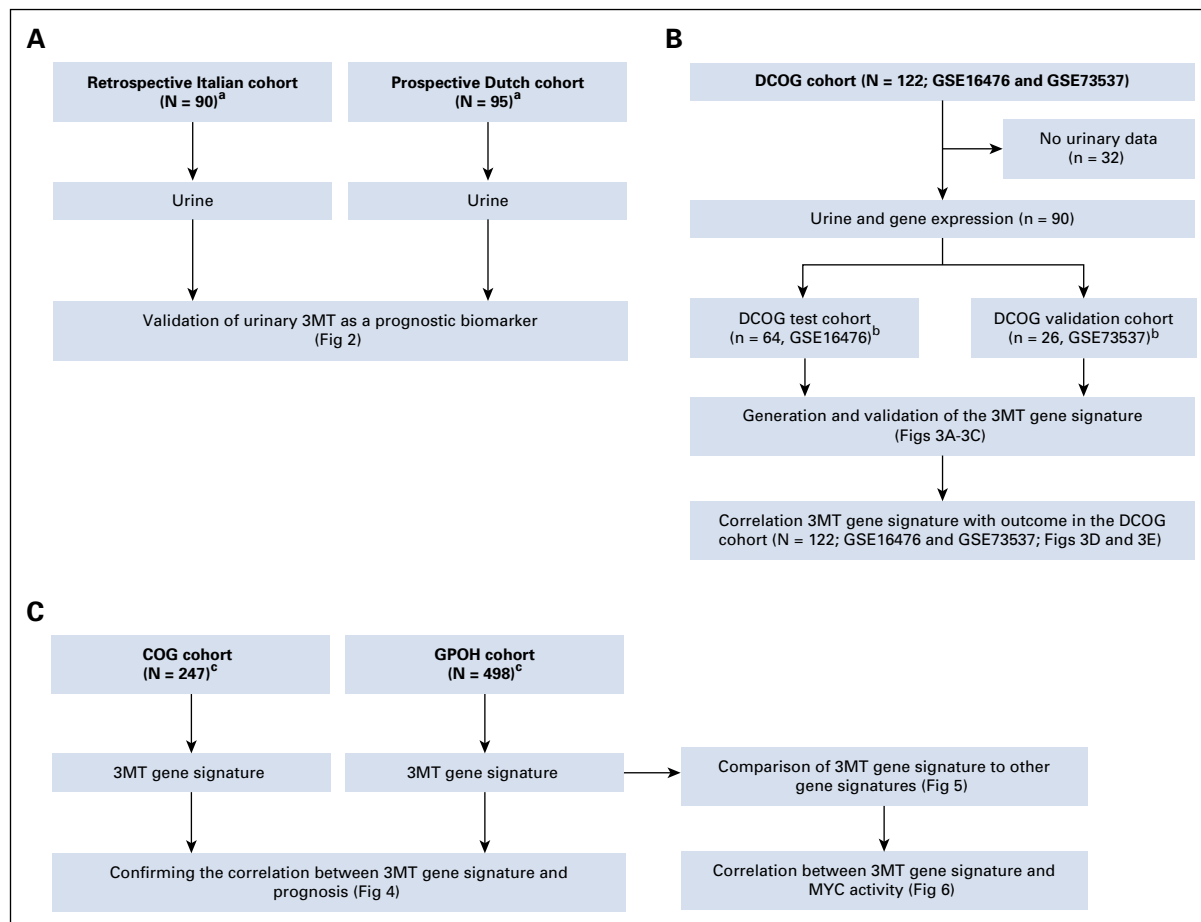
FIG 1. Flow chart depicting the analyses and cohorts included in this study. (A) The retrospective Italian cohort and the prospective Dutch cohort used to validate the putative association between elevated urinary 3MT levels and poor clinical outcome. (B) The DCOG cohort (N = 122; GSE16476 and GSE73537) that was used to generate the 3MT gene signature, by differential expression of genes in patients with and without elevated urinary 3MT levels. As in 32 patients urinary 3MT was not measured, the remaining 90 patients with neuroblastoma with urinary 3MT and gene expression data were allocated into a test cohort and a validation cohort on the basis of the expression data set that contained the profiling of their primary tumor (n = 64 GSE16476 and n = 26 GSE73537, respectively). The relation of the 3MT high and low signature, determined by k-means analysis, was next studied in this cohort. (C) The COG cohort[15] and the GPOH cohort (GSE62564) that were used to confirm the correlation between the 3MT gene signature with prognosis and MYC activity. The numbers of patients of each data set are given in parentheses. All data sets are available via the R2 platform, GEO web site, and Target web site (COG). [a]The Italian and Dutch cohorts were significantly different for age (*P* = .02), disease stage (*P* = .002), risk group (*P* = .013), MYCN status (*P* = .02), LOH1p (*P* ≤ .0001), and deceased patients (*P* = .01). [b]No statistically significant differences were observed between the DCOG test and validation cohorts. [c]The COG consisted of 87% high-risk and the GPOH of 37% high-risk patients. *P* values of all clinical characteristics are described in the Data Supplement. Statistical significance was evaluated using the chi-square test. 3MT, 3-methoxytyramine; COG, Children's Oncology Group; DCOG, Dutch Cancer Oncology Group; GPOH, German Pediatric Oncology Group; LOH1p, loss of heterozygosity of chromosome 1p.

prospective Dutch cohort, both EFS (Fig 2E) and OS (Fig 2G) were significantly lower among the patients with elevated urinary 3MT levels. Multivariate analysis in the prospective Dutch cohort (Figs 2F and 2H) confirmed that elevated urinary 3MT is a novel risk factor for poor EFS (*P* = .003), independent of expected risk factors like disease stage, the patient's age, MNA status, and loss of heterozygosity of chromosome 1p. These results support the association between elevated urinary 3MT levels and poor clinical outcome in neuroblastoma. Multivariate analyses in the retrospective Italian cohort could not confirm

the independence of 3MT as risk factor for EFS and OS (Figs 2B and 2D).

Urinary 3-MT Level Is Associated With a Specific Gene Expression Pattern

Next, we compared the gene expression profiles in the DCOG test cohort (n = 64 patients) between patients with elevated urinary 3MT and patients without elevated urinary 3MT, defined using our previously reported prognostic cutoff value.[11] We found that elevated urinary 3MT levels were significantly correlated (*P* < .01) with the

FIG 2. Patients with elevated 3MT have reduced EFS and OS compared with patients without elevated 3MT. (A) Kaplan-Meier curve of EFS and (B) forest plot on the basis of the results of the multivariate analysis for EFS for patients in the retrospective Italian cohort (N = 90). (C) Kaplan-Meier curve of OS and (D) forest plot on the basis of the results of the multivariate analysis for OS are plotted for patients in the retrospective Italian cohort (N = 90). (E) Kaplan-Meier curve of EFS and (F) forest plot on the basis of the results of the multivariate analyses of EFS for patients in the prospective Dutch cohort (N = 95). (G) Kaplan-Meier curve of OS and (H) forest plot on the basis of the results of the multivariate analysis for OS are plotted for patients in the prospective Dutch cohort (N = 95). Elevated urinary 3MT was defined as a > 2.9-fold increase at diagnosis. 3MT, 3-methoxytyramine; EFS, event-free survival; INSS, International Neuroblastoma Staging System; LOH1p, loss of heterozygosity of chromosome 1p; MNA, *MYCN* amplification; OS, overall survival.
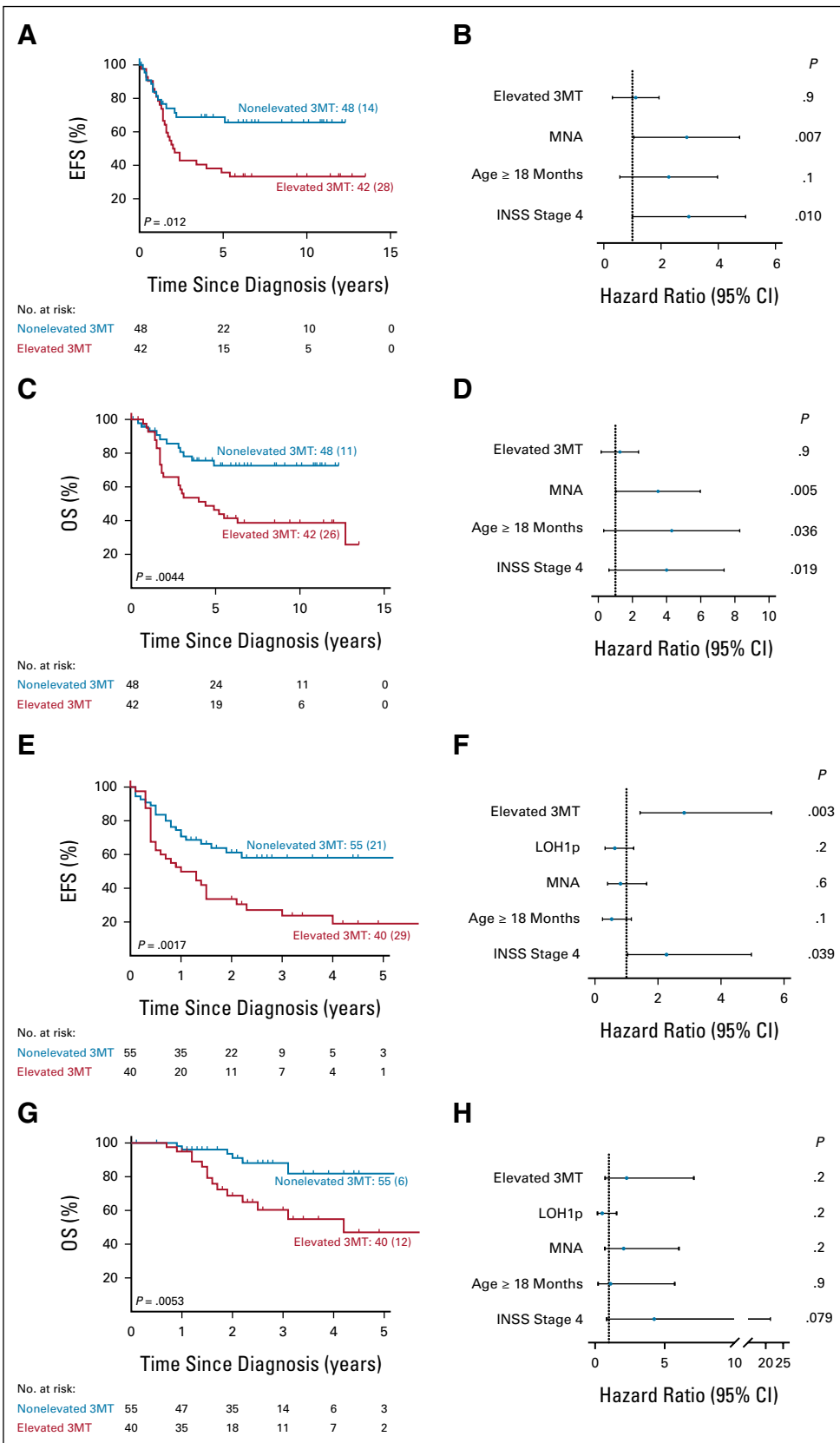
downregulation and upregulation of 76 and 86 genes, respectively (Data Supplement). After adjusting the cutoff value to achieve a *P* value < .001, a total of eight differentially expressed genes remained and were used establish a 3MT gene signature; specifically, the *SRR*, *PLXNC1*, *FBX O30*, and *CHD5* genes were downregulated, and the *UTP4*, *EEF1AKNMT*, *NCBP2AS2*, and *CA5BP1* genes were upregulated (Fig 3A; see the Data Supplement for the function of these genes). We then performed a k-means analysis on the basis of the 3MT signature scores to predict urinary 3MT level in the DCOG cohort (n = 26 and 64 patients in the validation and test cohorts, respectively). Sensitivity, specificity, and accuracy of the 3MT gene signature to predict urinary 3MT level of the test cohort and validation cohort are shown in the Data Supplement. We found that the 3MT signature correctly predicted urinary 3MT level in 80 of 90 cases (89%; Fig 3B). Given that tyrosine hydroxylase (TH, encoded by the *TH* gene) is the rate-limiting enzyme in catecholamine biosynthesis,[12] we then measured *TH* expression in the four patient clusters defined by their 3MT signature score and 3MT level (Fig 3B) and found that the group with high 3MT signature scores but without elevated urinary 3MT (ie, the five patients in the top-left quadrant in Fig 3B) had significantly lower TH expression compared with the other three patient groups (*P* < .05, Data Supplement). Moreover, we found a strong positive correlation between the 3MT signature scores and urinary 3MT levels (Fig 3C). Finally, we examined EFS and OS in the entire DCOG cohort (N = 122) stratified by 3MT signature scores. We found that the 50 patients with a high 3MT signature score had significantly poorer survival compared with the 72 patients with a low 3MT signature score (Figs 3D and 3E). These results suggest that the 3MT gene signature score may serve as a surrogate marker for urinary 3MT levels.

The 3MT Gene Signature Score As a Prognostic Factor

To confirm the putative association between a high 3MT signature score and poor outcome, we also performed a k-means analysis using the COG cohort (N = 247) and GPOH cohort (N = 498). In both the COG and GPOH cohorts, the patients with a high 3MT signature score had significantly poorer EFS (Figs 4A and 4C, respectively) and OS (Figs 4B and 4D, respectively) compared with patients with a low 3MT signature score (all *P* < .001). Multivariate analyses of both cohorts revealed that a high 3MT signature score is an independent risk factor for poor EFS (Figs 4I and 4K) and OS (Figs 4J and 4L). In addition, we repeated the analysis of EFS and OS separately for the subset of patients in the GPOH with localized disease (International Neuroblastoma Staging System stage 1-3) without MNA (Figs 4E and 4F) and for the subset of patients in the GPOH cohort with high-risk disease (Figs 4G and 4H). In both subsets, the patients with a high 3MT signature score had significantly poorer EFS and OS mortality compared with the patients with a low 3MT signature score. Similar results were obtained after we

excluded patients with MNA from the high-risk patient group in the GPOH cohort (Data Supplement), consistent with our results obtained for the COG cohort, which consists primarily of high-risk patients. The results provided in this article show the association between a high 3MT signature score and poor outcome. Analysis on the basis of different cohorts suggest an association between 3MT gene signature score and survival outcomes among patients with either low-risk or high-risk neuroblastoma.

The 3MT Gene Signature Score Can Predict MYC Activity

Performing a direct gene ontology analysis failed to link the 3MT gene signature with any specific biologic process (data not shown). Therefore, we looked for possible correlations between the 3MT gene signature and other known gene signatures from the Broad Institute (the Oncogenic, Hallmarks, and Curated databases) and the KEGG database and found, among other correlations (Data Supplement), several strong positive correlations with *MYC*-related processes (Fig 5A). We then examined the correlation between the 3MT gene signature and both a known *MYCN* activity signature[18] and the recently identified *MYC* activity signature.[19] We found strong positive correlations between our 3MT signature and both the *MYCN* (Fig 5B) and *MYC* (Fig 5C) signatures. Finally, we performed a k-means analysis to categorize the GPOH cohort into patients with high tumoral *MYC* activity and patients with low tumoral *MYC* activity. On the basis of their MYC activity status and 3MT signature, the patients were allocated into four distinct groups (Fig 5D), demonstrating that the 3MT signature accurately predicted *MYC* activity in the tumor in 436 of 498 patients, with a sensitivity of 87% and specificity of 88%.

Given that MNA can also provide an indication of high MYC activity in the tumor, we then focused on the subset of patients with MNA (indicated by the red symbols in Fig 5D). We found that MNA predicted high MYC activity tumor with a sensitivity of 59% and a specificity of nearly 100%; thus, the presence of a high 3MT gene signature score may be a more suitable biomarker than MNA with respect to high MYC activity in the tumor. As shown in Figure 5D, 8% of patients with a high 3MT signature score had low MYC activity, and 4% of patients with a low 3MT signature score high MYC activity. Strikingly, we also found that patients with a high 3MT signature score—regardless of their MYC activity status—had poorer EFS and OS compared with patients with a low 3MT signature score (Figs 5E and 5F). Interestingly, however, the OS of patients with a high 3MT signature score was significantly worse in the subset of patients with high MYC activity (*P* = .008; Fig 5F), whereas EFS was not affected by MYC activity in the subset of patients with a high 3MT signature score (Fig 5E).

Increased MYC Activity May Underlie the Increase in Urinary 3MT Levels

Previously, we reported that MNA was correlated with both elevated 3MT levels and decreased levels of the
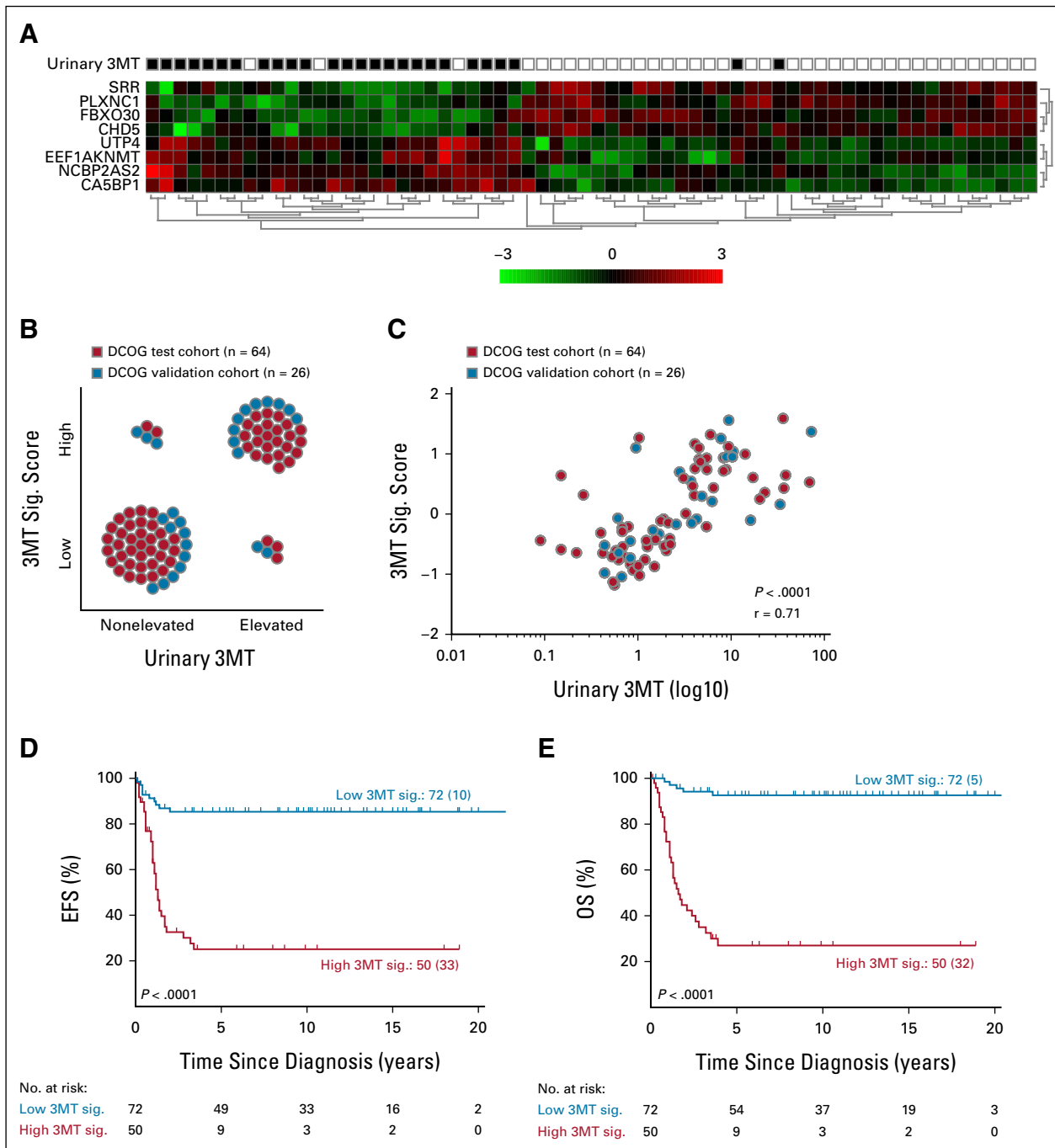
FIG 3. Elevated urinary 3MT levels are correlated with a unique tumoral 3MT gene sig. (A) The 3MT gene sig. generated on the basis of gene expression data obtained from patients in the DCOG test cohort (n = 64); gene expression was compared between patients with elevated urinary 3MT levels (black squares) and patients without elevated urinary 3MT (white squares), revealing that four genes (*UTP4*, *METTL13*, *NCBP2AS2*, and *CA5BP1*) were significantly upregulated, whereas four other genes (*SRR*, *PLXNC1*, *FBX O30*, and *CHD5*) were significantly downregulated in the patients with elevated urinary 3MT levels. (B) By applying k-means analysis to the DCOG validation cohort (n = 26; blue symbols), all 90 patients in the cohort were categorized as having either a high or low 3MT sig. score and are plotted against their corresponding binary urinary 3MT status (ie, elevated or nonelevated). (C) For each patient, the 3MT score is plotted against the corresponding urinary 3MT level; the correlation was determined using Pearson's correlation coefficient. (D) EFS and (E) OS are plotted for the subgroup of patients in the DCOG cohort with a low 3MT sig. and the subgroup of patients with a high 3MT sig. 3MT, 3-methoxytyramine; DCOG, Dutch Cancer Oncology Group; EFS, event-free survival; OS, overall survival; sig., signature.
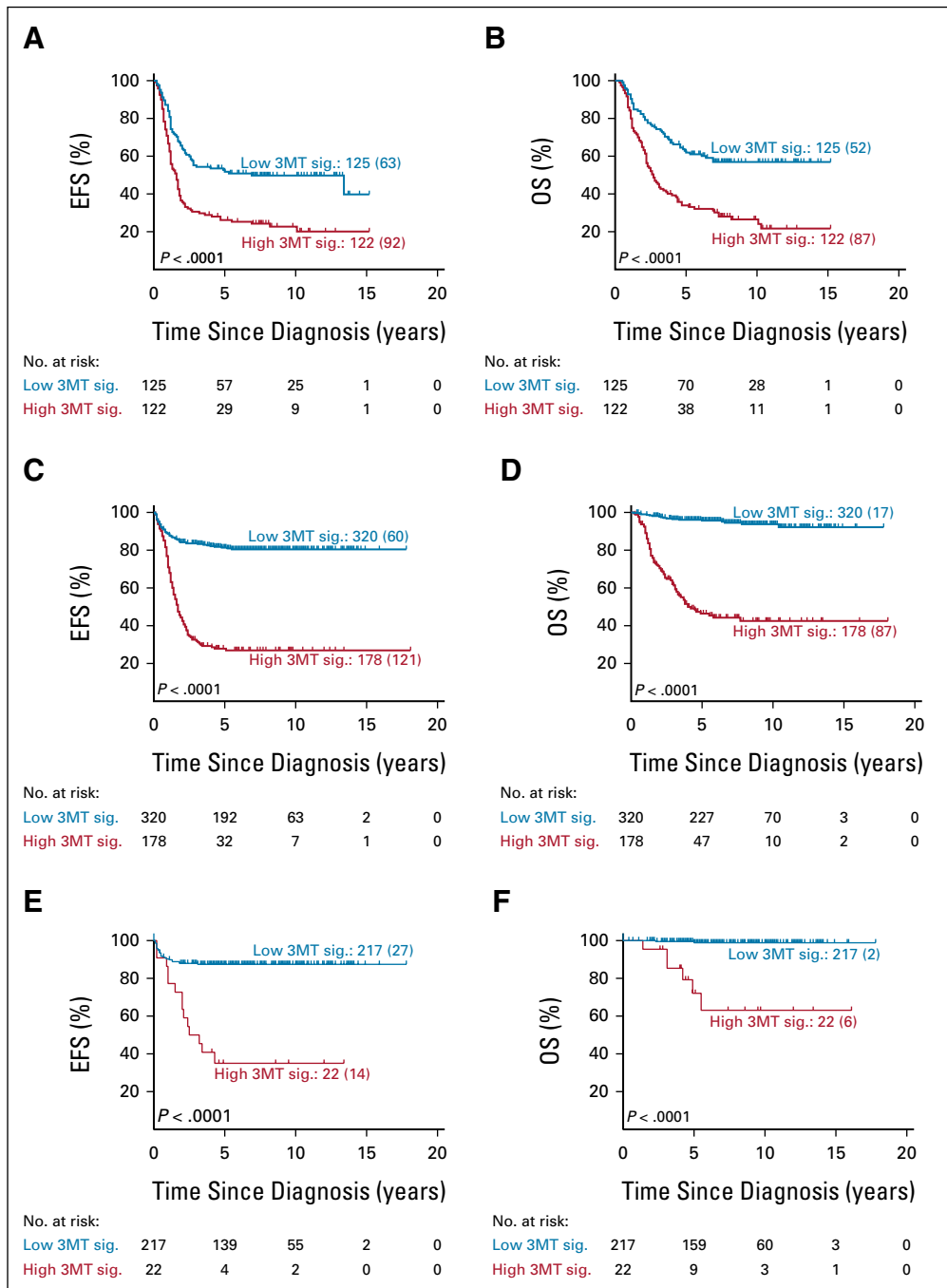
FIG 4. The 3MT gene sig. and clinical outcome. (A) EFS and (B) OS are plotted for the patients in the COG cohort, stratified by 3MT sig. (N = 247 patients). (C) EFS and (D) OS are plotted for the patients in the GPOH cohort, stratified by 3MT sig. (N = 498). (E) EFS and (F) OS are plotted for the subgroup of patients in the GPOH cohort with localized disease without MNA, stratified by 3MT sig. (n = 239). (G) EFS and (F) OS are plotted for the subgroup patients in the GPOH cohort with high-risk disease, stratified by 3MT sig. (n = 193). Forest plot on the basis of the results of the multivariate analysis for (I) EFS and (J) OS in the COG cohort. Forest plot on the basis of the results of the multivariate analysis for (K) EFS and (L) OS in the GPOH cohort. Stage was removed from the multivariate analysis in the COG cohort since 90% of the patients in this cohort had stage 4 disease. The 3MT gene sig. score was defined as high or low using k-means analysis on the basis of the expression of the eight genes that comprise the 3MT gene sig. 3MT, 3-methoxytyramine; COG, Children's Oncology Group; EFS, event-free survival; GPOH, German Pediatric Oncology Group; INSS, International Neuroblastoma Staging System; MNA, *MYCN* amplification; OS, overall survival; sig., signature.
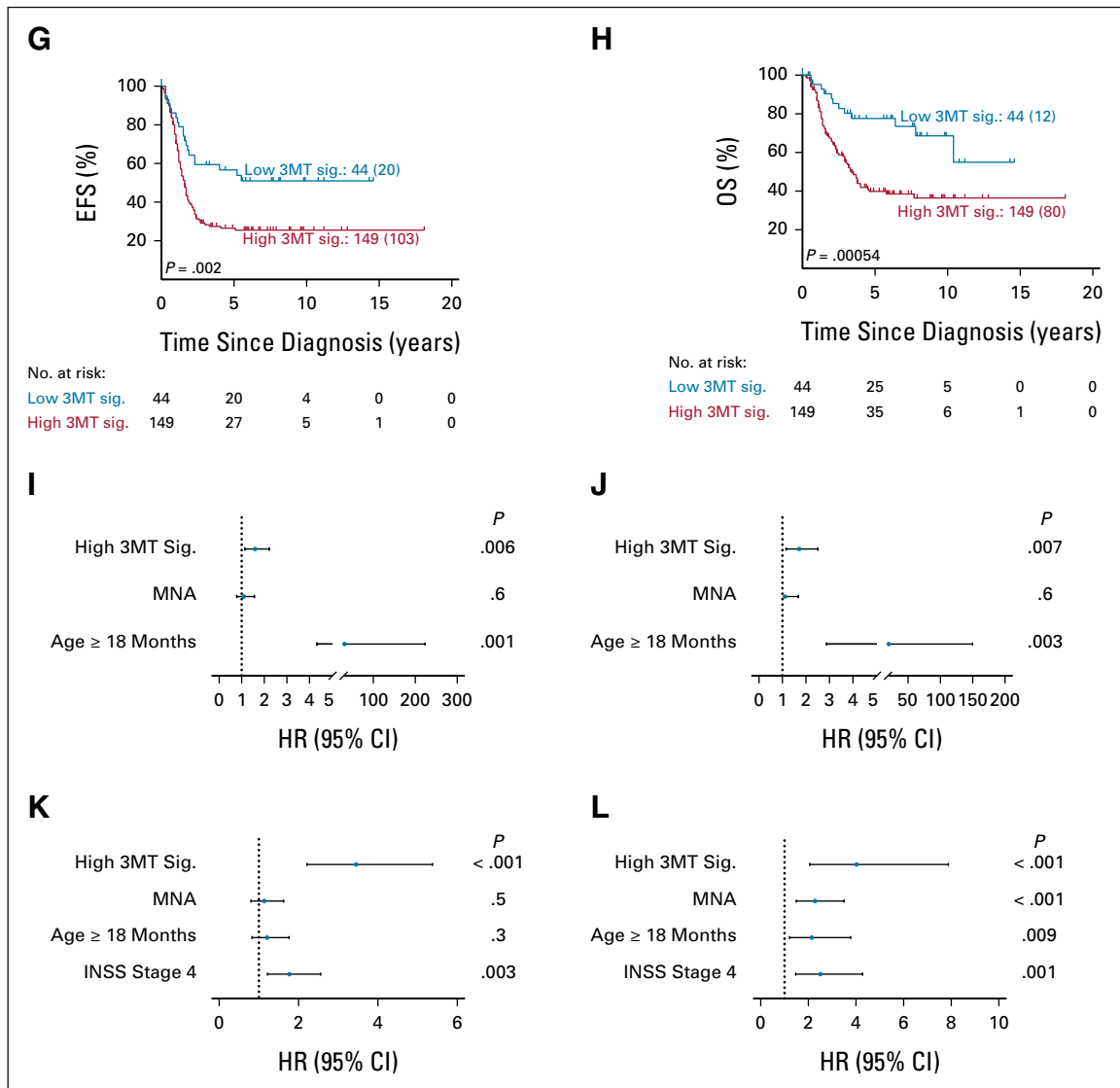
FIG 4. (Continued)

catecholamine metabolite vanillylmandelic acid in two in-dependent cohorts.[14] Given our finding that the 3MT gene signature is strongly correlated with MYC activity in the tumor, we investigated whether high MYC activity is linked to catecholamine metabolism and elevated urinary 3MT levels. We found that patients with high tumoral MYC activity had significantly higher levels of urinary dopamine and 3MT and significantly lower levels of vanillylmandelic acid and the metabolite normetanephrine compared with patients with low tumoral MYC activity (Fig 6A). Taken together, these results suggest that high MYC activity may affect the conversion of dopamine to norepinephrine by downregulation of the enzyme DBH (Fig 6B). To tests this theory, MYCN was overexpressed in MYCN single-copy cell lines. High MYCN protein expression was accompanied by a profound decrease in DBH protein expression (Data Supplement). The presence of doxycycline itself resulted in

only a small decrease in DBH suppression, which was much less profound when compared with that observed in clones with MYCN overexpression (Data Supplement). Classification of the DCOG, COG, and GPOH cohorts into high and low MYC activity groups using k-means analysis and subsequently comparing DBH expression between these two groups showed that high MYC activity was always accompanied by significantly lower DBH expression compared with the corresponding group with low MYC activity (Fig 6C). These results were confirmed by staining primary tumor samples for MYCN and DBH, which dem-onstrated that the majority of neuroblastoma tumors with high MYCN staining had low DBH staining, and vice versa (Fig 6E). For example, in patient 583, the tumor had strong MYCN staining and weak DBH staining, whereas the tumor in patient 105 had weak MYCN staining and strong DBH staining (Fig 6D). Thus, overall, the patients with elevated
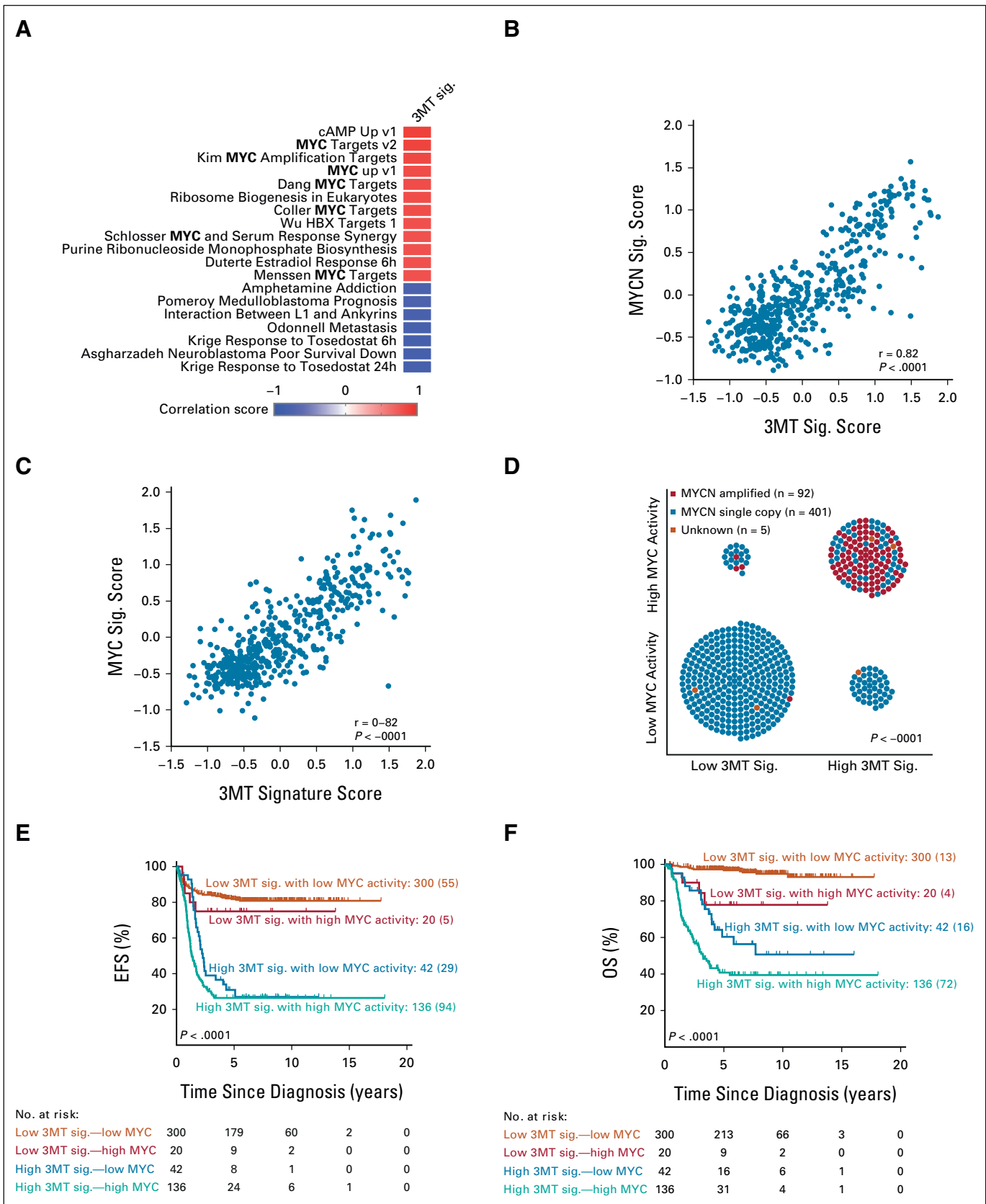
A

	3MT sig.
cAMP Up v1	
MYC Targets v2	
Kim **MYC** Amplification Targets	
MYC up v1	
Dang **MYC** Targets	
Ribosome Biogenesis in Eukaryotes	
Coller **MYC** Targets	
Wu HBX Targets 1	
Schlosser **MYC** and Serum Response Synergy	
Purine Ribonucleoside Monophosphate Biosynthesis	
Duterte Estradiol Response 6h	
Menssen **MYC** Targets	
Amphetamine Addiction	
Pomeroy Medulloblastoma Prognosis	
Interaction Between L1 and Ankyrins	
Odonnell Metastasis	
Krige Response to Tosedostat 6h	
Asgharzadeh Neuroblastoma Poor Survival Down	
Krige Response to Tosedostat 24h	

Correlation score −1 0 1

B

r = 0.82
P < .0001

MYCN Sig. Score vs 3MT Sig. Score

C

r = 0−82
P < −0001

MYC Sig. Score vs 3MT Signature Score

D

- MYCN amplified (n = 92)
- MYCN single copy (n = 401)
- Unknown (n = 5)

High MYC Activity / Low MYC Activity
Low 3MT Sig. / High 3MT Sig.

P < −0001

E

EFS (%) vs Time Since Diagnosis (years)

Low 3MT sig. with low MYC activity: 300 (55)
Low 3MT sig. with high MYC activity: 20 (5)
High 3MT sig. with low MYC activity: 42 (29)
High 3MT sig. with high MYC activity: 136 (94)

P < .0001

No. at risk:
Low 3MT sig.—low MYC	300	179	60	2	0
Low 3MT sig.—high MYC	20	9	2	0	0
High 3MT sig.—low MYC	42	8	1	0	0
High 3MT sig.—high MYC	136	24	6	1	0

F

OS (%) vs Time Since Diagnosis (years)

Low 3MT sig. with low MYC activity: 300 (13)
Low 3MT sig. with high MYC activity: 20 (4)
High 3MT sig. with low MYC activity: 42 (16)
High 3MT sig. with high MYC activity: 136 (72)

P < .0001

No. at risk:
Low 3MT sig.—low MYC	300	213	66	3	0
Low 3MT sig.—high MYC	20	9	2	0	0
High 3MT sig.—low MYC	42	16	6	1	0
High 3MT sig.—high MYC	136	31	4	1	0

FIG 5. (continued on following page)

FIG 5. (Continued). The 3MT sig. can accurately predict MYC activity in neuroblastoma. (A) The GPOH cohort (N = 498 patients) was used to compare the 3MT gene sig. to gene signatures from the Broad Institute (Oncogenic, Hallmarks, and Curated databases) and KEGG database. Only gene signatures that were correlated strongly with the 3MT sig. (defined as a correlation score > 0.75 or < −0.75) are shown. (B-C) The 3MT sig. score for each patient in the GPOH cohort was plotted against the corresponding MYCN sig. score[18] (B) and MYC sig. score[19] (C). (D) A k-means analysis was used to categorize the patients in the GPOH cohort as having a high 3MT sig. score or a low 3MT sig. score, and each patient is plotted against the corresponding MYC activity status. (E) EFS and (F) OS was plotted for the indicated patient subgroups. 3MT, 3-methoxytyramine; EFS, event-free survival; GPOH, German Pediatric Oncology Group; OS, overall survival; sig., signature. Elevated urinary 3-methoxytyramine (3MT) at diagnosis is an independent risk factor for poor event-free survival and poor overall survival in neuroblastoma. Our analysis including gene expression data and urinary 3MT showed that increased urinary 3MT corresponds with tumor MYC activity.

urinary 3MT levels had relatively low DBH expression, high MYC activity, and a generally poorer prognosis compared with patients without elevated urinary 3MT levels (Fig 6E).

DISCUSSION

In this study, we investigated the biologic basis underlying the putative association between elevated urinary 3MT levels and poor prognosis in patients with neuroblastoma and identified a gene expression pattern associated with increased urinary 3MT levels and high MYC activity.

Our results obtained from both retrospective Italian and prospective Dutch cohorts support our previous finding that an elevated urinary 3MT level above the prognostic cutoff defined as a > 2.9-fold change[11] is associated with poor prognosis. Our previous finding that elevated 3MT levels were associated with poor EFS[11] independent of disease stage, patient age, MNA status, and loss of heterozygosity of chromosome 1p was confirmed in our prospective Dutch cohort, but because of the relatively brief median follow-up time (2.2 years), this could not be demonstrated for OS. In the Italian cohort, with significantly more low-risk patients, the 3MT correlation with EFS and OS was demonstrated by Cox regression analyses only. Finally, because the Italian (SIOPEN) and Dutch (DCOG) protocols differ with respect to induction chemotherapy, consolidation chemotherapy, and radiotherapy dosage (Data Supplement), our results confirm our previous finding that the association between elevated urinary 3MT levels and poor prognosis is not therapy-dependent.[11]

Importantly, our analysis revealed that urinary 3MT levels correspond to a specific gene expression pattern in the primary tumor—the so-called 3MT gene signature—in 89% of cases. We previously reported that non–catecholamine-excreting neuroblastoma tumors do not express the enzyme TH.[12] Consistent with this finding, the five patients in our study who had a high 3MT signature score but nonelevated urinary 3MT levels also had significantly lower expression of tumoral TH. With respect to the five patients who had a low 3MT signature score but elevated urinary 3MT levels, it is conceivable that the entire tumor may not have been represented, as only a small portion of the tumor was used to isolate RNA.[9]

In using the 3MT gene signature as a proxy for urinary 3MT levels, we confirmed the association between elevated urinary 3MT levels and poor outcome in two international cohorts of patients with neuroblastoma. This association was

observed for the entire cohort, which included all risk groups, as well as among patients with high-risk neuroblastoma, regardless of MNA status; moreover, high 3MT signature scores were associated with poor prognosis independent of disease stage, the patient's age, and MNA status. Importantly, our results provide the first evidence that urinary 3MT level has prognostic value in patients with low-risk neuroblastoma. Interestingly, a recent study described 3MT as a promising marker for monitoring disease activity.[20] The presence of elevated urinary 3MT levels in patients initially classified as low-risk might allow the upstaging of such patients ensuring that they receive the most suitable therapy.[5,6] Currently, prospective validation of these findings is carried out in a European cohort with the aim to assess whether and how urinary 3MT levels at diagnosis should be integrated into our risk-stratification model.

Gene ontology analysis was unable to link the 3MT gene signature to any biologic process, likely because of the fact that the 3MT signature consists of only eight genes, of which only the *CHD5* and *PLXN1C* genes were studied previously in the context of neuroblastoma.[21,22] The *CHD5* gene is located on chromosomal region 1p36.31, which is frequently lost in high-risk neuroblastoma[21]; it is, therefore, conceivable that the 3MT gene signature reflects a specific chromosomal aberration. However, this is unlikely, given that all eight genes in the signature are located on different chromosomes and/or chromosomal arms. Moreover, comparing the 3MT gene signature to other cancer-related gene signatures revealed a strong positive correlation with MYC activity status in the tumor.

High MYC activity is generally associated with a more aggressive clinical phenotype.[18,19] However, in neuroblastoma, only MNA has been used as a biomarker for MYC activity in risk-assessment models,[2,3] and MNA occurs in approximately 50% of patients with neuroblastoma with high MYC activity.[18] This finding is consistent with our own finding that only 59% of patients with high MYC activity had MNA; the remaining patients with increased MYC activity might be explained by an amplification of *c-MYC*, although this is very rare,[23] and/or increased stability of the *MYCN* protein or other genetic aberrations.[18,24] Furthermore, although rare, MNA status can differ between cells in the primary tumor, as well as between the primary tumor and its metastases.[25,26] Taken together, these results indicate the need for a more sensitive biomarker for determining MYC activity in neuroblastoma. In this respect, increased urinary 3MT levels provide a far more
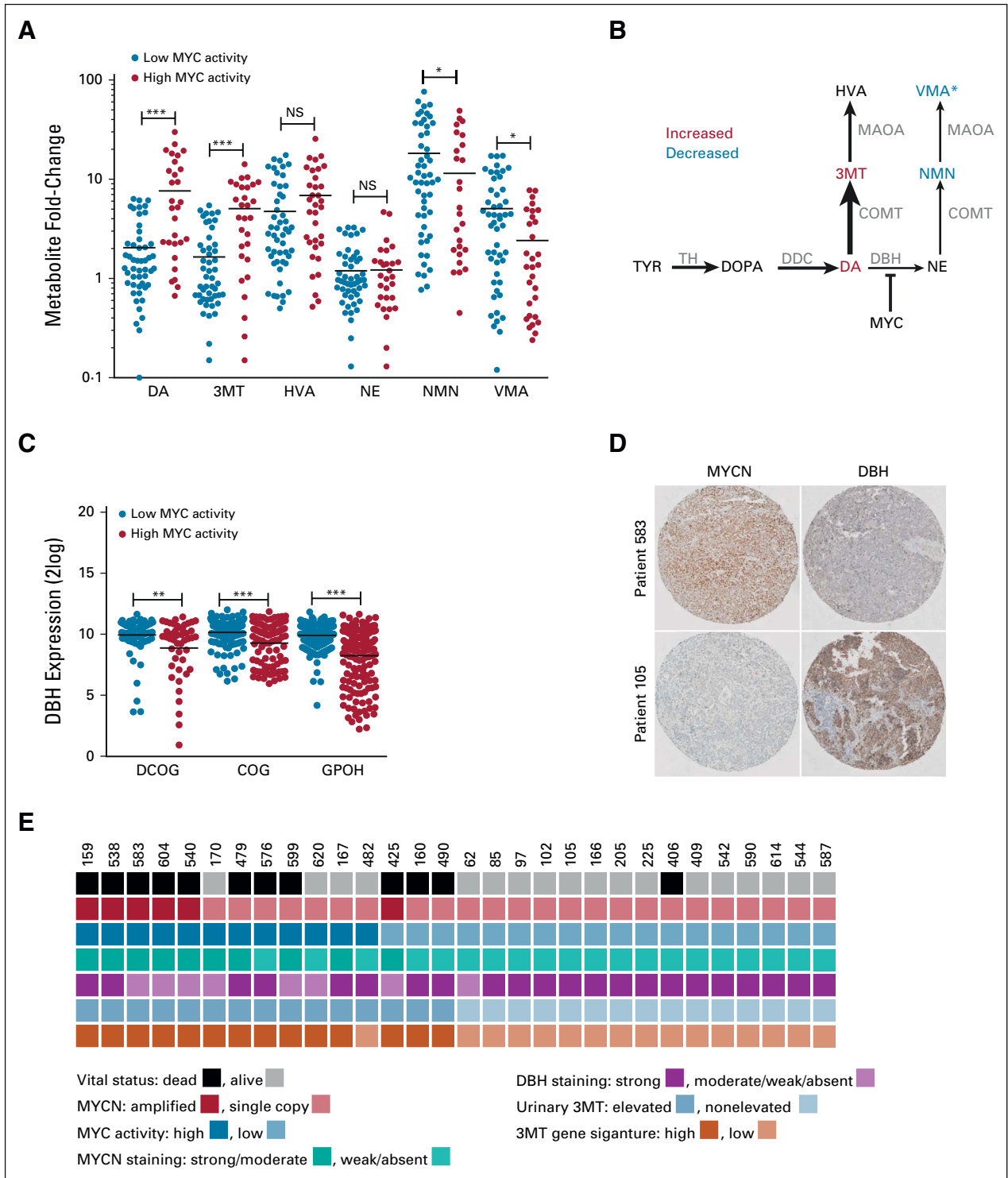
FIG 6. MYC activity affects catecholamine biosynthesis. (A) The indicated catecholamines and catecholamine metabolites levels were measured in the patients in the retrospective Dutch cohort with known MYC activity status and are plotted separately for patients with low MYC activity (blue) and patients with high MYC activity (red). Fold change is defined as metabolite concentration per mmol creatinine divided by the upper limit of the age-related reference range. *$P < .05$, ***$P < .001$, and NS. (B) Pathway depicting the biosynthesis of catecholamines from tyrosine, and the production of catecholamine metabolites. Note that the production of VMA from NMN requires additional steps not shown here. (C) *DBH* mRNA was measured in tumor samples obtained from the DCOG, COG, and GPOH cohorts, stratified for patients with low MYC activity (blue) or high MYC activity (red). **$P < .01$ and ***$P < .001$. (D) Representative examples of primary neuroblastoma samples obtained from patient 583 (a patient with elevated urinary 3MT) and patient 105 (a patient without elevated urinary 3MT) and stained for MYCN and DBH (scale bar: 100 μm). (E) Summary of 30 patients with neuroblastoma. For each patient, the vital status, MCA status, MYC activity, MYCN staining, (continued on following page)

FIG 6. (Continued) DBH staining, urinary 3MT status, and 3MT signature are indicated. 3MT, 3-methoxytyramine; COG, Children's Oncology Group; COMT, catechol-O-methyltransferase; DA, dopamine; DBH, dopamine β-hydroxylase; DCOG, Dutch Cancer Oncology Group; DDC, dopa decarboxylase; DOPA, 3,4-dihydroxy-L-phenylalanine ((L)-DOPA); GPOH, German Pediatric Oncology Group; HVA, homovanillic acid; MAOA, monoamine oxidase A; NE, norepinephrine; NMN, normetanephrine; NS, not significant; TH, tyrosine hydroxylase; TYR, tyrosine; VMA, vanillylmandelic acid.

sensitive biomarker of high MYC activity in neuroblastoma. Thus, circulating biomarkers such as urinary 3MT may reflect the biology of the entire tumor more accurately than a genetic profile obtained from a biopsy.[10-12] Future studies in which urinary 3MT levels are compared with MNA status and MYC activity—determined either from solid biopsies or liquid biopsies—are warranted to support this correlation. Furthermore, given the increasing availability of therapies that target MYC/MYCN signaling,[27] analyzing urinary 3MT levels may help identify patients who would likely benefit from these therapies.

Previously, MNA status was associated with specific patterns of urinary catecholamines[14,28] that were attributed to decreased DBH expression.[12,29] Here, we provide the first evidence that both MNA and high MYC activity are inversely correlated with DBH expression in neuroblastoma measured at both the mRNA and protein levels. Furthermore, we found that both elevated urinary 3MT levels and a high 3MT gene signature score were correlated with high MYC activity and inversely correlated with DBH expression, indicating that high MYC activity in neuroblastoma likely underlies the increase in urinary 3MT levels.

Although we found high concordance (88%) between MYC activity status and 3MT signature scores, we also identified a subset of patients (8%) who had a high 3MT gene signature score but a low MYC activity, and poor outcome. It is therefore conceivable that other oncogenic mechanisms may also underlie the increase in urinary 3MT levels. In this respect, it is interesting to note that several studies identified telomere maintenance as a key feature of high-risk neuroblastoma associated with poor outcome.[5,30,31] Telomeres are maintained by both MYC activity–related (eg, MNA) and MYC activity–independent (eg, rearrangement of the TERT gene) genetic alterations.[30,31] Taken together, it is therefore possible that elevated urinary 3MT levels—similar to telomere maintenance—is a general feature of high-risk neuroblastoma associated with multiple genetic aberrations.

In conclusion, using both retrospective and prospective cohorts, we found that an elevated urinary 3MT level at diagnosis predicts poor outcome in patients with neuroblastoma, independent of disease stage, the patient's age, and MNA status. This association can be attributed—at least to a large extent—to high MYC activity in the tumor. Thus, urinary 3MT levels, which can be readily measured using a variety of validated methods,[32] may provide a noninvasive method for determining MYC activity in the tumor, thereby improving risk assessment in neuroblastoma.

AFFILIATIONS
[1]Princess Máxima Center for Pediatric Oncology, Utrecht, the Netherlands
[2]Department of Clinical Chemistry, Cancer Center Amsterdam, Amsterdam Gastroenterology Endocrinology Metabolism, Amsterdam University Medical Center, University of Amsterdam, the Netherlands
[3]Department of Medical Statistics and Bioinformatics, Leiden University Medical Center, Leiden, the Netherlands
[4]Mathematical Institute, Leiden University, Leiden, the Netherlands
[5]Department of Oncogenomics, Amsterdam University Medical Center, University of Amsterdam, Amsterdam, the Netherlands
[6]Department of Pathology, Amsterdam University Medical Center, University of Amsterdam, Amsterdam, the Netherlands
[7]Central Laboratory of Analyses, IRCCS Istituto Giannina Gaslini, Genoa, Italy

CORRESPONDING AUTHOR
Godelieve A. M. Tytgat, MD, PhD, Princess Máxima Center for Pediatric Oncology, Heidelberglaan 25, 3584 CS, Utrecht, the Netherlands NLD528; e-mail: G.A.M.Tytgat@prinsesmaximacentrum.nl.

EQUAL CONTRIBUTION
I.R.N.V., Y.A.H.M., L.J.V., G.A.M.T., and A.B.P.v.K. shared first and last authorship.

Manuscript writing: All authors
Final approval of manuscript: All authors
Accountable for all aspects of the work: All authors

AUTHORS' DISCLOSURES OF POTENTIAL CONFLICTS OF INTEREST

The following represents disclosure information provided by authors of this manuscript. All relationships are considered compensated unless otherwise noted. Relationships are self-held unless noted. I = Immediate Family Member, Inst = My Institution. Relationships may not relate to the subject matter of this manuscript. For more information about ASCO's conflict of interest policy, please refer to www.asco.org/rwc or ascopubs. org/po/author-center.

Open Payments is a public database containing information reported by companies about payments made to US-licensed physicians (Open Payments).

Jan Koster

Consulting or Advisory Role: Frame Therapeutics

No other potential conflicts of interest were reported.

REFERENCES

1. Park JR, Bagatell R, Cohn SL, et al: Revisions to the international neuroblastoma response criteria: A consensus statement from the National Cancer Institute clinical trials planning meeting. J Clin Oncol 35:2580-2587, 2017

2. Cohn SL, Pearson AD, London WB, et al: The International Neuroblastoma Risk Group (INRG) classification system: An INRG Task Force report. J Clin Oncol 27:289-297, 2009

3. Pinto NR, Applebaum MA, Volchenboum SL, et al: Advances in risk classification and treatment strategies for neuroblastoma. J Clin Oncol 33:3008-3017, 2015

4. Schleiermacher G, Janoueix-Lerosey I, Ribeiro A, et al: Accumulation of segmental alterations determines progression in neuroblastoma. J Clin Oncol 28:3122-3130, 2010

5. Ackermann S, Cartolano M, Hero B, et al: A mechanistic classification of clinical phenotypes in neuroblastoma. Science 362:1165-1170, 2018

6. Oberthuer A, Juraeva D, Hero B, et al: Revised risk estimation and treatment stratification of low- and intermediate-risk neuroblastoma patients by integrating clinical and molecular prognostic markers. Clin Cancer Res 21:1904-1915, 2015

7. Brodeur GM, Pritchard J, Berthold F, et al: Revisions of the international criteria for neuroblastoma diagnosis, staging, and response to treatment. J Clin Oncol 11:1466-1477, 1993

8. Ambros PF, Ambros IM, Brodeur GM, et al: International consensus for neuroblastoma molecular diagnostics: Report from the International Neuroblastoma Risk Group (INRG) Biology Committee. Br J Cancer 100:1471-1482, 2009

9. Marusyk A, Almendro V, Polyak K: Intra-tumour heterogeneity: A looking glass for cancer? Nat Rev Cancer 12:323-334, 2012

10. Combaret V, Audoynaud C, Iacono I, et al: Circulating MYCN DNA as a tumor-specific marker in neuroblastoma patients. Cancer Res 62:3646-3648, 2002

11. Verly IRN, van Kuilenburg ABP, Abeling N, et al: 3-Methoxytyramine: An independent prognostic biomarker that associates with high-risk disease and poor clinical outcome in neuroblastoma patients. Eur J Cancer 90:102-110, 2018

12. Verly IRN, Leen R, Meinsma JR, et al: Catecholamine excretion profiles identify clinical subgroups of neuroblastoma patients. Eur J Cancer 111:21-29, 2019

13. Goldstein DS: Catecholamines 101. Clin Auton Res 20:331-352, 2010

14. Verly IR, van Kuilenburg AB, Abeling NG, et al: Catecholamines profiles at diagnosis: Increased diagnostic sensitivity and correlation with biological and clinical features in neuroblastoma patients. Eur J Cancer 72:235-243, 2017

15. National Cancer Institute Office of Cancer Genomics: TARGET Data Matrix, https://ocg.cancer.gov/programs/target/data-matrix

16. Su Z, Fang H, Hong H, et al: An investigation of biomarkers derived from legacy microarray data for their utility in the RNA-seq era. Genome Biol 15:523, 2014

17. Koster J: Genomics analysis and visualization platform, http://r2.amc.nl

18. Valentijn LJ, Koster J, Haneveld F, et al: Functional MYCN signature predicts outcome of neuroblastoma irrespective of MYCN amplification. Proc Natl Acad Sci USA 109:19190-19195, 2012

19. Jung M, Russell AJ, Liu B, et al: A myc activity signature predicts poor clinical outcomes in Myc-associated cancers. Cancer Res 77:971-981, 2017

20. Lam L, Woollard GA, Teague L, et al: Clinical validation of urine 3-methoxytyramine as a biomarker of neuroblastoma and comparison with other catecholamine-related biomarkers. Ann Clin Biochem 54:264-272, 2017

21. Fujita T, Igarashi J, Okawa ER, et al: CHD5, a tumor suppressor gene deleted from 1p36.31 in neuroblastomas. J Natl Cancer Inst 100:940-949, 2008

22. Lebedev TD, Vagapova ER, Popenko VI, et al: Two receptors, two isoforms, two cancers: Comprehensive analysis of KIT and TrkA expression in neuroblastoma and acute myeloid leukemia. Front Oncol 9:1046, 2019

23. Westermann F, Muth D, Benner A, et al: Distinct transcriptional MYCN/c-MYC activities are associated with spontaneous regression or malignant progression in neuroblastomas. Genome Biol 9:R150, 2008

24. Wang LL, Suganuma R, Ikegaki N, et al: Neuroblastoma of undifferentiated subtype, prognostic significance of prominent nucleolar formation, and MYC/MYCN protein expression: A report from the Children's Oncology Group. Cancer 119:3718-3726, 2013

25. Theissen J, Boensch M, Spitz R, et al: Heterogeneity of the MYCN oncogene in neuroblastoma. Clin Cancer Res 15:2085-2090, 2009

26. Marrano P, Irwin MS, Thorner PS: Heterogeneity of MYCN amplification in neuroblastoma at diagnosis, treatment, relapse, and metastasis. Genes Chromosomes Cancer 56:28-41, 2017

27. Fletcher JI, Ziegler DS, Trahair TN, et al: Too many targets, not enough patients: Rethinking neuroblastoma clinical trials. Nat Rev Cancer 18:389-400, 2018

28. Zambrano E, Reyes-Mugica M: Hormonal activity may predict aggressive behavior in neuroblastoma. Pediatr Dev Pathol 5:190-199, 2002

29. Izbicki T, Izbicka E, Mazur J: Prognostic significance of biochemical heterogeneity of catecholaminergic clones in neuroblastoma. J Pediatr Surg 41:1506-1512, 2006

30. Hertwig F, Peifer M, Fischer M: Telomere maintenance is pivotal for high-risk neuroblastoma. Cell Cycle 15:311-312, 2016

31. Peifer M, Hertwig F, Roels F, et al: Telomerase activation by genomic rearrangements in high-risk neuroblastoma. Nature 526:700-704, 2015

32. Plenis A, Oledzka I, Kowalski P, et al: Recent trends in the quantification of biogenic amines in biofluids as biomarkers of various disorders: A review. J Clin Med 8:640, 2019

■ ■ ■