Mémoire de Maîtrise en médecine

Evaluation rétrospective de la signification pronostique de la méthoxytyramine plasmatique dans le suivi d'enfants atteints de neuroblastome de risque bas et intermédiaire

Retrospective study of the prognostic value of the plasma methoxytyramine in the follow-up of children with low or intermediate risk neuroblastoma

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1. Abstract

Background and objective: Neuroblastoma (NB) accounts for 10% of the malignancies in children and is responsible for 15% of cancer related child mortality. Urine vanillylmandelic acid (VMA) and homovanillic acid (HVA) are the current gold standard for the diagnosis of NB. Urine harvesting might be particularly challenging considering the early childhood occurrence of NB, and this technique provides only suboptimal diagnostic sensitivity (70-80%). Based on previous studies, there was evidence for a superior sensitivity of methoxylated plasma catecholamine derived metabolites called metanephrines. This project was designed to give a first retrospective insight on the use of plasma metanephrines and, particularly the free methoxytyramine as a biomarker for a diagnosis and follow-up approach of NB.

Patients and methods: Retrospective study of a panel of 15 patients with low to intermediate risk NB. Patients were reviewed for clinical data and follow-up of imaging and laboratory results. None had renal dysfunction. The plasma total and free metanephrine (MN), normetanephrine (NMN) and 3-methoxytyramine (MT) were analyzed and compared with age and gender-based reference percentiles established for 191 healthy pediatric controls by Franscini et al. (2015).

Results: Follow up data up suggested a great variability in biomarker pattern with a broad spectrum of pathologies. Some tumors were still metabolically active although radiologically stable, which makes biomarkers interpretation challenging. Other patients still exhibited tumoral tissue without any metabolic activity. In a vast majority of cases, we observe normalisation of both metanephrines and catecholamines after treatment.

At diagnosis, the combination of VMA and HVA urinary markers had a sensitivity of 90%. Plasma free MT had only 46% sensitivity with 0.1 nmol/l as cut-off. Plasma total MT and free NMN for NB diagnosis as single markers showed an 87% and 93% sensitivity respectively. Interestingly sensitivity increased up to 100% when plasma total MT and free NMN were combined.

Conclusion and perspectives: In this retrospective observational design, our follow-up data underlies the broad clinical presentation of NB correlated with great variability of both catecholamines and metanephrines patterns. This underlies the need of considering the patient as a whole clinical and biological picture. The main outcome measure was 100% sensitivity for NB diagnosis with combined use of plasma total MT and free NMN in low to intermediate risk patients. In contrast, fMT seems not to be a satisfying parameter at diagnosis for low risk to intermediate risk specific NB disease. We confirmed better sensitivity of Franscini et al. partitioning by age and gender percentiles for plasma metanephrines as compared with the current reference limits. These promising results need further assessment and confirmation in a prospective large national multicentric study including low to high-risk NB patients.

2. Résumé

Contexte et objectif: Le neuroblastome (NB) est une néoplasie qui provoque de 10% des tumeurs malignes chez l'enfant et est responsable de 15% de la mortalité infantile liée au cancer. La mesure de l'acide vanillylmandélique (VMA) et l'acide homovanillique (HVA) urinaires est la référence actuelle pour le diagnostic du NB. La récolte urinaire est particulièrement difficile chez l'enfant et cette technique permet uniquement un diagnostic avec une sensibilité sous-optimale (70-80%). Basé sur des études antérieures, une sensibilité supérieure a été attribuée à la mesure plasmatique de métabolites dérivés des catécholamines, les métanephrines. Ce projet a été conçu pour donner un premier aperçu rétrospectif sur l'utilisation des métanéphrines plasmatiques, en particulier de la méthoxytyramine libre comme biomarqueur pour une approche diagnostique et de suivi du NB.

Patients et méthodes: Étude rétrospective d'un panel de 15 patients présentant un neuroblastome de risque faible à intermédiaire. Les données cliniques et de suivi recueillies comprenaient des résultats d'imagerie et de laboratoire. Aucun patient ne présentait d'insuffisance rénale. Les rechutes cliniques ont été définies comme la progression de la maladie sur les modalités d'imagerie. Les métabolites plasmatiques totaux et libres de la métanéphrine (MN), normétanéphrine (NMN) et 3-méthoxytyramine (MT) ont été analysés et comparés avec les percentiles de référence selon l'âge et le genre établis sur une base de 191 contrôles pédiatriques en bonne santé par Franscini et al. (2015).

Résultats: Les données du suivi des patients suggèrent une grande variabilité à la fois dans les valeurs de biomarqueurs et dans les pathologies. Certaines tumeurs étaient métaboliquement actives, alors que radiologiquement stables, ce qui rend l'interprétation des biomarqueurs difficile. D'autres patients avaient encore des résidus tumoraux non métaboliquement actifs. Dans la plupart des cas, on observe une normalisation des métanéphrines après traitement.

Lors du diagnostic, la combinaison des marqueurs urinaires VMA et HVA avait une sensibilité de 90%. La sensibilité de la fMT était seulement de 46% (cut-off 0.1 nmol/l). La sensibilité isolée de la MT totale et de la NMN libre pour le diagnostic NB était respectivement de 87% et 93%. La sensibilité augmente à 100% lorsque la MT totale plasmatique et la NMN libre sont combinées.

Conclusion and perspectives: Dans ce modèle observationnel rétrospectif, les données suggèrent une présentation clinique large avec une grande variabilité des patterns de catecholamines et métanéphrines. Ceci souligne l'utilité de considérer le patient comme un tout, biologique et clinique. Le résultat principal fait état d'une sensibilité de 100% pour le diagnostic au NB avec l'utilisation combinée de la MT totale plasmatique et de la NMN libre. Nous avons confirmé une meilleure sensibilité de la répartition des percentiles par âge et genre de Franscini et al. pour les métanéphrines plasmatiques par rapport aux limites de référence actuellement utilisées. Ce résultat prometteur doit être évalué et confirmé dans le cadre d'une vaste étude multicentrique nationale prospective portant sur des patients présentant des neuroblastomes à risque faible à élevé de rechute.

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4. Introduction

4.1. Neuroblastoma

Neuroblastoma (NB) is a neuroendocrine tumour of the peripheral sympathetic nervous system, arising from multipotent neural crest cells. It is the most frequent extracranial solid tumour in children, represents 8-10% of malignancies during childhood (1), and is believed to be responsible of 15% of cancer related mortality in childhood (2). This disease has a broad spectrum of clinical behavior with contrasting pattern of outcome, ranging from spontaneous regression to life-threatening disease resistant to intense multimodal therapy (3). It presents most commonly as an abdominal mass, with the adrenal gland as a frequent primary site.

The diagnosis of neuroblastoma is based on the convergence of clinical, imaging, histological and biological features. An international consensus defined the diagnostic elements of neuroblastoma, among which the measurement of urinary catecholamines and their metabolic derivatives, which are discussed in more details later (4). More recently, the International Neuroblastoma Risk Group Staging System (INRGSS) used age, stage, histology and molecular pathology [DNA ploidy, MYCN amplification] to stratify patients into low, medium and high risk groups (5) for recurrence. The correct risk-stratification of patients is particularly critical for the adequate therapeutic approach with well-defined protocols as well as differential survival rates. Multimodal treatment is based upon this risk-stratification and can include chemotherapy, surgery, autologous cells transplant, immunotherapy with or without radiotherapy.

Various clinicopathological and biological factors are known that correlate with patients' outcome and prognosis. Age > 18 months at diagnosis, unfavourable histology according to Shimada, *MYCN* amplification and segmental chromosomal aberrations (SCA) such as 17q gain and 11q loss on a molecular level, and alteration in DNA ploidy such as diploidy or tetraploidy are associated with poor prognosis and outcome (6-8).

4.2. Catecholamines and peptides

One hallmark of current diagnosis and follow-up of neuroblastoma is the quantification of urine catecholamines with a spot test based on a 24 hour urinary collection or normalization by creatininuria. The understanding of the metabolic pathway of catecholamines provides the rationale for the biochemical method proposed in our study. Catecholamines are hormones or neurotransmitters synthesized from the amino acid tyrosine. Among them are adrenaline (EPI), noradrenaline (NE) and dopamine (DA).

There are basically two multistep enzymatic pathways through which the catecholamines are produced, a major neuronal route and a minor extraneuronal route. The neuronal route occurs through postganglionic fibers of the sympathetic nervous system. The extraneuronal pathway leads to the formation of O-methylated metabolites through the action of catechol-O-methyltransferase enzyme (COMT). This specific enzyme is expressed within the adrenal medullary cells, in the liver and in the kidneys, and is not present in the catecholamine producing neurons.

The O-methylated derivatives are 3-methoxytyramine (MT) for DA, normetanephrine (NMN) for NE, and metanephrine (MN) for EPI. MN and NMN can be sulfated or further metabolised by monoamine oxidases (MAO) in vanillylmandelic acid (VMA) and 3-MT in homovanillic acid (HVA). These end products are then typically excreted in the urine. The extensive sulfate-conjugation can occur in cells that express the monoamine-preferring phenolsulfotransferase

(SULT1A3) such as mesenteric organs. MN, NMN and MT are classified as free (unconjugated) or total (sum of sulfate-conjugated plus free).

Formerly, dosage of catecholamines and their metabolites in urine was carried out by fluorometric methods (6). Recently, the specificity has been increased by high-pressure liquid chromatography methods, allowing each metabolite to be separately identified. However, despite this upgrade in the measuring methods, it has been shown that the sensitivity in the diagnosis and follow-up of neuroblastoma remains poor. Strenger et al. showed sensitivity of DA, VMA and HVA of respectively 61.3%, 80.7% and 71.9% (7). Interestingly, sensitivity increases to 91.2% when all parameters are combined. The determination of VMA and HVA is currently considered as the gold standard for the diagnosis and monitoring of neuroblastoma.

Urinary measurement of catecholamines can be technically challenging, as neuroblastoma occurs in young children. The secretion is highly variable over time. There are strict recommendations for urine sampling, as values can be influenced by environmental factors such as type of food, nutritional status or drugs. Urinary spot on a 24 hours urine collection is used but the urine harvesting in young patients is often incomplete and only partially corrected by the creatinine value obtained. Creatinine values also rely on variable parameters such as muscular mass, nutritional status and physical activity.

Recently it has been shown that plasma catecholamines which are intratumorally converted by catechol-O-methyltransferase (COMT) into metanephrines, have become the gold standard test for the diagnosis of pheochromocytoma, a rare neuroendocrine tumor in children (8–10). Lenders et al. found that plasma free metanephrines provide the best test for excluding or confirming pheochromocytoma compared to plasma catecholamines and urinary metanephrines in a multicenter cohort of patients (8). Furthermore Grouzmann et al showed that in absence of renal failure, the combination of free and total (i.e. free.sulfated metanephrines) metanephrines in plasma improved the diagnostic specificity for pheochromocytoma. This has later also been confirmed for pediatric patients with pheochromocytoma (11). In order to study the role of plasma metanephrines in pediatric neuroendocrine tumors such as neuroblastoma, we established in 2015 (12) normal values in healthy children from birth to 18 years. Our study will focus on evaluation of plasma metanephrines that have been measured in a limited number of patients with known diagnosis of NB in parallel to the establishment of normal plasma metanephrine values in children. The study protocol has been approved by the local ethics committee (No 2017-00382), and written informed consent has been obtained from all the families.

5. Materials and Methods

5.1. Patient recruitment

The study cohort included 15 pediatric patients (mean age at diagnosis 1.91 years) who have been treated and followed at the Unit of Pediatric Hematology and Oncology at the Centre Hospitalier Universitaire Vaudois (CHUV), between 2003 and 2017. Two of 15 patients presented a ganglioneuroma and 13 a neuroblastoma proven by biopsy. They were treated according to the current SIOPEN protocol for non-high risk neuroblastoma (LINES) and assigned to very low, low or intermediate risk group categories according to the INRGSS used in the protocol which comprises age at diagnosis, disease extension, histology and molecular markers. They had no renal failure and had received no anti-hypertensive drugs during 2 weeks prior to the blood sampling.

Each patient file was reviewed for clinical documentation including age, weight, height, diagnosis, histology, biology (N-MYC amplification, 1p-, 17q+ or other segmental abnormalities), stage, treatment subgroup according to risk stratification. Radiological investigations were reviewed with modalities including conventional radiography (RX), magnetic resonance imaging (MRI), ultrasound (US), CT scan, metaiodobenzylguanidine (MIBG) scintigraphy.

5.2. Biochemical assays

Blood for metanephrines measurements was collected on heparine coated tubes from all participants and immediately transferred to the catecholamine/peptide laboratory. A maximum 30 minutes delay before centrifugation was considered as acceptable; blood was centrifuged at 4°C to 3000G, then aliquoted (2 aliquots of 0.2ml per analysis) and quickly frozen (in liquid nitrogen ideally). (10). The analysis comprised the measurement of plasma free and total MT, NMN, MN.

Urines samples included when possible a 24 hours collection. Analysis of urine catecholamines and their derivatives were centralised at the CHUV laboratory (dopamine, HVA, VMA, creatinine) except for the first sample of patient No 5 (done at another external laboratory).

The blood and urinary measurements were performed at time points required by the treatment protocol, which is at diagnosis, during follow-up, end of treatment and follow-up controls. The frequency of the analysis was defined by the risk group and treatment received which extended from observation only up to 6-8 months chemotherapy combined with surgery and occasionally radiotherapy.

5.3. Data analysis

The repeated measurements of free and total plasma metanephrines (MT, NMN, MN) at diagnosis, during and at the end of treatment and during follow-up were based on the published age- and gender-adjusted reference values (12). They were compared to the urinary catecholamines, VMA and HVA, considered currently as « gold standard » for the diagnosis and monitoring of neuroblastoma. The validated reference values established by the laboratory of catecholamines/peptides of the CHUV (appendix 3) were used to determine normal and pathological values at diagnosis and follow-up. Results were considered as abnormal if equal to or higher than the upper reference limit. Relative fold change was expressed as a value on the basis of the upper reference limit for each variable. The results of catecholamine and metanephrine measurements were represented graphically over time in parallel to disease response to treatment, follow-up, which included also clinical observation, treatment modalities and imaging. Response to treatment was defined according to RECIST criteria (13) and divided in complete remission, partial remission, stable disease or progression.

Sensitivity at diagnosis was assessed and compared with both validated reference values of the CHUV (appendix 3) and >97.5th percentile of age/gender reference values in healthy children by Franscini et al (2015).

Statistics: The comparison between plasma and urine markers values among risk groups was performed by Student t-test. The values of fMT that were under the lower limit of detection (<0.03 nmol/l) were replaced by 0 to facilitate comprehension. All statistical tests were two-sided and a *P* value less than 0.05 was considered as statistically significant. Matlab software (2017, version 9.2) has been used for all the statistics.

6. Results

6.1. Clinical characteristics

Between 2003 and 2016, 15 patients with very low, low or intermediate risk NB were treated according to the SIOPEN protocols. They were eligible for our review. There were 5 boys and 10 girls with a mean age at diagnosis of 1.91 years (range 0.01 – 7.98) (appendix 1). A majority of patient (8/15) were above 18 months old at diagnosis. All patients were from the very low, low or intermediate risk group. The INRG stage assignment showed a majority of L2 stage (8/15), whereas 4/15 were L1 stage and 3/15 MS stage. Two patients of L2 stage displayed ganglioneuroblastoma as histology (patient 1 and 13). Primary tumor site was abdominal in 13/15 patients, and thoracic in 2/15 patients. We reported 3 patients with MS stage displaying liver metastasis. Among these, patient No 6 presented also orbitar, intramuscular and subcutaneous metastasis.

No bone marrow invasion was reported. None of the patient presented MYC amplification at diagnosis and 4 patients were identified with triploidy. Other segmental aberrations were found in 4 patients. These included mutations like 11q loss or 17q gain in 2/4 patients.

6.2. Clinical history and follow-up of urinary catecholamines and plasma metanephrines

The following graphs present the patients' clinical findings, treatment, response to treatment, plasma metanephrines and urinary catecholamines at the various time points. Values are expressed as the upper reference limits (in red) with the relative fold change in brackets (appendix 3). As explained earlier, free MT is expressed as 0 if <0.03 nmol/l and values over the reference limit displayed in red circles. More details about clinical history can be found in the appendix.

Treatments acronyms are CADO for cyclophosphamide, doxorubicine and vincristine and C/VP-16 for caboplatin-topotecan (VP-16). Response to treatment is defined as complete remission (CR), partial remission (PR) or very good partial remission (VGPR).

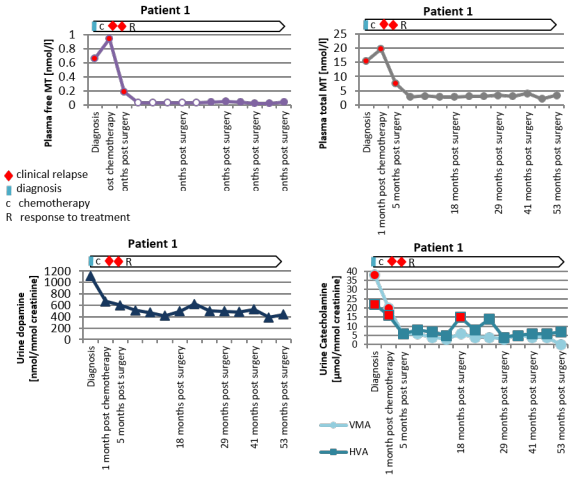
6.2.1. L2 stage patients, intermediate risk group

Patient 1

Age: 18 months
Localisation: intrathoracic
Extension: Local with intracanalar tumoral extension (dumbbell tumor)
Histology: ganglioneuroblastoma intermixed further maturated to ganglioneuroma after chemotherapy
Biology: no N-MYC amplification, triploid
Treatment: 2 C/VP-16 + 4 CADO (partial response) + 2 steps surgery
Response to treatment: CR
Diagnosis: increased catecholamines (VMA and HVA) and plasma metanephrines (fMT and tMT)
Clinical relapse: two events. One without data, one with increased metanephrines (fMT, tMT) and catecholamines except dopamine.
Follow-up: isolated increase in HVA 2 years after surgery, not significant in this context. Progressive normalisation pattern of both metanephrine and catecholamines.

Conclusion: tMT and fMT normalized later than urinary catecholamines reflecting possibly a residual microscopic tumor activity

Patient 1		Diagnosis	1 month post chemotherapy	5 months post surgery	18 months post surgery	29 months post surgery	41 months post surgery	53 months post surgery
urine	dopamine [nmol/mmol creatinine]	1112	670	603	494	497	529	440
	VMA [µmol/mmol creatinine]	38 (3.8N)	20 (2N)	6	6	3	4	0
	HVA urine [µmol/mmol créat]	22 (1.3N)	16 (1.1N)	6	15 (1N)	4	6	7
plasma	tMT [nmol/l]	15.27 (3.6N)	19.74 (4.7N)	7.44 (1.8N)	2.88	3.44	4.12	3.42
	fMT nmol/l	0.66 (11N)	0.94 (15.7N)	0.18 (3N)	0	0.05	0.02	0.04



Patient 2

Age: 1 month

Localisation: Retroperitoneal

Extension: Local with intracanalar tumoral extension (dumbbell tumor)

Histology: neuroblastome no otherwise specified (NOS). Poorly differentiated, poor stroma

Biology: MYCN not amplified

Treatment: 6 courses cyclophosphamide/ vincristine + 2 courses C/VP-16. No surgery on remained suprarenal mass (too risky)

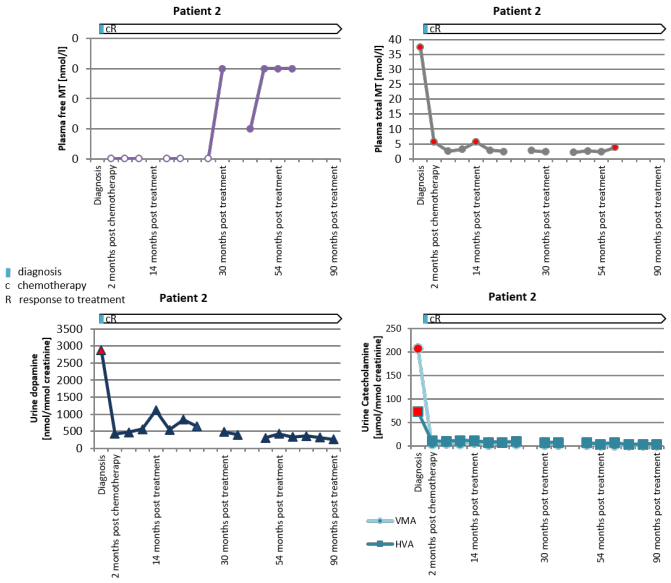
Response to treatment: VGPR

Diagnosis: increased urine catecholamines (Dopamine, VMA,HVA) and tMT. No values for fMT obtained

Clinical relapse: none

Conclusion: tMT remained still elevated while catecholamines and fMT were already normalized. Not tumor related value?

Patient 2		Diagnosis	2 months post chemotherapy	14 months post treatment	30 months post treatment	54 months post treatment	90 months post treatment
urine	dopamine [nmol/mmol creatinine]	2874 (1.9N)	421	1113	481	426	261
	VMA [µmol/mmol creatinine]	208 (14.9N)	5	7	4	2	2
	HVA urine [µmol/mmol créat]	73 (3.7N)	11	11	7	4	4
plasma	tMT [nmol/l]	37.46 (9N)	5.62 (1.3N)	5.6 (1.3N)	2.4	2.4	
	fMT [nmol/l]		0		0.03	0.03	



Patient 3

Age: 4 years 9 months

Localisation: Retroperitoneal

Extension: Local into the L4-L5 foramina

Histology: neuroblastoma

Biology: MYCN not amplified, triploid

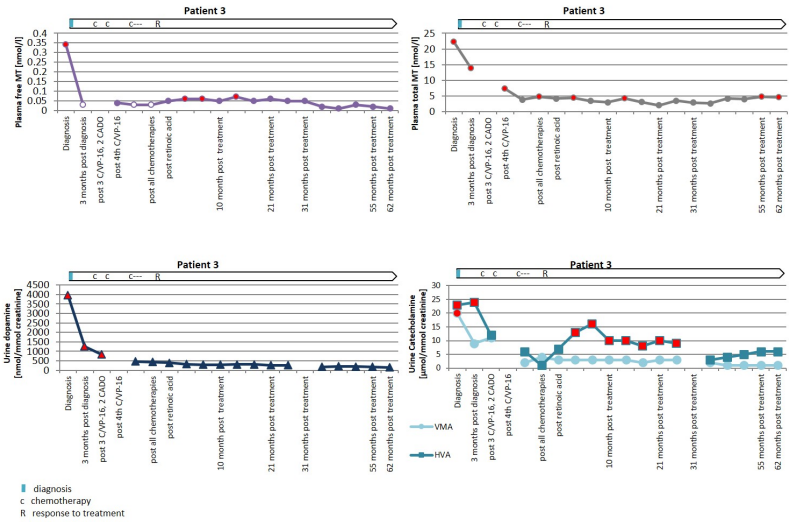
Treatment: 4 Carboplatin/VP-16 + 2 CADO + partial resection surgery + 2 adjuvant therapy with Topotecan vincristine doxorubicine + high dose chemotherapy with CEM (carboplatin, etoposide, melphalan) with autologous stem cell reinfusion + adjuvant retinoic acid

Response to treatment: PR

Follow-up: slightly elevated tMT and HVA values with progressive normalisation of fMT and HVA. The 3 slightly elevated fMT values were 0.06-0.07 nmol/l, which corresponds to the upper reference limit.

Clinical relapse: none

Patient 3		Diagnosis	3 months post diagnosis	post 3 C/VP-16, 2 CADO	post 4th C/VP-16	post all chemotherapies	post retinoic acid	10 month post treatment	21 months post treatment	31 months post treatment	55 months post treatment	62 months post treatment
urine	dopamine [nmol/mmol creatinine]	3962 (5.4N)	1254 (1.7N)	837 (1.1N)		436	397	292	266		188	148
	VMA [µmol/mmol creatinine]	20 (2N)	9	11 (1.1N)		4	3	3	3		1	1
	HVA urine [µmol/mmol créat]	23 (1.5N)	24 (1.6N)	12		1	7	10 (1.4N)	10 (1.4N)		6	6
plasma	tMT [nmol/l]	22.18 (5.3N)	13.86 (3.3N)		7.24 (1,7N)	4.67 (1.1N)	4.13	3	2.06	2.88	4.79 (1.1N)	4.63 (1.1N)
	fMT nmol/l	0.34 (5.6N)	0		0.04	0	0.05	0.05	0.06	0.05	0.02	0.01



Conclusion: metabolically active tumor with non-growing residual lesions. In presence of a residual tumor and among plasma measurements, the fMT seemed to be the most reliable value

Patient 4

Age: 4 years 9 months

Localisation: Right paravertebral

Extension: Local with invasion of the spinal canal (dumbbell tumor)

Histology: poorly differentiated neuroblastoma (NOS)

Biology: NMYC not amplified, no genetic abnormalities

Treatment: 4 C/VP-16

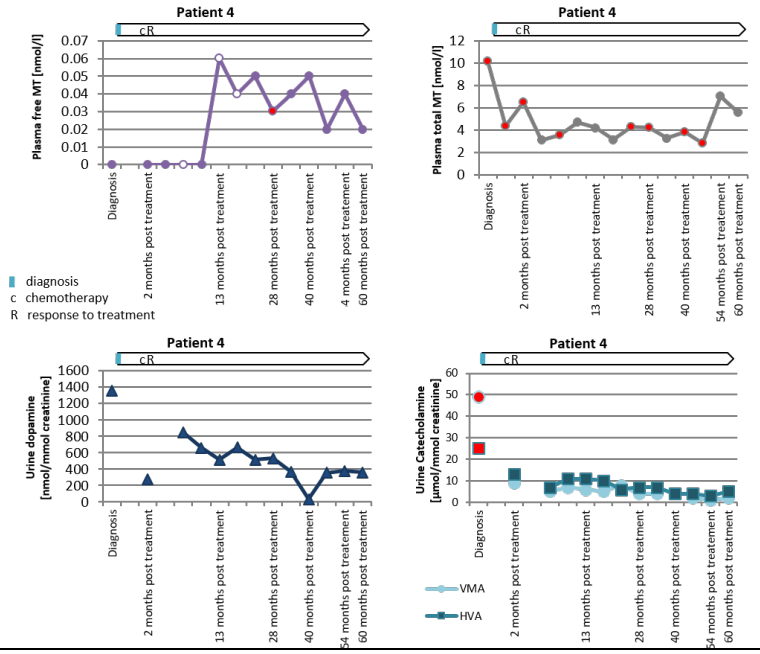
Response to treatment: CR

Follow-up: Radiological exams during 3 years without signs of recurrence. Elevated of tMT at the end of the follow-up not significant in this context.

Clinical relapse: none

Patient 4		Diagnosis	2 months post treatment	13 months post treatment	28 months post treatment	40 months post treatment	54 months post treatement	60 months post treatment
urine	dopamine [nmol/mmol creatinine]	1361	277	515	534	33	377	362
	VMA [µmol/mmol creatinine]	49 (3.5N)	9	6	4		1	2
	HVA urine [µmol/mmol créat]	25 (1.5N)	13	11	7	4	3	5
plasma	tMT [nmol/l]	10.16 (2.4N)	6.5 (1.6N)	4.24 (1N)	4.24 (1N)	3.86	7.04 (1.7N)	5.58 (1.3N)
	fMT nmol/l	0	0	0.06 (1N)	0.03	0.05	0.04	0.02

Conclusion: good concordance between fMT and catecholamines.



Patient 5

Age: 13 months

Localisation: hemiabdomen

Extension: Local with intra-ductal extension and compression of medullary cone (dumbbell tumor)

Histology: neuroblastoma NOS type with poor stroma, low MKI

Biology: MYCN not amplified, tetrasomy 2, 11q-, 1p-, 17q-

Treatment: 2 C/VP-16 + 2 CADO + 2 partial surgery (first intraductal, then abdominal component) + 2nd look complement surgery

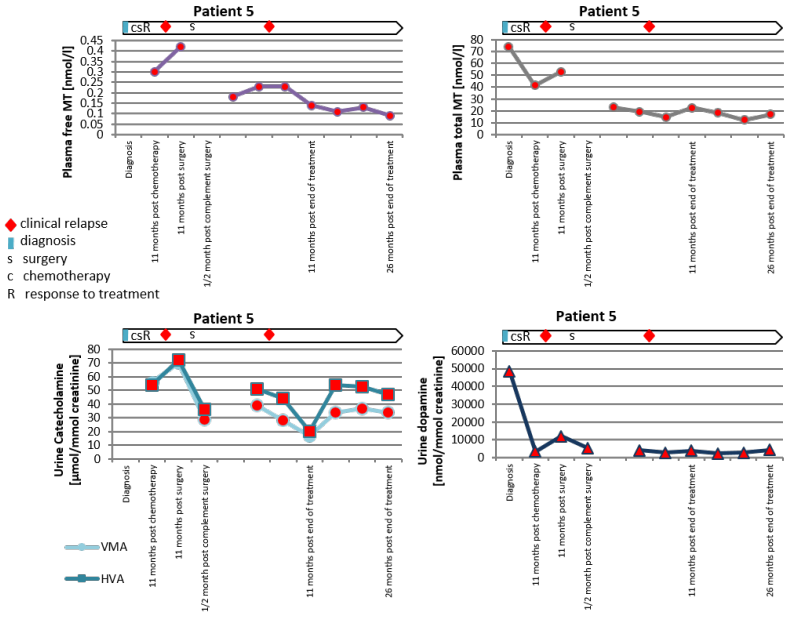
Response to treatment: PR

Follow-up: progressive decline in both urinary and plasma biomarkers. Stability of the lesions during radiological exams after surgery.

Clinical relapse: 2 events, without increased urinary or plasma parameters

Conclusion: persistence of a metabolically active but radiologically stable disease.

Patient 5		Diagnosis	11 months post chemotherapy	11 months post surgery	1/2 month post complement surgery	11 months post end of treatment	26 months post end of treatment
urine	dopamine [nmol/mmol creatinine]	48500 (32.3N)	3437 (2.6N)	12144 (16.4N)	5562 (7.5N)	3874 (7.2N)	4427 (6N)
	VMA [µmol/mmol creatinine]		56 (5.6N)	70 (7N)	29 (2.9N)	17 (1.7N)	34 (3.4N)
	HVA urine [µmol/mmol créat]		54 (3.2N)	72 (4.8N)	36 (2.4N)	20 (1.3N)	47 (3.1N)
plasma	tMT [nmol/l]	74 (17.6N)	41.28 (10N)	52.94 (12.6N)		22.63 (5.9N)	17.13 (4N)
	fMT nmol/l		0.3 (5N)	0.42 (7N)		0.14 (2.3N)	0.09 (1.5N)



Patient 10

Age: 26 months

Localisation: Left paravertebral

Extension: Local with intraspinalextension and spinal compression (dumbbell tumor)

Histology: low differentiated neuroblastoma (NOS)

Biology: MYCN not amplified, tetraploidy, SCA as 11q-, 17q+, 12q+, X chromosome loss

Treatment: 2 C/VP-16 + 2 CADO + 1 C/VP-16 + 1 CADO + partial surgery + adjuvant radiotherapy + retinoic acid

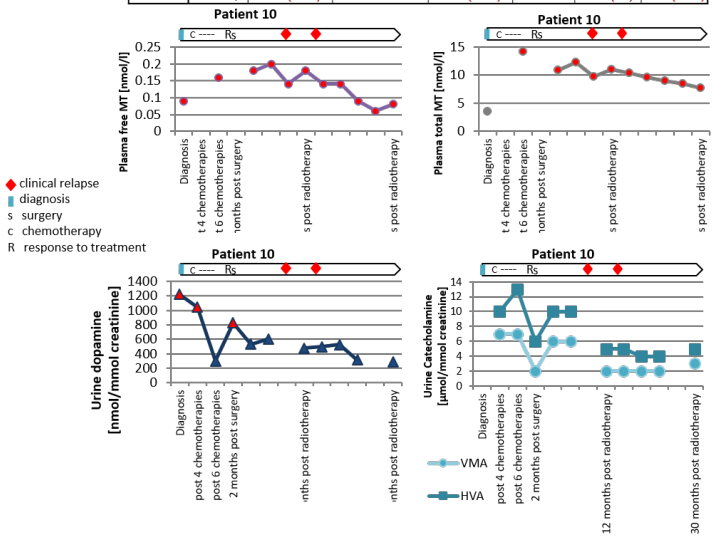
Response to treatment: VGPR

Diagnosis: elevated dopamine and fMT

Follow-up: elevated metanephrines 3 years after the end of the treatment.

Clinical relapse: 2 events, first without direct increase in metanephrine (catecholamines not measured). 3 months later, slight increase in plasma metanephrines with normal catecholamines correlated with an increase tumoral volume.

Patient 10		Diagnosis	post 4 chemotherapies	post 6 chemotherapies	2 months post surgery	12 months post radiotherapy	30 months post radiotherapy
urine	dopamine [nmol/mmol creatinine]	1216 (1.6N)	1040 (1.4N)	299	825 (1.6N)	478	291
	VMA [µmol/mmol creatinine]		7	7	2	2	3
	HVA urine [µmol/mmol créat]		10	13	6	5	5
plasma	tMT [nmol/l]	3.62		14.1 (3.4N)		11 (2.6N)	7.64 (1.8N)
	fMT nmol/l	0.09 (1.5N)		0.16 (2.7N)		0.18 (3N)	0.08 (1.3N)



Conclusion: residual tumor seems to be still active, although not growing

Patient 12

Age: 18 months

Localisation: Retroperitoneal

Extension: Local at diagnosis with further paravertebral and paramediastinum lesion development

Histology: neuroblastoma (differentiating subtype)

Biology: MCYN not amplified, SCA on 1,2,3,5 and 10 chromosomes, deletion 1p

Treatment: 2 CT + 2 CADO

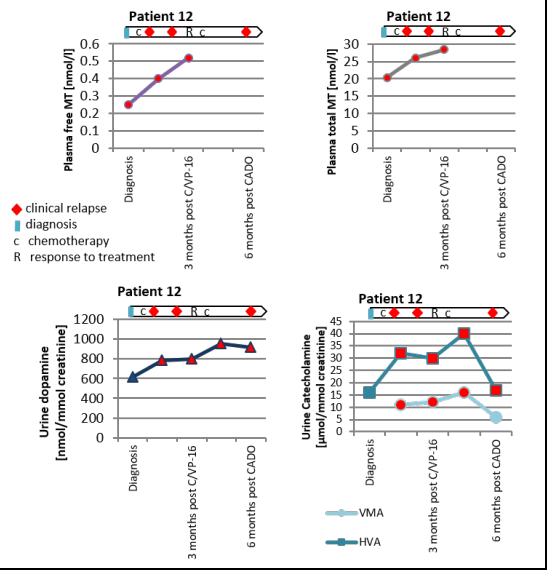
Response to treatment: PR

Follow-up: high levels of catecholamines 7 months post-treatment (normalized VMA). Missing values for plasma metanephrines

Clinical relapses: 3 events. First without biological assay performed. Second one with increase in dopamine, VMA, HVA, tMT and fMT. Third with radiologic extension without biological correlation on catecholamines (missing values for metanephrines).

Conclusion: metabolic active disease with abnormal catecholamines and metanephrines with a progressive growing of the retroperitoneal mass.

Patient 12		Diagnosis	3 months post C/VP.16	6 months post CADO
urine	dopamine [nmol/mmol creatinine]	617	801 (1N)	913 (1.2N)
	VMA [µmol/mmol creatinine]		12 (1.2N)	6
	HVA urine [µmol/mmol créat]	16	30 (2N)	17 (1.1N)
plasma	tMT [nmol/l]	20.34 (4.9N)	28.47 (6.8N)	
	fMT nmol/l	0.25 (4.2N)	0.52 (8.7N)	



Patient 13

Age: 8 years

Localisation: Abdominal

Extension: Local

Histology: ganglioneuroblastoma

Biology: MYNC not amplified

Treatment: 2 Carbo/VP-16 + 2 CADO + surgery

Response to treatment: CR

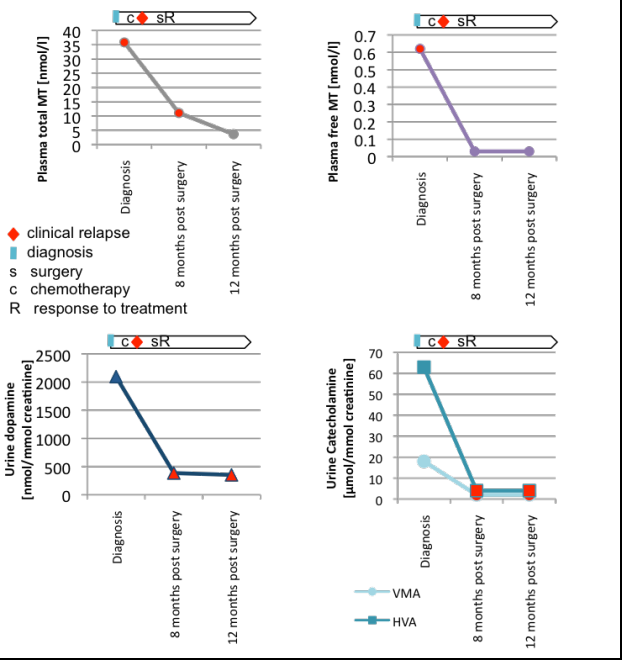
Diagnosis: elevated catecholamines (dopamine, VMA, HVA) and metanephrines (tMT, fMT)

Follow-up: isolated elevated tMT not significant in this context with normalisation after 2 months.

Clinical Relapse: 1 event, without data for urinary or plasma test.

Conclusion: **12 months post treatment the patient remains in both biological and radiological complete remission.**

Patient 13		Diagnosis	8 months post surgery	12 months post surgery
urine	dopamine [nmol/mmol creatinine]	2096 (4.2N)	386	354
	VMA [µmol/mmol creatinine]	18 (2.6N)	2	2
	HVA urine [µmol/mmol créat]	63 (9N)	4	4
plasma	tMT [nmol/l]	35.84 (8.6N)	11.07 (2.6N)	3.63
	fMT [nmol/l]	0.62 (10.3N)	0.03	0.03



6.2.2. MS stage patients

Patient 6

Age: 7 months

Localisation: right adrenal gland

Extension: multiple metastasis including intraorbital, subcutaneous, muscular, cervical, mediastinal, hepatic

Histology: neuroblastome (NOS)

Biology: NMYC not amplified, triploid tumor

Treatment: 4 C/VP-16

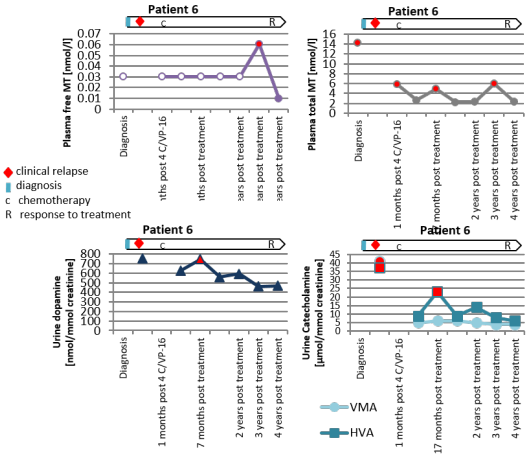
Response to treatment: VGPR

Follow-up: Stability and/or improvement of the oncological status during the following radiological exams. During follow-up, one isolated increase in dopamine, HVA and tMT and one isolated increase in tMT and fMT, not significant in these contexts.

Clinical relapse: 1 event, without data for urinary or plasma test.

Conclusion: progressive normalisation of both catecholamines and metanephrines without signs of progressive disease

Patient 6		Diagnosis	1 months post 4 C/VP-16	17 months post treatment	2 years post treatment	3 years post treatment	4 years post treatment
urine	dopamine [nmol/mmol creatinine]			743 (1N)	595	462	468
	VMA [µmol/mmol creatinine]			6	5	4	4
	HVA urine [µmol/mmol créat]			23 (1.5N)	14	8	6
plasma	tMT [nmol/l]	14.13 (3.8N)	5.84 (1.4N)	4.90 (1.2N)	2.28	6.00 (1.4N)	2.38
	fMT nmol/l	0	0	0	0	0.06 (1N)	0.01



Patient 7

Age: 5 days

Localisation: Abdominal (left surrenalian)

Extension: hepatic nodules

Histology: Neuroblastoma (NOS) with poor stroma, MKI index low

Biology: NMYC not amplified

Treatment: 4 C/VP-16

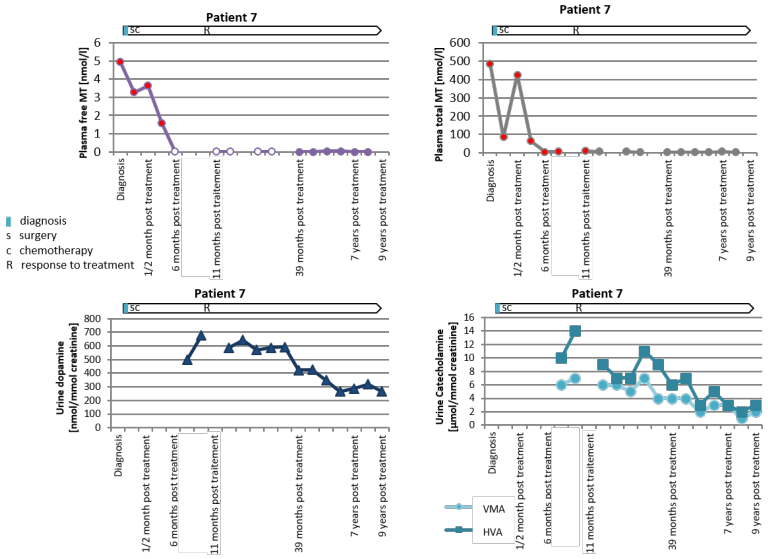
Response to treatment: VGPR

Follow-up: Missed urinary values during the beginning of the follow-up are non-reported measurement due to unnormalized values by creatinemia. No clinical relapse was highlighted.

Clinical relapse: None

Conclusion: 9 years radiological follow-up demonstrated a stable tumoral residue without signs of recurrent disease, together with progressive decline of tMT and fMT.

Patient 7		Diagnosis	1/2 month post treatment	6 months post treatment	11 month post treatment	39 months post treatment	7 years post treatment	9 years post treatment
urine	dopamine [nmol/mmol creatinine]					424	288	265
	VMA [µmol/mmol creatinine]					4	3	2
	HVA urine [µmol/mmol créat]					6	3	3
plasma	tMT [nmol/l]	485.00 (115.8N)	425.78 (101.6N)	5.00 (1,2N)	9.26 (2.2N)	2.4	3.88	
	fMT [nmol/l]	4.92 (82N)	3.64 (60.7N)	0	0	0	0	



Patient 14

Age: 43 days

Localisation: right adrenal

Extension: hepatic metastasis

Histology: poorly differentiated NB, favourable histology

Biology: no MYCN amplification, no SCA

Treatment: no treatments, wait and see strategy

Response to treatment: VGPR

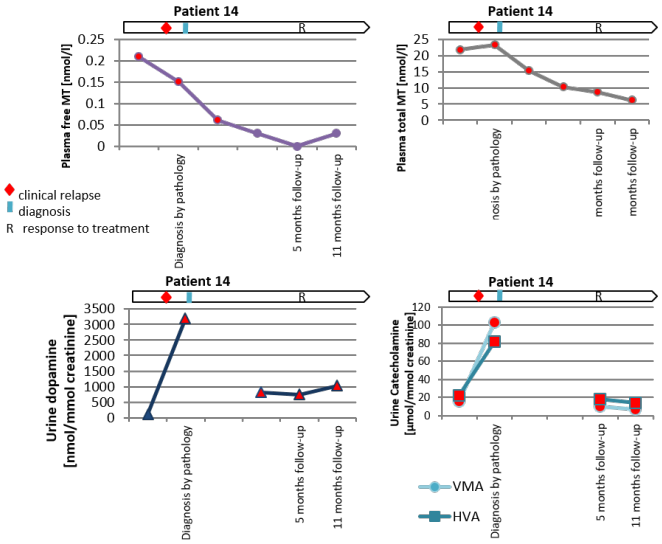
Diagnosis: all biomarkers elevated

Follow-up: Radiology follow-up showed constant regression of the tumoral residue, together with a progressive decrease of urinary dopamine, VMA, HVA and plasma tMT.

Clinical relapses: 1 event before diagnosis confirmed by pathology, with highlight of hepatic metastasis (progression into Ms stage) and increased tumoral mass with correlated with high level of urinary dopamine, VMA, HVA and tMT

Conclusion: fMT corresponds perfectly to the clinical follow-up, which shows less and less disease.

Patient 14		Diagnosis by pathology	5 months follow-up	11 months follow-up
urine	dopamine [nmol/mmol creatinine]	3186 (4.3N)	742 (1N)	1025 (1.4N)
	VMA [µmol/mmol creatinine]	103 (10.3N)	10 (1.4N)	7 (1N)
	HVA urine [µmol/mmol créat]	82 (5.4N)	18 (2.6N)	14 (2N)
plasma	tMT [nmol/l]	23.26 (5.6N)	8.67 (2.1N)	6.23 (1.5N)
	fMT nmol/l	0.15 (2.5N)	0	0.03



6.2.3. L1 stage patients

Patient 8

Age: 45 days

Localisation: surrenalian / Extension: local and further hepatic nodules development during follow-up

Histology: low differentiated neuroblastoma with favorable histology, poor stroma, MKI index low

Biology: triploid aneuploidy, MCYN negative

Treatment: complete surgery

Response to treatment: CR

Diagnosis: Increased dopamine, VMA, HVA (values not normalized by creatinine) and plasma tMT

Follow-up: 7 years of radiological exams showed no sign of tumor recurrence

Clinical relapse: none

Patient 8		Diagnosis
urine	dopamine [nmol/24h]	1318 (1.8N)
	VMA [µmol/24h]	123 (8.8N)
	HVA urine [µmol/24h]	87 (4.4N)
plasma	tMT [nmol/l]	21.50 (5.1N)
	fMT nmol/l	0

Conclusion: increased tMT at diagnosis with normal fMT. No follow-up of urinary or plasma values were recorded for this patient.

Patient 9

Age: 18 months

Localisation: Retroperitoneal paravertebral

Extension: local, no foraminal extension

Histology: neuroblastoma NOS

Biology: MYCN not amplified, SCA (13+, 3-, 4-, 14-, 19-, 21-)

Treatment: complete resection surgery

Response to treatment: CR

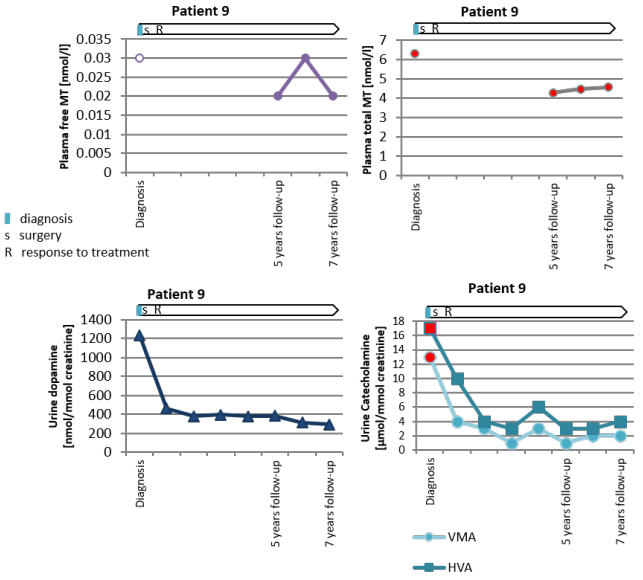
Diagnosis: High values of VMA, HVA and plasma tMT

Follow-up: absence of tumoral recurrence after surgery during 7 years of radiologic exams. tMT in progressive decreased along with the absence of disease recurrence. fMT levels remains normal during follow-up.

Clinical relapse: none

Conclusion: Is normal fMT level correlated with low tumor aggressiveness?

Patient 9		Diagnosis	5 years follow-up	7 years follow-up
urine	dopamine [nmol/mmol creatinine]	1231	383	295
	VMA [µmol/mmol creatinine]	13 (1.3N)	1	2
	HVA urine [µmol/mmol créat]	17 (1N)	3	4
plasma	tMT [nmol/l]	6.32 (1.5N)	4.27 (1N)	4.57 (1.1N)
	fMT [nmol/l]	0	0.02	0.02



Patient 11

Age: 21 months
Localisation: left thoracic
Extension: local
Histology: Neuroblastoma, differentiating subtype, MKI low, stroma poor
Biology: no MYCN amplification
Treatment: resection surgery
Response to treatment: CR
Diagnosis: elevated VMA, tMT and fMT
Follow-up: 2 years of radiological exams showing stable tumoral residues.
Clinical relapse: none

Patient 11		Diagnosis	24 months follow-up
urine	dopamine [nmol/mmol creatinine]	642	
	VMA [µmol/mmol creatinine]	10 (1N)	
	HVA urine [µmol/mmol créat]	10	
plasma	tMT [nmol/l]	11.94 (2.8N)	7.14 (1.7N)
	fMT nmol/l	0.06 (1N)	0

Conclusion: progressive decrease of tMT and fMT in correlation with stable radiological and clinical state. More follow-up values are needed.

Patient 15

Age: 59 months
Localisation: Abdominal (paravertebral)
Extension: local
Histology: Peripheral neuroblastic tumor (NOS)
Biology: MYCN not amplified, no SCA
Treatment: subtotal excision surgery
Response to treatment: CR
Diagnosis: elevated dopamine with normal VMA and HVA levels. Elevated tMT, normal level of fMT.
Follow-up: Radiological stable residual tumor with an increase of tMT and fMT, not significant in this context.
Clinical relapse: None

Patient 15		Diagnosis	6 months post surgery
urine	dopamine [nmol/mmol creatinine]	771 (1N)	
	VMA [µmol/mmol creatinine]	8	
	HVA urine [µmol/mmol créat]	13	
plasma	tMT [nmol/l]	5.84 (1.4N)	6.78 (1.6N)
	fMT nmol/l	0.03	0.09 (1.5N)

Conclusion: stable but metabolically active tumor with elevated plasma metanephrines. More data are needed to interpret this follow-up.

6.3. Dopamine, HVA and VMA concentrations

At diagnosis, 24h urine output corrected with creatininemia of dopamine showed a sensitivity of 46%, 73% for HVA and 90% for VMA. The combination of HVA and VMA didn't increase sensitivity compared with VMA alone, with a sensitivity reaching 90%. We found 18% of urinary catecholamine (dopamine, HVA or VMA corrected to creatininemia) negative tumors at diagnosis for the 3 molecules.

At diagnosis, urine concentration of VMA were increased from 1 to 14.9 fold time, HVA from 1 to 9 fold time and dopamine from 1 to 32.3 fold time adapted with an age dependant upper reference value.

VMA, HVA and dopamine levels at diagnosis were compared among L1, L2 and MS groups. No significant difference between these groups was highlighted ($p(VMA_{L1/L2}) = 0.15$, $p(VMA_{L1/MS}) = 0.08$, $p(VMA_{L2/MS}) = 0.16$, $p(HVA_{L1/L2}) = 0.11$, $p(HVA_{L1/MS}) = 0.06$, $p(HVA_{L2/MS}) = 0.34$, $p(Dopa_{L1/L2}) = 0.16$, $p(Dopa_{L1/MS}) = 0.33$, $p(Dopa_{L2/MS}) = 0.82$).

6.4. Methoxytyramine concentrations

Plasma concentration of tMT was increased from 1.4 to 115.8 fold time at diagnosis (L1: 1.4-5.1 fold ; L2: 2.4-17.6 fold ; MS: 3.8-15.8 fold), with an upper reference value of 4.19 nmol/l. Only one patient of L2 stage didn't exhibit increased concentration of tMT at diagnosis. Mean tMT at diagnosis weren't significantly different among stages ($p(tMT_{L1/L2}) = 0.2$, $p(tMT_{L1/MS}) = 0.27$, $p(tMT_{L2/MS}) = 0.13$). tMT at diagnosis had a 94% sensitivity with > 4.19 as upper reference value.

At diagnosis, fMT was below the lower limit of quantification in 31% of the patients (Patient no 8, 9, 4, 6). These patients didn't display normal urine parameters at diagnosis, with data missing for patient 8. Plasma concentration of fMT exhibit an increase from 1 to 82 fold time at diagnosis with an upper reference value of 0.06 nmol/l. We reported 62% sensitivity of fMT at diagnosis with 0.06 nmol/l as an upper reference value. The fMT concentration mean at diagnosis was screened and compared among stage, without any significant difference in mean being highlighted. However, mean fMT of L2 group compared with L1 group were at the limit of significance (Student t-test/ p: 0.059).

6.5. Normetanephrine concentrations

Plasma concentration of tNMN was increased from 1.19 to 174.36 fold time at diagnosis with an upper reference value of 36.65 nmol/l (Appendix 2). Median time fold increase was 2.29 (Patient 7 displayed an outlier of 174.36 time fold the reference with 6373 nmol/l). We report 10/15 patients with increased tNMN at diagnosis. Median value at diagnosis was 48.19 nmol/l, mean 508.15 nmol/l. Mean tNMN was not significantly different among stages $p(tNMN_{L1/L2}) = 0.88$, $p(tNMN_{L1/MS}) = 0.27$, $p(tNMN_{L2/MS}) = 0.10$). Sensitivity for this biomarker at diagnosis of NB is 67%. One patient displayed a value under the actual reference limit.

Plasma concentration of fNMN was increased from 1.27 to 61.25 fold time at diagnosis with an upper reference value of 1.39 nmol/l (Appendix 2). Median time fold increase was 2.78 (with patient 7 with an outlier of 61.25 time fold the reference with 86.14 nmol/l). Median value at diagnosis was 3.7 nmol/l, mean 66.15 nmol/l. Mean fNMT was not significantly different among stages $p(fNMN_{L1/L2}) = 0.46$, $p(fNMN_{L1/MS}) = 0.22$, $p(fNMN_{L2/MS}) = 0.64$). 10/14 of the patients had increased fNMN at diagnosis, which corresponds to a 71% sensitivity of the biomarker with a 1.39 nmol/l cut off.

6.6. Clinical relapse

A total of 12 clinical relapse situations were identified based on radiological modalities. Among them, we didn't have urinary or plasma data performed close to the radiological exam to correlate with 5 occurrences. No increase in dopamine, HVA, VMA, tMT and fMT was observed in 2 situations. Two clinical relapses were associated with an isolated increased in tMT and fMT. Two relapses displayed increased dopamine, VMA and HVA associated with either tMT and fMT or tMT elevation alone. One occurrence had tMT, fMT, HVA and VMA over the upper reference limit, with normal dopamine levels (Patient No 1).

6.7. Sensitivity of catecholamine metabolites at diagnosis using different cut-offs

	DA	VMA	HVA	VMA + HVA	tMT	fMT	tMN	fMN	tNMN	fNMN	tMT + fNMN
Fixed reference value (appendix 3)	46%	90%	73%	90%	94%	62%	0%	0%	67%	71%	93%
> 97.5th percentile by Franscini et al. (2015)	-	-	-	-	87%	-	34%	43%	74%	93%	**100%**

Table 1: Sensitivity of catecholamines metabolites according to different cut-offs. fMT partitionning model was not built by Franscini et al. since a majority of patients were under the lower limit of quantification for this parameter.

In this patient cohort, the use of the current fixed reference value for plasma is more sensitive for tMT. More specifically, tMT (>4,19 nmol/l) provides a benefit with a sensitivity of 94%, with one more patient with low risk neuroblastoma being highlighted.

According to the percentile provided by Franscini et al. (2015), plasma total methoxytyramine overlaps with controls population in 2 patients out of 15, which indicate a sensitivity of 87% (appendix 2). Increase in plasma tMN, fMN, tNMN and fNMN over the 97.5th percentile display a sensitivity of 34%, 43%, 74%, and 93% respectively.

If we look at fNMN, percentiles by age and gender by Franscini et al. are more sensitive (93%) as compared with the actual reference value of <1.39 nmol/l (71% sensitivity). Same observation is made for tNMN (74% vs 67%), fMN (43% vs 0%) and tMN (34% vs 0%). Interestingly, a combined measure composed of tMT and fNMN is able to reach 100% sensivity in this cohort of NB patients.

Franscini et al. (2015) suggested a best cut-off level of 0.1 nmol/l for fMT independently of age and gender with a sensitivity of 100%. With this cut-off, we report only 46% sensitivity of fMT.

7. Discussion

This study involved a small cohort of 15 patients of low to intermediate risk neuroblastoma. We assessed both urine catecholamines and plasma metanephrines sensitivity based on different cut-offs. Reference intervals have a central role in the evaluation of laboratory results. As the usefulness of a tumor marker is related to the amount of its increase over the reference interval, relative fold change to the upper reference limit were also assessed.

We found 18% of urinary catecholamine (dopamine, HVA or VMA corrected to creatininemia) negative tumors at diagnosis. Urinary VMA and HVA sensibility were 90% and 73% respectively, with 90% for their combined measure. This result was similar to other studies

such as Strenger et al. showing sensitivity of VMA and HVA of respectively 80.7% and 71.9% (7).

The fMT marker was particular to assess since there were patients presenting values under the lower limit of quantification for this parameter (<0.03 nmol/l). Due to this technical issue, Franscini et al (2015) suggested a best cut-off level of 0.1 nmol/l for fMT independently of age and gender with a sensitivity of 100% on their NB population. With this cut-off, we report only 46% sensitivity of fMT. Both studies differ in terms of NB population, as Franscini et al. study didn't included any L1 stage, which could explain our decreased sensitivity level. It could be related to the fact that less aggressive tumors, as such we find in the low to intermediate risk group, secrete less in a general way and fMT isn't a satisfying parameters for this category of disease. In this case, we cannot expect to be a valuable indicator of relapse. Indeed, the increased secretion by more aggressive tumor is supported by the observation that plasma fMT absolute value at diagnosis was at the limit of significance higher in patients from L2 stage as compared with L1 stage. We underline the strong dispersal of the values with important interindividual variability, which can be secondary to an insufficient cohort population. It would be interesting to further test the hypothesis of an increased synthesis of this plasma marker in the intermediate form of NB with a more substantial sample size. We could also evaluate by a broader assessment if low, intermediate or high risk disease can be discriminated by this marker, providing a hint of the prognosis of the underlying disease that could help define aggressive treatments. Moreover, in further studies, we should re-evaluate this cut-off settled at 0.1 nmol/l with a larger sample size.

The best diagnosis performance was obtained with free NMN. As a single metabolite, it allowed to reach 93% sensitivity according to age and gender over the 97.5% percentile. 71% sensitivity was reached with the actual reference value used by the laboratory. This result is consistent with demonstrated evidence that the adrenals provide the largest single source of NMN (14).

In contrast, we could highlight very low sensitivity of free (43%) and total MN (34%) according to gender and age-related percentiles from Franscini et al. Moreover, all patients also displayed values under the actual upper reference limit of the CHUV laboratory. This result speaks in favor of the lack in neuroblastoma tumor of phenylethanolamine N-methyl transferase, enzyme needed for the transformation of norepinephrine into epinephrine.

The only parameter with increased sensitivity with the upper reference value as compared with the 97.5th percentile is the tMT. One more patient with low risk neuroblastoma is highlighted with the first mentioned cut-off. In this study, we were also interested in comparing diagnosis performance of both tMT and fMT. At diagnosis, tMT seems to give better sensitivity than fMT in these specific categories of low to intermediate risk patients. This result has to be further confirmed in a prospective study with a bigger cohort of patients.

To summarize, the implementation of reference values of plasma metanephrines by age and gender according to the distribution based on 191 healthy children obtained by Franscini et al. (2015) could significantly increase sensitivity for neuroblastoma diagnosis as concentrations of metanephrines display variation along life for each gender. Indeed, fNMN as a single marker has a 93% sensitivity of NB diagnosis and a combined measure composed of tMT and fNMN is able to reach 100% sensitivity in our cohort of patients. These results are supported by Franscini et al. conclusions.

Concerning clinical and biological follow-up of this cohort of 15 patients, we can observe that plasma metanephrines and urine catecholamines are frequently following the same trend

over time, with decreasing values speaking in favour of partial or total remission. We report many biological laboratory tests with elevation of either plasma and/or urine markers that are false positive events in the context of radiological remission. The marker that seems elevated in most of the identified clinical relapse is tMT. As bias, the number of clinical relapse events is highly dependent on the frequency of the radiological exams, as well as their performance in concluding in an increased tumoral mass. Indeed, a slight increase in marker might reflect a little tumoral grow, which might be considered unsignificant on radiological exams. A well-defined prospective protocol with controlled timing of radiological exams could minimize the potential bias of frequency.

Moreover, follow-up data up suggested a great variability in biomarker pattern with a broad spectrum of pathologies. Some tumors were still metabolically active although radiologically stable, which makes biomarkers interpretation challenging. Other patients still exhibited tumoral tissue without any metabolic activity. In a vast majority of cases, we observe normalisation of both metanephrines and catecholamines after treatment. This underlies the need of considering the patient as a whole clinical and biological picture. We need to underlie that little variations around the upper reference limit over time in markers should be balanced with studies demonstrating the physiologic variability of secretion over time that occur in healthy children. These urinary and plasmatic biomarkers are used in combination with other parameters to help decision of treatment of a residual disease, or for the *wait and see strategy* in case of low risk tumor, and also to monitor disease activity in response to therapy. However, we still do not have the perfect parameter that can certify the absence of the disease.

About the clinical characteristics of the patients, patient number was insufficient for accurate analysis of mean difference by age subgroup. Two patients (1 and 13) displayed ganglioneuroblastoma, which is a more mature tumor than neuroblastoma. This specific etiology wasn't linked to outlier values of DA, VMA, HVA, tMT or fMT.

Urine catecholamines (HVA and VMA) are the actual gold standard for the diagnosis of neuroblastoma. This technique is widely used, cost-effective and non-invasive. Pediatric reference intervals for urine metabolites are highly variable among laboratories. The measurement of urine is often inaccurate because of difficulties of correct sampling in young children. The use of creatininemia is used to standardise as well as possible the measure, but often only partially corrects the sampling. However, creatinine depends itself on various factors such as muscular mass, nutrition state or drugs. Plasma metanephrines could provide a convenient alternative to urinary sampling, as well as better sensitivity at diagnosis. Nevertheless, catecholamine in urine analysis is easier to measure compared to plasma metanephrines offering a limited availability for plasma metanephrines assay. In the near future, screening with affordable *point of care testing* with for example capillary plasma spot sampling would be highly suitable for developing countries and neonates that have a limited supply.

8. Conclusions and future perspectives

Catecholamines and its derivatives in urine have been considered as the gold standard for diagnosis and follow-up of patients with neuroblastoma. Urine sampling is complicated in young children with regard to completeness of urine collection and a variating creatinine corrective factor depending on many parameters. Giving these difficulties, the use of plasma markers could be helpful. Many laboratories cannot invest yet into plasma assays, urinary analysis being performed with less demanding laboratory techniques, but it would provide an important increase in sensitivity in the diagnosis of neuroblastoma that can be more comfortably relied on by the clinician.

With this retrospective study we could demonstrate follow-up data underlying the broad clinical presentation of NB correlated with great variability of both catecholamines and metanephrines patterns. The main outcome was a 100% sensitivity for NB diagnosis with combined use of plasma total MT and free NMN with age and gender appropriate reference limits, providing better sensitivity than the actual urinary gold standard. In contrast, fMT seems not to be a satisfying parameter at diagnosis for low risk to intermediate risk specific NB disease. We confirmed better sensitivity at diagnosis of Franscini et al. partitioning by age and gender percentiles for plasma metanephrines as compared with the actual reference limits. This promising result needs further assessment and confirmation in a prospective large national multicentric study including low to high-risk NB patients.

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11. Appendix

Patient	Sex	Age at Dg [years]	Stage at Dg	Pretreatment risk group	Histology	Biology	Primary localisation	Metastasis at Dg	Treatment protocol	response
1	F	1.87	L2	intermediate	Ganglioneuroblastoma (mixed)	N-MYC not amplifed, triploid	left thorax	No	SIOPEN unresectable	27.04.2009 CR
2	M	0.10	L2	intermediate	Neuroblastoma, NOS	N-MYC not amplifed	right adrenal gland	No	INES	27.01.2009 VGPR
3	F	4.82	L2	intermediate	Neuroblastoma, NOS	N-MYC not amplified, Triploid	abdomen	No	SIOPEN	03.10.2010 PR
4	F	0.16	L2	intermediate	Neuroblastoma, NOS (poorly differenciated)	N-MYC not amplifed	right paraspinal (D10-11 to L3-L4)	No	INES 99.1	29.01.2010 CR
5	F	0.81	L2	intermediate	Neuroblastoma, NOS (differentiating NBL)	N-MYC not amplified ; trisomy 2 tretrasomy 2 (02.03.2015), 11q- 1p- 17q-	left hemiabdomen	No	LINES (4 courses)	21.02.2014 PR
6	F	0.66	MS	very low	Neuroblastoma, NOS	N-MYC not amplified, Triploid	right adrenal gland	left orbitar, subcutaneous and intramsucular, hepatic	INES 99.2 (4 courses)	04.09.2008 VGPR
7	F	0.01	MS	very low	Neuroblastoma, NOS	N-MYC not amplified	left adrenal gland	Liver	INES	13.01.2012 CR
8	M	0.12	L1	low	Neuroblastoma, NOS	N-MYC not amplifed, triploid	right adrenal gland	No	Surgery only	13.03.2008 CR
9	F	1.58	L1	low	Neuroblastoma, NOS	N-MYC not amplified +13 3- 4- 14- 19- 21-	retroperitoneum	No	Surgery only	29.01.2010 CR
10	F	2.19	L2	intermediate	Neuroblastoma, NOS (poorly differentiated)	N-MYC not amplified, near tetraploidy, , 11q- +17q 10q- +12q X chromosome loss	left retroperitoneum	No	LINES	07.10.2014 VGPR
11	M	1.71	L1	low	Neuroblastoma, differentiating subtype	N-MYC not amplified	right thorax	No	Surgery only	23.02.2016 CR
12	M	1.44	undefined	intermediate	Neuroblastoma (differentiating)	N-MYC not amplified / not conclusive ; SCA : 1,2,3,5 and 10 chromosomes; del 1p	left retroperitnoeal	No	LINES	04.10.2016 PR
13	F	7.98	L2	intermediate	Ganglioneuroblastoma (without histotype)	N-MYC not amplified	intra-abdominal site	No	LINES	15.02.2017 CR
14	F	0.12	MS	very low	Neuroblastoma	N-MYC not amplified	right adrenal	hepatic lesions	LINES	20.12.2016 VGPR
15	M	5.13	L1	low	Peripheral Neuroblastic Tumor, NOS	N-MYC not amplified, no SCA	abdominal (left retroperitoneal mass)	No	Surgery only	23.01.2017 CR

Appendix 1 : Patient characteristics

SCA: segmental chromosomal abnormalities.

CR: complete remission

PR: partial remission

VGPR: very good partial remission

NOS: no otherwise specified

INES: Infant neuroblastoma protocole (former protocol)

LINES: current very low, low and intermediate risk protocol

Patient	Gender	Age at Dg [months]	Stage	Pretreatment risk group	plasma tMT at Dg [nmol/l]	Percentile of plasma tMT at Dg	plasma tMN at Dg [nmol/l]	Percentile of plasma tMN at Dg	plasma fMN at Dg [nmol/l]	Percentile of plasma fMN at Dg	plasma tNMN at Dg [nmol/l]	Percentile of plasma tNMN at Dg	plasma fNMN at Dg [nmol/l]	Percentile of plasma fNMN at Dg
1	F	23	L2	intermediate	15.27	> P 97.5	0.92	< P 2.5	0.11	P 25-50	55.93	>P 97.5	7.85	>P 97.5
2	M	1	L2	intermediate	37.46	> P 97.5	3.16	P 75-90	0.07	P 25-50	441.6	>P 97.5	24.12	>P 97.5
3	F	59	L2	intermediate	22.18	> P 97.5	2.28	P 50-75	0.11	P 10-25	43.4	>P 97.5	3.62	>P 97.5
4	F	2	L2	intermediate	10.16	> P 97.5	7.28	> P 97.5	0.26	> P 97.5	83.68	>P 97.5	3.78	>P 97.5
5	F	10	L2	intermediate	74	> P 97.5	7.2	> P 97.5	NA	NA	1.94	< P 2.5	NA	NA
6	F	8	MS	very low	14.13	> P 97.5	1.79	P 25-50	0.14	P 50-75	87	>P 97.5	8.32	>P 97.5
7	F	0.17	MS	very low	485	> P 97.5	10.08	> P 97.5	0.26	> P 97.5	6373	>P 97.5	86.14	>P 97.5
8	M	1.5	L1	low	21.5	> P 97.5	5.34	P 95-97.5	0.68	> P 97.5	255.9	>P 97.5	3.86	>P 97.5
9	F	19	L1	low	6.32	P 75-90	3.34	P 75-90	0.14	P 25-50	15	>P 97.5	1.06	>P 97.5
10	F	27	L2	intermediate	3.62	P 50	1.8	P 25-50	0.15	P 25-50	8.92	P 90-95	0.87	>P 97.5
11	M	21	L1	low	11.94	> P 97.5	9.5	> P 97.5	0.37	> P 97.5	31.8	P 90-95	1.31	>P 97.5
12	M	18	undefined	intermediate	20.34	> P 97.5	3.22	P 75-90	0.16	P 50-75	35.36	>P 97.5	2.01	>P 97.5
13	F	97	L2	intermediate	35.84	> P 97.5	6.59	> P 97.5	0.18	> P 97.5	48.19	>P 97.5	1.76	>P 97.5
14	F	1.5	MS	very low	23.26	> P 97.5	1.6	P 10-25	0.03	> P 97.5	133.8	>P 97.5	3.86	>P 97.5
15	M	59	L1	low	5.84	> P 97.5	1.1	P 2.5-5	0.05	P 50-75	6.84	P 50-75	0.42	P 75-90

Appendix 2 : Values of tMT, tMNT, fMNMN, tNMN and fNMN at diagnosis according to different cut-off. Age and Gender related distribution over the 97.5th percentile by Franscini et al. (2015) is displayed in red color. In blue, patient over the actual fixed upper reference value of the catecholamine laboratory lab of the CHUV (appendix 3).

PLASMA	pg/ml	nmol/l	Lim. sup. asymptomatiques	info masse mol
E	4 à 225	0,02 - 1,23		183.2
NE	108 à 1108	0,64 - 6,55		169.2
DA	1.5 à 58	0,01 - 0,38		153.1
MN libres	6 à 168	0,03 - 0,85	0.56	197.2
NMN libres	7 à 255	0,04 - 1,39	0.71	183.2
MT libres	< 5	< 0,06		167.2
MN totales		0,66 - 13,45	7.49	197.2
NMN totales		2,14 - 36.65	13.03	183.2
MT totales		0,59 - 4,19		167.2

URINES		VMA	HVA	5-HIAA
0 - 1 ans	µmoles/24 h	< 14	< 16	
	µmoles/mmoles créat	< 14	< 20	
1 - 2 ans	µmoles/24 h	< 16	< 18	
	µmoles/mmoles créat	< 10	< 17	
2 - 6 ans	µmoles/24 h	< 16	< 21	
	µmoles/mmoles créat	< 10	< 15	
6 - 16 ans	µmoles/24 h	< 21	< 28	
	µmoles/mmoles créat	< 7	< 7	
Adultes	µmoles/24 h	< 25	< 34	<34
	µmoles/mmoles créat	< 3	< 4	

URINES		NE	E	DA	NMN	MN	MT
0 - 3 mois	nmol/24h	< 60	< 14	< 750	< 540	< 150	< 1250
	nmol/mmol creatinine	< 280	< 45	< 1500	<2732	<496	< 1500
3 - 6 mois	nmol/24h	< 60	< 14	< 750	< 540	< 150	< 1250
	nmol/mmol creatinine	< 280	< 45	< 1500	<1681	<389	< 1500
6-12 mois	nmol/24h	< 60	< 14	< 750	< 540	< 150	< 1250
	nmol/mmol creatinine	< 280	< 45	< 1500	<1164	<268	< 1500
1 - 2 ans	nmol/24h	< 100	< 14	< 1000	< 750	< 320	< 325
	nmol/mmol creatinine	< 80	< 45	< 1300	<593	<216	< 750
2 - 3 ans	nmol/24h	<100	< 19	< 1200	< 750	< 320	< 390
	nmol/mmol creatinine	< 80	< 35	< 740	<456	<213	< 355
3 - 4 ans	nmol/24h	<100	< 19	< 1200	< 750	< 320	< 390
	nmol/mmol creatinine	< 80	< 35	< 740	<456	<213	< 355
4 - 5 ans	nmol/24h	< 170	< 33	< 1200	< 750	< 320	< 390
	nmol/mmol creatinine	< 60	< 20	< 740	<456	<213	< 355
5 - 6 ans	nmol/24h	< 170	< 33	< 1200	< 750	< 320	< 390
	nmol/mmol creatinine	< 60	< 20	< 740	<287	<178	< 355
6 - 7 ans	nmol/24h	< 170	< 33	< 1800	< 1100	< 860	< 1000
	nmol/mmol creatinine	< 60	< 20	< 500	<287	<178	< 355
7 - 8 ans	nmol/24h	< 266	< 55	< 1800	< 1100	< 860	< 1000
	nmol/mmol creatinine	< 60	< 20	< 500	<287	<178	< 355
8 - 9 ans	nmol/24h	< 266	< 55	< 1800	< 1100	< 860	< 1000
	nmol/mmol creatinine	< 60	< 20	< 500	<287	<178	< 355
9 - 10 ans	nmol/24h	< 266	< 55	< 1800	< 1100	< 860	< 1000
	nmol/mmol creatinine	< 60	< 20	< 500	<287	<178	< 355
10 - 16 ans	nmol/24h	< 472	< 110	< 1800	< 1100	< 860	< 1000
	nmol/mmol creatinine	< 55	< 15	< 500	<209	<131	< 355
Adulte	nmol/24h	< 610	< 130	< 3300	< 3800	< 1880	< 1900
	nmol/mmol creatinine	< 45	< 22	< 340	< 250	< 200	< 150

Selon Pussard & al. Clin.Biochem.2009;42(6):536-9. et Grouzmann & al. Eur J Endocrinol 2010; 162(5)951-60

Appendix 3: References values of the catecholamines and derivatives of the CHUV laboratory based on Pussard & al. Clin. Biochem. 2009; 42(6);536-9. and Grouzmann & al. Eur J Endocrinol 2010; 162(5)951-60. E: epinephrine, NE: norepinephrine, DA: dopamine, MN: metanephrine, NMN: normetanephrine, MT: methoxytyramine, libre: free, totales: total.

11.1. Clinical history follow-up

Patient 1

18 months old child presenting a localized intrathoracic mass with intracanalar tumoral extension to the controlateral side (dumbbell tumor) and medullar compression. MIBG scintigraphy showed no metastasis at diagnosis. Bone marrow biopsy showed no tumor infiltration. A surgical tumor biopsy revealed a ganglioneuroblastoma intermixed without *N-MYC* amplification. The tumor responded little to chemotherapy with 2 courses of Carboplatine/VP-16 and 4 courses of combined regimen of cyclophosphamide, doxorubicine and vincristine (CADO) with the intracanalar portion unchanged. One month after the end of the chemotherapy, MRI showed an increase of the thoracic tumoral mass coupled with increasing values of tMT and fMT. After an observation period of 2 more months, the child started to present symptomatologic medullar compression with inferior limbs weakness and walking difficulties. Semi-urgent surgery was carried out in two steps with posterior laminectomy D7-D11 with complete intracanalar resection and resection of the intrathoracic mass. Histology of the operated tumor showed ganglioneuroma which was probably due to a maturation effect of chemotherapy. The patient was classified in complete remission afterwards.

Patient 2

1 month-old patient with an unresecable retroperitoneal mass (L2) that encompassed the right kidney provoking a hydronephrosis. Urinary catecholamines and plasma metanephrines were increased confirming the diagnosis of neuroblastoma as did a tumor biopsy. After 8 courses of chemotherapy, the surprarenal mass remained unchanged on CT whereas the perirenal component disappeared almost completely. As surgery was still considered too risky the child was followed clinically and radiologically during 8 years without signs of reccurence.

Patient 3

4 years and 9 months old patient, presenting with a heterogeneous and calcified retroperitoneous mass occupying the whole abdomen with extension. An MRI excluded any spinal compression. Catecholamines and plasma metanephrine dosage followed by MIBG scintigraphy suggested neuroblastoma, which was confirmed by a biopsy. A compete work-up excluded metastatic disease. The child needed 4 courses of carboplatine/VP-16 CT followed by and 2 courses CADO, incomplete surgery with numerous resected local lymph nodes that showed to be positive followed by 2 courses of adjuvant CT with Topotecan-Vincristine-Doxorubicine. At this point, MIBG scintigraphy showed decreased capture of renal hilum tumoral residue and intense but stable capture of the pelvic tumoral residue. Finally the patient received high dose chemotherapy with CEM (carboplatin, etoposide, melphalan) with autologous stem cell reinfusion, followed by 6 months oral retinoic acid. Partial remission was achieved in December 2010. No progression occurred since then. 2 years after response to treatment assessment, MIBG scintigraphy showed decreased capture of the known residual lesions.

Patient 4

7 weeks old patient with a solid right paravertebral mass pushing the kidney and invading the spinal canal. A paraspinal biopsy showed poorly differentiated neuroblastoma and radiology investigations confirmed a dumbbell tumor with extension into the D10-D11 to L3-L4 foramina. MIBG scintigraphy allowed excluding metastatic disease. Chemotherapy by 4 courses of Carboplatine/ VP-16 led to complete clinical, biological and radiological remission at the end of the treatment. 4 years followed up didn't show signs of radiologic recurrence of the disease.

Patient 5

13 months old patient, with a dumbbell neuroblastoma (poor stroma, low MKI) with intra-ductal extension from T11 to L1 with compression of the medullary cone and of the fibers of the cauda equina (L2). Emergency chemotherapy is started with 2 courses of Carboplatine/VP-16 followed by 2 courses of CADO. After a total of 4 chemotherapy courses, the tumor remained unchanged on MRI. Because of persisting urinary problems surgery was done in two parts, first for the intraductal portion and then for the abdominal mass. During follow-up, MIBG scintigraphy showed an increased tumor capture and a second-look surgery was performed after three months. 2 years follow-up showed no signs of disease progression.

Patient 6

Seven months old patient, presenting since 1 month a superior left eyelid swelling with ptosis and a progressive shift of the eyeball downwards, was diagnosed with an atypical stage IVS neuroblastoma with multiple metastasis (subcutaneous, muscular, hepatic, cervical and subcarinal mediastinal lymph nodes), without medullar infiltration or bone metastasis. Pathology showed a triploid tumor with favourable histology. After an initial observation for 1 month, the primary tumor and the metastasis progressed without symptoms and the child received a treatment of 4 courses of carboplatine/VP-16 with a very good partial response. Since then the child has been observed and remained with stable disease.

Patient 7

A newborn girl born on term presented a palpable mass on the left side of the abdomen crossing the midline. Because of an hemorrhagic shock due to haemoperitoneum in the context of an abdominal mass, investigations performed urgently revealed a mass suggesting neuroblastoma with disseminated liver tumoral infiltration. Total surgical resection was performed in emergency with an intraoperative status of multiple hepatic nodules. Pathology confirmed low differentiated neuroblastoma (poor stroma, MKI index low). In summary, this patient presents a congenital left surrenalian neuroblastoma stage MS. The fast progressing hepatomegaly, secondary to the tumoral infiltration and sign of hepatocellular dysfunction made chemotherapy necessary 1 month after surgery by 2 courses of C/VP16. She went progressively into very good partial remission 4 years later. Follow-up included 5 years without signs of radiological disease recurrence and with progressive decline of tMT and fMT.

Patient 8

Newborn born at term whose clinical examination during the first day of life showed a hepatic mass, suspicious of a congenital neuroblastoma. An abdominal US at day 2 of life showed a surrenalian hepatic mass without metastasis. Urinary catecholamines were increased. The patient was asymptomatic and stable. Imaging investigation pursued with an MIBG scintigraphy (07.02.08) with an intense capture of the abdominal lesion, without any metastasis.
A clinical and US follow-up was first organised considering the young age of the patient and the localised tumor, as the neuroblastic tumor may regress spontaneously. During this follow-up, two hepatic nodules were highlighted on a MRI and an increase in tumoral volume on a US. This radiological deterioration motivated a surgical intervention on this grade 4S neuroblastoma. Surrenalectomy with complete excision of primary tumor was performed. Pathology exam showed a low differentiated neuroblastoma with favorable histology, poor stroma, MKI index low, triploid aneuploidy, MCYN negative. There is a high probability of spontaneous regression of the hepatic lesions and expectative strategy was chosen. 7 years radiologic follow-up with US showed an absence of local tumor recurrence, an absence of metastasis in particular no more hepatic lesions.

Patient 9

18 months old patient, who was incidentally diagnosed with a retroperitoneal heterogeneous paravertebral mass without foraminal extension. Urinary VMA, HVA and plasma tMT were increased at diagnosis. Work-up did not show any disease extension. The tumor was completely resected by laparotomy and didn't need any adjuvant treatment. Pathology showed a neuroblastoma of unfavourable histology with favourable biology. 7 years follow-up radiologic exams showed an absence of tumoral recurrence.

Patient 10

Patient of 26 months presenting a localized left paravertebral dumbbell retroperitonal mass, confirmed by CT scan, with an intraspinal component and symptomatic spinal compression extending from L1 to L4. On biopsy pathology showed a low differentiated tumor with unfavorable histology. Urinary catecholamines, plasma metanephrines were elevated at diagnosis. The child was treated by 3 courses of carboplatine/VP-16 and 3 courses CADO. Partial surgical excision was performes 6 months after diagnosis. Treatment was competed by 2 months of adjuvant radiotherapy for a total dose of 21 Gy in 15 fractions of 1.5 Gy followed by retinoic acid. One year after radiotherapy, MRI showed discrete increase in epidural left lateral component. Partial remission was achieved 10 months after diagnosis with residual elevated tMT and fMT.

Patient 11

Since birth this patient, born prematured at 33 weeks of pregnancy, displayed a left apical thoracic opacity, first thought to be a calcified hematoma after setup of a central venous access. The plasma metanephrine values

were normal, except an isolated elevation of the total methoxytyramine. A MIBG scintigraphy showed a capture of the mass without any other sign of metastasis. It was decided to proceed to a resection of the thoracic mass. No adjuvant treatment was proposed. Tumoral residue was stable on the 2 years follow-up MRI.

Patient 12

Patient was first diagnosed with an abdominal mass by an ultrasound in Moldavia in the context of following for a hepatic hemangioma. Initially addressed to Moskow and Turkey for the treatment, the patient got a biopsy that confirmed the diagnosis of differentiated neuroblastoma in Turkey. Due to financial issues, the family first got back to Moldavia. Then, a second biopsy was performed in the CHUV where the patient finally received treatment.
Chemotherapy was started with two courses of C/VP-16. During this treatment and 3 months post diagnosis, a MRI showed a 20% progression of the retroperitoneal mass. At the end of the chemotherapy, three new lesions appeared on MRI (right high paravertebral thoracic, right paravertebral lombar, T5-T6 prevertebral paramediastinum). A biopsy confirmed metastasis with same histology as primary tumor. Two courses CADO were then performed. 6 months later, the patient presented an increased size of retroperitoneal mass without biological correlation.

Patient 13

Eight year old in good general health, who was diagnosed with a L2 abdominal neuroblastoma. abdominal tumor biopsy by laparotomy showed ganglioneuroblastoma. Unrinary catecholamines and plasma metanephrines were increased at diagnosis.
Treatment was initiated by two courses of C/VP-16 and two courses CADO (06.2016 to 09.2016) to shrink the tumor. The response to treatment was minimal with a CT scan showing progression of the mass (10%), without data for urinary or plasma biomarker. Considering this poor response and the persistence of arterial hypertension, the child finally underwent surgery of the abdominal tumor 8 months post diagnosis. Post surgery complications occurred with abdominal aorta lesion requiring angioplasty. 2 months later she was in complete remission with post-surgery radiological exams excluded any tumoral recurrence. Progressive decreased in urinary dopamine, VMA, HVA and plasma tMT, fMT

Patient 14

Antenatal discovery by US control during pregnancy of a right adrenal mass, confirmed at day 2 of life to be a well vascularised and well delimited mass suspicious of neoplasia. US abdominal showed mass on the right surrenal, with increased volume. Because of increase in size, investigations were completed revealing hepatic metastasis, confirming a MS disease.
Biopsy of the primary lesion was done showing a poorly differentiated NB corresponding to a favourable histology, and no bone marrow infiltration was found. The biology of the lesion showed no without segmental abnormalities. Since then the patient has been clinically and radiologically closely observed.

Patient 15

It is a patient, born preterm at 27 weeks of pregnancy with severe bronchopulmonary dysplasia, also known for a Beckwith-Wiedemann syndrome (macrosomia, ombilic hernia, macroglossia) diagnosed at birth. In the context of this syndrome, he was closely followed for an increased oncological risk. A routine US at 5 years of age showed an abdominal mass in the context of abdominal pain without B symptoms. A second US 2 months later showed a similar pattern with MRI confirmation. A MIBG scintigraphy excluded any bone involvement with a strong capture of the paravertebral mass. Two subsequent biopsies (one fine-needle and one by laparotomy) confirmed the diagnostic of peripheral neuroblastic tumor. Due to the paravertebral localization, the tumor surgical resection was challenging and subtotal excision was performed. Radiological follow-up showed stable post-excision residues.