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CAT
Critically Appraised Topic

Laboratory diagnostics of neuroblastoma and pheochromocytoma

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CLINICAL BOTTOM LINE

Neuroblastoma, paraganglioma, and pheochromocytoma are uncommon tumors originating from the neural crest and known for their catecholamine production (1,2). Neuroblastoma stands as the most prevalent extracranial solid tumor in paediatric patients (1,2). Pheochromocytoma and paraganglioma (PPGL) exhibit an annual incidence of six cases per million inhabitants and can lead to catecholamine-induced hemodynamic instability if left undiagnosed (3,4).

Traditionally, the diagnostic evaluation of **neuroblastomas** involved the measurement of vanillylmandelic acid (VMA) and homovanillic acid (HVA) levels in a 24-hour urine sample (5). However, due to the suboptimal diagnostic performance of this approach, alternative catecholamine metabolites in urine and plasma have been investigated. In a study conducted by the SIOPEN Catecholamine Working Group in 2023, an eight-catecholamine metabolite panel (VMA, HVA, dopamine (DA), 3-methoxytyramine (3MT), norepinephrine (NE), normetanephrine (NMN), epinephrine (E), and metanephrine (MN)) was compared to VMA plus HVA in random spot urine and a 24-hour urine collection. The findings indicated that the panel of eight catecholamine metabolites exhibited superior diagnostic accuracy, with similar diagnostic sensitivities observed in spot urine and 24-hour urine samples (5). Additionally, the same research group is conducting a multicenter prospective study comparing the diagnostic performance of urine and plasma catecholamines and catecholamine metabolites (HVA, VMA, E, NE, DA, MN, NMN, 3MT). Despite these advancements, international guidelines for biochemical testing in patients with (suspected) neuroblastoma are still warranted.

Guidelines for managing **PPGLs** recommend initial biochemical testing to entail the measurement of plasma free or urinary fractionated metanephrines, including normetanephrine (NMN), metanephrine (MN), and, in select cases, 3-methoxytyramine (3MT) (6). Plasma 3MT offers additional diagnostic value when suspecting a dopamine-secreting chromaffin tumor or in hereditary syndromes (e.g., SDHx mutation carriers) associated with PPGL. Moreover, it serves as a prognostic indicator for metastases (3,6–8). Strict adherence to preanalytical precautions is crucial for obtaining accurate results from plasma samples, necessitating a supine rest for a minimum of 20 minutes, an overnight fast (particularly for plasma 3MT), and the use of an indwelling intravenous catheter to minimize the false-positive rate (9,10). Plasma free metanephrines appear to offer superior diagnostic accuracy compared to urinary metanephrines (3,10). However, in certain scenarios, the need for preanalytical precautions with plasma samples may lead to the preference for urinary samples.

European guidelines advocate for a comprehensive evaluation of hormone excess in patients with adrenal incidentaloma, involving a 1-mg overnight dexamethasone suppression test and measurement of plasma or urinary fractionated metanephrines (11).

This Critically Appraised Topic provides an overview of the biochemical modalities available for the diagnosis of neuroblastoma and PPGL in clinical laboratory settings.

CLINICAL/DIAGNOSTIC SCENARIO

Neuroblastoma, paraganglioma and pheochromocytoma are catecholamine-producing tumors derived from the neural crest. Neuroblastomas are derived from immature cells of the neural crest destined for the adrenal medulla and sympathetic nervous system, while PPGLs are derived from more mature chromaffin cells (6,12). Neuroblastomas are a heterogeneous group of neuroblastic tumors with a widely variable clinical course and degree of differentiation (13). Pheochromocytomas arise from adrenal chromaffin cells and paragangliomas from extra-adrenal chromaffin cells of the sympathetic paravertebral ganglia of the thorax, abdomen, and pelvis (6,12).

The catecholamines dopamine (DA), norepinephrine (NE), and epinephrine (E) are monoamine neurotransmitters that are derived from the amino acid tyrosine. They can be metabolized either by methylation by catechol-O-methyltransferases (COMT) or by deamination by monoamine oxidases (MAO). Metanephrine (MN), normetanephrine (NMN) and 3-methoxytyramine (3MT) are derived from epinephrine, norepinephrine, and dopamine respectively by membrane-bound catecholamine O-methyltransferase (COMT) within chromaffin cells. These metabolites are produced continuously by catecholamine-producing tumors. Homovanillic acid (HVA) is an end-product of dopamine metabolization and vanillylmandelic acid (VMA) is the main urinary end-product of norepinephrine and epinephrine metabolization (Figure 1) (6,12).

The diagnostic work-up for neuroblastomas traditionally consists of measuring VMA and HVA in a 24-hour urine collection or a random urine collection. Neuroblastomas produce catecholamines but lack the catecholamine storage vesicles, present in mature chromaffin cells. Catecholamines are mostly metabolized within tumor cells and hence the measuring of VMA and HVA is part of the diagnostic testing in neuroblastoma (12,14,15). The difficulty in biochemically diagnosing a neuroblastoma is the complex biology and clinical heterogeneity of these tumors. Different catecholamine excretion profiles are described (16). The final diagnosis is made by histopathology (12).

The initial biochemical approach for diagnosing pheochromocytomas and paragangliomas (PPGLs) involves assessing the levels of plasma free metanephrines or urinary fractionated metanephrines. (6–8). The flowchart made by ARUP Laboratories even differentiates between high-risk and low-risk patients. Plasma free metanephrines are recommended for high-risk patients (better sensitivity) and urinary fractionated metanephrines for low-risk patients (better specificity) (Figure 2) (3,17,18). The recommendations for biochemical testing for PPGL of the Mayo Clinic Laboratories are similar, plasma free fractionated metanephrines (more sensitive assay) should be used for screening and a 24-hour urinary fractionated metanephrines can be used in patients with low suspicion (more specific assay) (19). A patient with an undiagnosed pheochromocytoma is at risk of having a pheochromocytoma crisis, an acute severe presentation of a catecholamine-induced haemodynamic instability causing end-organ damage and sometimes shock. This crisis has a mortality of 6% and if the patient is in shock the mortality rate goes up to 28% (4). All positive tests should result in a follow-up that consists of either an additional biochemical test, a wait-and-retest approach, or imaging (6). Especially in the case of borderline elevated values, serial assessment can be useful (3,4,7). False negative results are iuncommon and can be observed in small asymptomatic tumors and tumors with a metabolic defect (“nonfunctional” or “nonsecretory” PPGL) (7,20). Chromogranin A, a secretory protein located in secretory granules of neuroendocrine cells, can be elevated in patients with neuroendocrine tumors and used as biochemical analysis in silent PPGL. However, the diagnostic accuracy of this biomarker is lower than metanephrines in plasma or urine for PPGL (sensitivity 83%) (3,7,8,21). When chromogranin A was used as a follow-up test in case plasma fractionated metanephrines were borderline elevated, a sensitivity of 87% was reached with a specificity of 73% to 89% depending on the used cutoff (22).

Genetic testing should be considered in all patients with PPGL and patients should be engaged in shared decision making. At least 30-40% of patients with PPGL have disease-causing germline mutations. Syndromic PPGL must be suspected in patients with multiple, multifocal and/or recurrent PPGL, an extra-adrenal PPGL, early onset of the disease and/or a positive family history for syndromic PPGL. Patients with an SDHB germline mutation present more frequently with metastatic disease and risen methoxytyramine levels (4,6–8).

Long-term surveillance is strongly recommended for at least 10 years, for high-risk patients lifelong follow-up should be considered (7,12,21).

QUESTION(S)

1) *Which urine and plasma biomarkers are recommended for the diagnosis of neuroblastoma?*

2) *Which biomarkers and sample types are recommended for the diagnosis of PPGL?*

3) *What is the diagnostic performance of the above-mentioned biomarkers in urine compared to blood in patients with PPGL?*

4) *What is the diagnostic performance of the above-mentioned biomarkers in patients with an adrenal incidentaloma?*

SEARCH TERMS

1) *MeSH Database (PubMed): MeSH term: "Neuroblastoma", "Metanephrine", "Diagnosis", "Pheochromocytoma", "Paraganglioma", "Guidelines as Topic", "Biomarkers"*
2) *PubMed (Medline; from 1966): "Adrenal incidentaloma" [Supplementary Concept], hereditary syndromes paraganglioma, pheochromocytoma, neuroblastoma, metanephrines, HVA, VMA, incidentaloma, biochemical*
   Cochrane (https://www.cochranelibrary.com/): ("Neuroblastoma"[Mesh]) AND "Diagnosis"[Mesh], ("Pheochromocytoma"[Mesh]) AND "Diagnosis"[Mesh], ("Paraganglioma"[Mesh]) AND "Diagnosis"[Mesh]
3) *International organizations: Mayo Clinic Laboratories, ARUP Laboratories, National Cancer Institute (NIH), American Cancer Society, Richtlijnendatabase (https://richtlijnendatabase.nl/)*

\+ *reference list of the selected literature was reviewed.*

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1) Which urine and plasma biomarkers are recommended for the diagnosis of neuroblastoma?

Neuroblastomas are the most common extracranial solid tumors in children (1,2). As mentioned above, urinary VMA plus HVA are classically used in the diagnostic work-up, besides histopathology and imaging (12,16,23–25). During the latter part of the twentieth century, the screening of infants for neuroblastoma using the measurement of urinary vanillylmandelic acid (VMA) in combination with homovanillic acid (HVA) was implemented in Germany, Japan, the United States, and Canada. However, these screening programs were discontinued due to their failure to reduce mortality and the occurrence of overdiagnosis, leading to unnecessary treatment (26).
In the last 10 to 15 years other biomarkers have been proposed for the diagnosis of neuroblastoma such as catecholamines (E, NE, DA) and metanephrines (MN, NMN, 3MT) in urine or plasma (5,15,16,23,27–29). More recently cystathionine (CTN), cortisol (COR), vanillactic acid (VLA) and 3-methoxytyramine sulfate (3-MTS) were also proposed as possible biomarkers (23,24).

Urinary catecholamines and catecholamine metabolites
More than 90% of the neuroblastomas excrete catecholamines and consequently, VMA and HVA are produced in significant quantities by these tumors. Unfortunately, the diagnostic performance of urinary VMA plus HVA is suboptimal (15,28–30). Most studies report a diagnostic sensitivity of urinary VMA plus HVA of 73%-92% with a specificity of 96%-100% (15). The Italian cooperative group for neuroblastoma reported better diagnostic performance with a sensitivity for urinary VMA plus HVA in spot urine samples of 74.5-100% and specificity of 88.2-99.7% depending on the age of the patient and the staging of the tumor (31). Given the suboptimal diagnostic performance of urinary VMA plus HVA, researchers have been looking for other metabolites.
Verly et al. reported in 2017 that a panel of eight urine catecholamine metabolites (VMA, HVA, E, NE, DA, free MN, free NMN, free 3MT) had a significantly higher sensitivity compared to urinary VMA plus HVA (92% versus 80% with a cutoff of 3 SD) in a retrospective cohort of 301 neuroblastoma patients (29). Only 58% of stage 1 patients had an increased VMA plus HVA, compared to 93% of stage 4 patients (29). Urine NMN increased from stage 1 to stage 4 and 71% of patients who had a normal VMA plus HVA excreted other catecholamine metabolites. The sensitivity of VMA plus HVA in patients with MIBG-negative tumors was only 33% compared to 89% for the eight-metabolite panel (29). MIBG, an analogue of NE, accumulates in neurosecretory granules (4). In a follow-up study by the SIOPEN Catecholamine Working Group, the study population was expanded to 400 neuroblastoma patients and a control group of 571 patients without neuroblastoma was included to assess the diagnostic performance of the panel of eight catecholamine metabolites (5). The overall diagnostic performance of the panel with eight metabolites was significantly higher than VMA plus HVA (area under the receiver operator curve of 0.952 versus 0.920, p=0.02), but the specificity of the eight-metabolite panel was significantly lower than VMA plus HVA (75% versus 92% in spot urine samples). The specificity of each catecholamine metabolite varied between 94% and 96% (5). HVA and NMN were the most commonly elevated metabolites in neuroblastoma and had the highest specificity of the eight markers (5).
The current standard clinical practice recommendations of the European Society for Paediatric Oncology include urinary catecholamine metabolites (VMA and HVA minimum) as part of the initial evaluation at diagnosis (32)

Spot urine versus 24-hour urine collection
Historically, a 24-hour urine collection was preferred to measure the catecholamine to exclude the circadian fluctuations in the excretion of the metabolites. Neuroblastoma is generally diagnosed in children, the median age at diagnosis is 18 months (2,5). In young children, however, a 24-hour urine collection can be difficult (5). A spot urine sample with results normalized per creatinine has been proposed as an alternative. A potential disadvantage of a spot urine sample is that the creatinine

concentration depends on the muscular mass, disease state and the concomitant intake of some drugs (27,33).
The SIOPEN Catecholamine Working Group looked at the difference between spot urine (62 patients) and a 24-hour urine collection (171 patients) and found a similar diagnostic sensitivity in a 24-hour collection and spot urine samples for VMA plus HVA or an eight-metabolite panel (VMA, HVA, DA, 3MT, NE, NMN, E and MN) (5). No parallel 24-hour collection and spot urine samples were available from the same patients. Of note, the planned 24-hour urine collection was not completed in more than 25% of the children with neuroblastoma with a similar distribution in all ages. Other studies showed comparable results when comparing a 24-hour urine collection to spot urine (31,34). Barco et al. found a specificity of 96.3% and a sensitivity of 81.9% for the measurement of VMA plus HVA in spot urine (31).

Plasma free catecholamines and catecholamine metabolites
The disadvantage of measuring plasma catecholamines and catecholamine metabolites is the instability compared to acidified urine samples (28). Paediatric reference ranges for plasma free 3MT and free NMN were established by Franscini et al. and Peitzsch et al. (27,35). Both found age-related changes in plasma free metanephrines with an important drop in the plasma levels of 3MT, NMN and 3-O-methyldopa in the first years of life (Figures 3 and 4) (12,27,35).
Peitzsch et al. looked at the diagnostic performance of 3-O-methyldopa, 3MT and NMN in plasma in a retrospective cohort of 96 patients with neuroblastoma and 553 patients without neuroblastoma and observed that plasma free 3MT plus free NMN had a better sensitivity (97.9% versus 82.2%) and better specificity (95.1% versus 84.8%) than urinary VMA plus HVA (15). The plasma free 3MT concentration showed a 22.3-fold increase above the median values of the control group and NMN an 8.2-fold increase. The increase in urinary VMA and urinary HVA was less pronounced (6.3-fold and 3.6-fold increase, respectively). In this retrospective study, the sensitivity was the highest for 3MT (96.8% of the cases had elevated levels) followed by HVA, VMA and NMN (72.9%, 75.6% and 73.2%, respectively). The authors also studied the diagnostic performance of 3-O-methyldopa and concluded that this parameter could be useful as a complement to the panel for tumors with limited production of downstream catecholamine metabolites (15). Barco et al. found in a small retrospective cohort of 38 neuroblastoma patients that the sensitivity and specificity of urinary VMA plus HVA and plasma free 3MT plus free NMN were comparable (90% and 79%, respectively, for both) (28). A drawback of both studies is that no other urinary metabolites including urine 3MT and urine NMN were measured. The SIOPEN Catecholamine Working Group is currently performing a multicentre prospective study that will compare the diagnostic performance of urine and plasma catecholamines and catecholamine metabolites (HVA, VMA, E, NE, DA, MN, NMN, 3MT).

Other potential biochemical biomarkers
Cystathionine (CTN) showed an 11-fold increase in patients with neuroblastoma and cortisol a 10-fold increase (23). This can be considered as metabolites as part of an extensive panel. However further research is needed to see what the exact benefit is.
Other researchers proposed the usefulness of vanillactic acid (VLA) and 3-methoxytyramine sulfate (3-MTS) (23,24). HVA, VMA, VLA and MTS were all good markers to distinguish high-risk neuroblastomas, but VLA and MTS were the only metabolites able to determine low-risk neuroblastomas. Furthermore, VLA and MTS had higher diagnostic accuracy in a scoring model than VMA and HVA (24).

2) Which biomarkers and sample types are recommended for the diagnosis of PPGL?

Catecholamines, metanephrines and VMA have all been evaluated for the diagnosis of PPGL in the last 50 years. Multiple studies have shown that catecholamines, which are less stable than metanephrines, and only secreted intermittently by PPGL have a lower diagnostic performance than metanephrines (3,9). Measurement of metanephrines in urine or plasma is currently considered the main biochemical test in the diagnostic work-up for PPGL. 3MT in plasma can be beneficial as an

additional metabolite in the diagnostic work-up for dopaminergic PPGL or as a prognostic factor for metastasis (3,6).
The Endocrine Society Clinical Practice Guideline from 2014 recommended that measurement of plasma free or urinary fractionated metanephrines should be performed as the initial biochemical testing using liquid chromatography with tandem mass spectrometry (LC-MS/MS) or liquid chromatography with electrochemical detection (LC-ECD) (6). The Joint Position Statement of the Korean Pheochromocytoma and Paraganglioma Task Force and a Spanish guideline, both from 2021, gave the same recommendations (7,8). The more recent clinical practice guideline on the management of adrenal incidentalomas of the European Society of Endocrinology also recommends excluding pheochromocytoma by measuring urinary fractionated metanephrines or plasma free metanephrines (11). American and Canadian guidelines on adrenal incidentalomas strongly recommend excluding pheochromocytoma by measuring plasma or 24-hour urinary metanephrines (36,37).

In a prospective study with 2056 patients screened for PPGL, 236 patients were diagnosed with a chromaffin cell tumor. The free metanephrines (NMN and MN) in plasma and a 24-hour urine collection had a good diagnostic performance (sensitivities of 96.6% and 92.9%, and specificities of 94.9% and 94.5%, respectively) (10). Plasma and urinary free NMN showed a 9.6- to 10.4-fold increase in comparison to patients without PPGL. Plasma and urinary MN gave smaller increases (3.9- and 4.0-fold increases, respectively). When 3MT in plasma is added, the sensitivity rose to 97.9% without any significant decrease in specificity (94.2%) (10). Urinary 3MT is less useful because of the metabolization pathway. Urinary dopamine has multiple sources such as diet and production in diffuse autocrine/paracrine systems of the gut and other tissues. Furthermore, DOPA is metabolized to 3MT in the kidneys (Figure 1) (3,12). In the prospective study by Eisenhofer et al., urine samples were analyzed with and without the acid hydrolysis step. Differences were seen in the diagnostic performance, urinary free metanephrines had a comparable sensitivity (92.9% versus 92.9%) but had a higher specificity (94.5% versus. 92.8%) (10). The acid hydrolysis step converts the sulfate-conjugated metabolites into the free form, so the sulfate-conjugated metabolites are measured and the free metabolites. Sulfate-conjugated metabolites are produced in various parts of the body (3,10).

Even with measuring plasma free metanephrines, there is still a false-negative rate of 2.1%. Most of those patients had small tumors and tested positive on follow-up. It was rarely due to a nonsecretory PPGL (1 in 236 patients with diagnosed PPGL) (3,10). Tumor size is positively correlated to metanephrines (only a weak relationship between tumor size and catecholamine levels was described). A small elevation of NMN in plasma or urine in patients with an adrenal tumor of over 5 cm can be a false positive result or a nonsecretory PPGL (Figure 5) (3,20).

As mentioned above 3MT in plasma can be useful in addition to NMN and MN when a dopamine-secreting chromaffin tumor is suspected or in hereditary syndromes (e.g. SDHx mutation carriers) associated with PPGL. Furthermore, this biomarker can be a prognostic marker for metastases (3,6–8).

Chromogranin A, a secretory protein located in secretory granules of neuroendocrine cells and co-released with catecholamines or other monoamines, can be seen as an additional test in the diagnostic work-up for PPGL. Plasma chromogranin A is generally used as a prognostic marker in neuroendocrine tumors. In screening for PPGL, this protein had a lower diagnostic performance (sensitivity 83%) (3,7,8,21). This biomarker also had limited specificity due to possible elevation in several clinical conditions linked to increased adrenergic activity or impaired renal function, with some medications (e.g. proton pump inhibitors), and can be elevated in other neuroendocrine tumors and other neoplasms (3,38). The multidisciplinary practice guidelines of the Spanish Societies and the Korean Pheochromocytoma and Paraganglioma Task Force proposed to measure chromogranin A when plasma or urinary metanephrines are negative, especially in patients with high clinical suspicion, (7,8).

The clonidine suppression test has been proposed as an additional test for borderline elevated NMN levels (3,6,7,39). Borderline elevated metanephrines levels are defined as anything between one and three times the upper limit of the reference interval (4). Clonidine is a centrally acting alpha$_2$-adrenoreceptor agonist and mediates sympathoinhibitory responses. It reduces the NE release in sympathetic nerves but has no influence on the exocytosis of NE by PPGL. When clonidine is administered in patients with PPGL, elevated NMN levels in plasma persist. In patients without PPGL NMN levels drop below 40% of baseline values (3,6,7). Drugs that act on the NE reuptake or the alpha$_2$-adrenoreceptor should be withdrawn at least 48 hours before testing (3,6).

The incidence of PPGL is low (2-8 cases per million inhabitants) (7) with a pretest prevalence of only 0.6% to 0.8% (3). In patients with an adrenal incidentaloma, pretest prevalence is higher (4-7%), also patients with a hereditary predisposition have a higher pretest prevalence depending on the type of genetic mutation (patients with MEN2 have a penetrance up to 50% for a pheochromocytoma, for NF1 carriers, on the other hand, the penetrance of pheochromocytoma is estimated between 0.1% to 7.7%). In these patients, measuring plasma free metanephrines is recommended because of the higher sensitivity (3,17,18). Patients after surgical resection of a PPGL should be followed up because of the risk of a new PPGL (estimated prevalence of 3%) (40). Pre-test prevalence can also be influenced by the signs and symptoms of the patient at presentation. The classic triad of symptoms in patients with PPGL was sometimes not present, palpitations were seen in 65% of the cases, hyperhidrosis in 55% and headache in 46%. When more features of the triad were present, the likelihood of a PPGL rose (3). In hypertensive outpatients, the prevalence of a PPGL was estimated at 0.2-0.6%, but in patients with a PPGL hypertension was reported in 85-95% of the cases (3,4). Depending on the pretest prevalence and the indication used for biochemical testing, positive results should be interpreted differently (3,4). Post-test probabilities of PPGL were estimated between 75% and 95% when a 2-fold elevation above the upper reference limit of either plasma free NMN or plasma free MN was measured for pretest probabilities of 0.5% to 5.0%. When patients were tested due to signs and symptoms of catecholamine excess, 3-fold increases in concentration of metanephrines could be considered. False-positive results for plasma free metanephrines are usually seen when only one of the metabolites is elevated (70% of the patients with PPGL have at least two increased metabolite levels) (3).

3) What is the diagnostic performance of the above-mentioned biomarkers in urine compared to blood in patients with PPGL?

One of the first studies comparing plasma samples and 24-hour urine collection for the measurement of metanephrines was done almost 30 years ago. 1003 patients were included of which 214 patients were diagnosed with a pheochromocytoma. The authors concluded that plasma free metanephrines had the best diagnostic performance and should be the first choice (sensitivity of 99% and specificity of 89% versus sensitivity of 97% and specificity of 69%) (9).

20 years later another study looked at the difference in diagnostic performance between plasma free metanephrines and urinary free metanephrines (24-hour urine collection). Sensitivity was higher for plasma free metanephrines than urinary free metanephrines (96.6% versus 92.9%). Specificity was comparable in both tests (94.9% for plasma free metanephrines and 94.5% for urinary free metanephrines). Urinary total (deconjugated) metanephrines were less performant with a sensitivity of 92.9% and a specificity of 92.8%. As mentioned before, adding plasma 3MT to the plasma free metanephrines (MN and NMN) improved the diagnostic performance (sensitivity of 97.9% and specificity of 94.2%) (10).

The above-mentioned studies warned that adherence to preanalytical precautions is important. Precautions include a supine rest for at least 20 minutes, an overnight fast (especially for plasma 3MT), and the use of an indwelling intravenous catheter to lower the false-positive rate (9,10). A recent prospective study in ten European tertiary referral centres in 3147 patients who were screened for PPGL (including 278 patients in whom PPGL was diagnosed) confirmed the importance of the

preanalytical phase for the measurement of plasma metanephrines (MN and NMN) and plasma 3MT. Blood collection for plasma metanephrines should be carried out after 20 minutes in a comfortable supine position through an indwelling catheter and on cold days patients should stay prolonged in a warm indoor environment. The authors saw lower plasma metanephrines levels in inpatients compared to outpatients (39). A Canadian study also warned about the difficult interpretation of urinary metanephrines (24-hour urine collection) in hospitalized patients who were acutely ill due to the activation of the sympathoadrenal function. 974 inpatients were compared to 6802 outpatients and 58 patients with a confirmed PPGL retrospectively. An upward shift of urinary NMN in patients with a critical illness was seen (41). In those cases, imaging can be preferred as a first step in diagnosing PPGL when PPGL is suspected, biochemical testing can be postponed in those cases (3,41).

Metanephrines concentrations are unstable in whole blood at room temperature. Samples should be collected onto ice or cold packs before centrifugation. After centrifugation and separation of the plasma metanephrines are stable for 6 hours at 4°C and can be frozen for longer storage (42). Urinary metanephrines are more stable and it is advisable not to use a strong acid preservative for metanephrines due to the possible deconjugation of those catecholamine metabolites (3).

Kline et al. looked at the correlation between urinary metanephrines levels in 24-hour urine collection and impaired kidney function. Only in the most advanced stages of renal impairment (eGFR < 15 mL/min/m2) falsely low levels of urinary metanephrines levels were seen (43). Plasma deconjugated metanephrines can be elevated among patients with renal impairment since sulfate-conjugated metabolites are primarily cleared by the kidneys, plasma free metanephrines are less susceptible to renal function. For patients with end-of-stage renal disease, disease-specific reference intervals should be considered (3).

Situations that activate the sympathoadrenal system cause higher levels of metanephrines and potentially false-positive results. As aforementioned, a supine position is preferred in the measurement of plasma metanephrines. Even a 24-hour urine collection could be influenced by posture (3). Peitzsch et al. studied if sample collection after a night of sleep resulted in fewer false-positive results. Plasma free metabolites had the best diagnostic performance compared to a 24-hour urine collection and an overnight urine sample (sensitivities of 97.5%, 87.3% and 93.7%, respectively and specificities of 94.7%, 94.7% and 94.3%). An overnight urine sample had a better diagnostic performance than a 24-hour urine collection (44). Sbardella et al. found similar diagnostic power by comparing random spot urine to 24-hour urine collection and plasma samples (45). Other situations that activate the sympathoadrenal system are a cold environmental temperature, acute emotional stress and the direct venipuncture itself. Those situations have more impact on the false-positive rate in the measurement of plasma metanephrines (3,9).

Dietary constituents and some medications can influence plasma or urinary catecholamines and catecholamine metabolites. Especially dopamine and its metabolites can be impacted by foods containing tyramine or L-DOPA. An overnight fast before blood sampling can be a solution for the measurement of 3MT in plasma (3). Davison et al. described a much higher false-positive rate in non-fasting ambulant patients (up to 30%) compared to a 5% false-positive rate observed in patients who underwent overnight fasting and provided samples in a supine position (4). Nicotine and caffeine are known to stimulate sympathoadrenal secretion of catecholamines (3,9). An overview of interfering substances on the sympathoadrenal system is given by Eisenhofer et al. (Table 1) (3). Phenoxybenzamine, a nonselective alpha-adrenoreceptor blocker, is frequently used as an antihypertensive drug in patients with pheochromocytoma but it interferes with the measurement of catecholamines and their metabolites. Tricyclic antidepressants and venlafaxine are among the pharmaceuticals commonly used by individuals exhibiting false-positive outcomes (3,9).

Plasma free metanephrines seem to have a better diagnostic accuracy than urinary metanephrines. Nevertheless, due to the preanalytical precautions needed for measuring plasma free metanephrines, a urinary sample can be preferred.

4) What is the diagnostic performance of the above-mentioned biomarkers in patients with an incidentaloma?

An adrenal incidentaloma is defined as an adrenal mass detected when imaging is performed for an indication not related to an adrenal disease (11,46). Prevalence of adrenal incidentalomas increases with age, 3% in 40-year-old patients and 10% among those aged 70 years (46). The aetiology of adrenal masses varies, in most cases, it is benign (85-95%) and non-functioning (50-70%) but adrenal masses can in some cases secrete hormones such as cortisol, aldosterone and metanephrines (Table 2) (46–48).

In case of an incidentaloma, a pheochromocytoma, Cushing's syndrome and primary aldosteronism should be excluded. In 2-12% of the cases, an adrenal incidentaloma is diagnosed as an adrenocortical carcinoma (46). Up to 50% of the patients with an adrenal incidentaloma had mild autonomous cortisol secretion (48) and approximately 7% of the adrenal incidentalomas are pheochromocytomas (49).

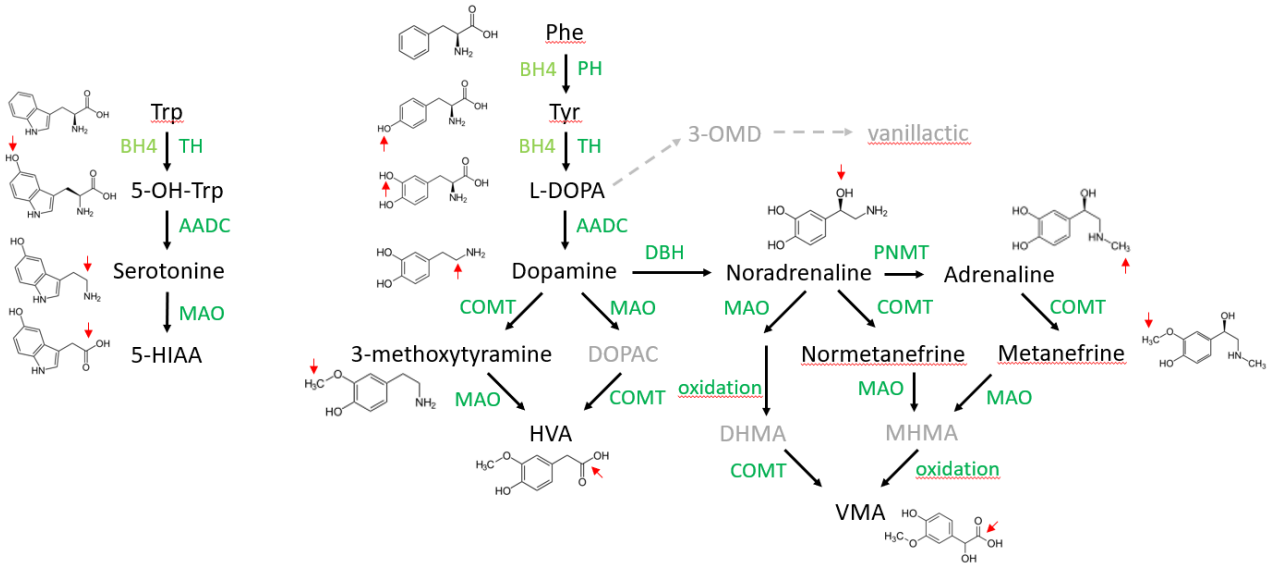
In 2023 the European Society of Endocrinology published clinical practice guidelines on the management of adrenal incidentalomas (11). Patients should undergo a thorough assessment of the hormone excess. A 1-mg overnight dexamethasone suppression test should be performed in all patients (except in some frail patients with limited life expectancy) and plasma or urinary fractionated metanephrines to exclude pheochromocytoma should be measured in patients with adrenal lesions with features not typical for a benign adenoma (11). An adrenal tumor with Hounsfield Units less than or equal to 10 in unenhanced CT is almost certainly not a pheochromocytoma (less than 0.5% of cases) (11,48). In case of concomitant hypertension or unexplained hypokalaemia, measurement of the aldosterone/renin ratio to evaluate primary aldosteronism is advised (11). A 2021 clinical practice article in the New England Journal of Medicine proposed an algorithm with the same biochemical analyses (dexamethasone suppression test and metanephrines in urine or plasma) as the European guidelines of 2023 for the hormonal evaluation in adrenal incidentalomas (Figure 6) (49).

COMMENTS

TO DO/ACTIONS

1) Implement the metanephrines (NMN, MN and 3MT) and catecholamines (E, NE, D) in plasma in the laboratory of UZ Leuven.

ATTACHMENTS



5-HIAA: 5-OH-indolacetic acid; 5-OH-tryptamine; 3-O-methyldopa; AADC: L-aromatic aminoacid decarboxylase; BH4: tetrahydrobiopterine; COMT: catechol-O-methyl-transferase; DBH: dopamine-beta- hydroxylase (co-factor: ascorbic acid); DHMA: dihydroxymandelic acid; DOPA: 3,4diOH-phenylalanine; DOPAC: 3,4-diOH-phenylacetic acid; HVA: homovanillic acid; MAO: monoamine oxidase (cofactor: FAD); MHMA: 3-methoxy-4-OH-mandelic aldehyse; PH: phenylalanine hydroxylase, PNMT: phenylethanolamine N-methyltransferase (co-factor: S-adenosyl-L-methionine); TH: tyrosine hydroxylase; VMA: vanillylmandelic acid

Figure 1: TH is the rate-limiting enzyme in the catecholamine synthesis, this enzyme can be found in the cytoplasm of a chromaffin cell. AADC has wide tissue distribution. DBH is located in secretory granules and PNMT is a cytoplasmatic enzyme in chromaffin cells, so a translocation of dopamine into a secretory granule is needed, followed by passive leakage in the cytoplasm of norepinephrine (noradrenaline). Epinephrine (adrenaline) is translocated back into an adrenergic secretory granule. Exocytosis of those granules is an active calcium-dependent process. Norepinephrine secreted by sympathetic nerves acts locally and over 90% is removed back into the nerves. In sympathetic nerves there is a MAO, but no COMT enzyme. COMT is present in chromaffin cells and the membrane-bound isoenzyme is responsible for the majority of metanephrines production. DHMA and DOPAC are further metabolized by soluble COMT isoenzymes, found in most tissues. Circulating metanephrine is mostly (>90%) derived from epinephrine secreted by chromaffin cells. On the other hand, circulating normetanephrine is in 25% produced within chromaffin cells and 75% is formed extraneuronal from norepinephrine excreted by sympathetic nerves. VMA is mostly produced in the liver, only a small portion is derived from norepinephrine secreted by chromaffin cells. Unlike VMA, HVA does not need oxidation in the liver. Metanephrine and normetanephrine metabolites undergo sulfate conjugation in varying degrees, the enzyme responsible for sulfatation is located in the gastrointestinal tissues and primarily removed by renal excretion. Metanephrines measured in urine after acid hydrolysis mainly reflect the sulfated metabolites. Urinary dopamine has multiple sources such as the diet and production in diffuse autocrine/paracrine systems of the gut and other tissues. Furthermore, DOPA is metabolized to methoxytyramine in the kidneys (3,12).

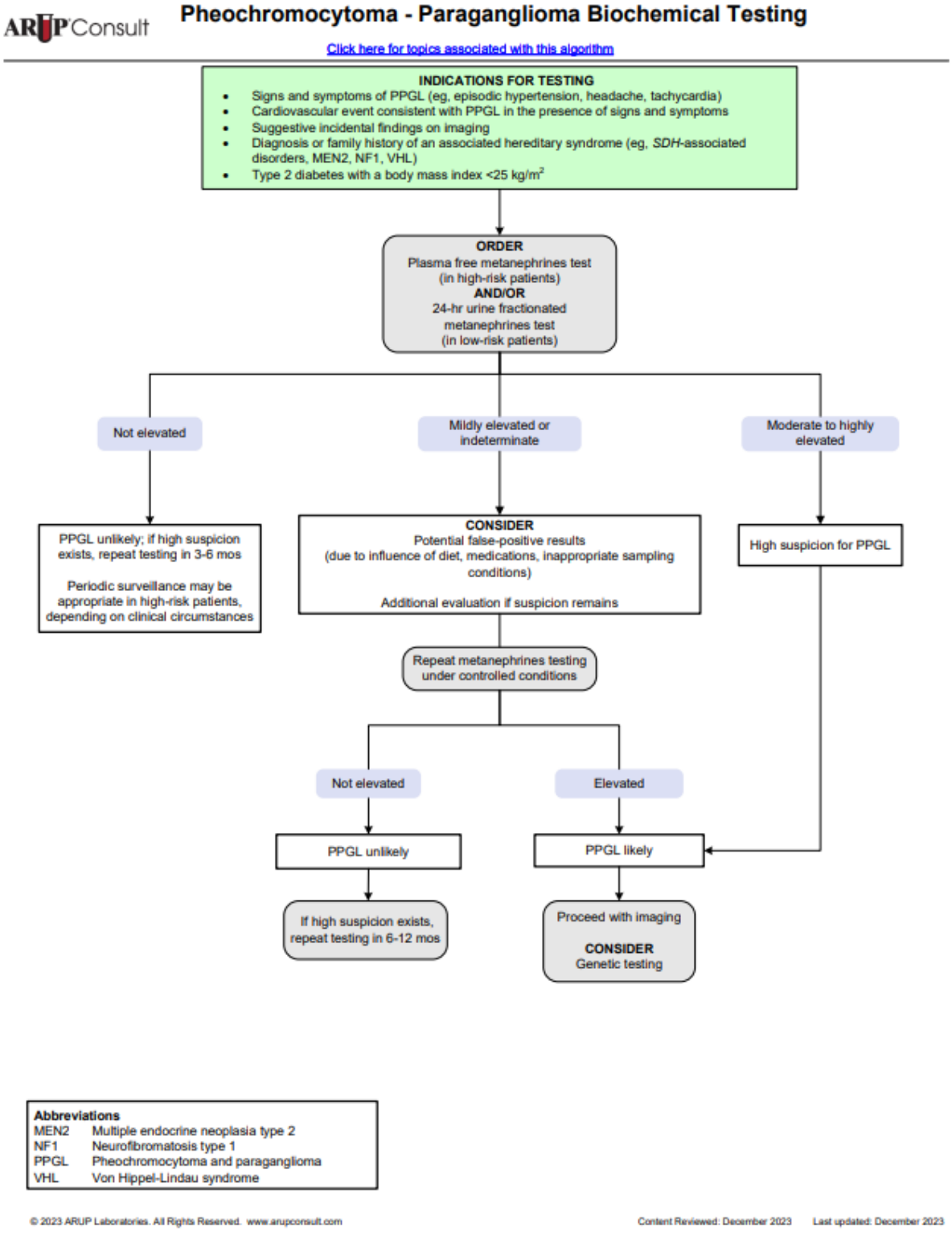


Figure 2: ARUP Laboratories Algorithm for pheochromocytoma and paraganglioma biochemical testing.

High-risk patients: Individuals or family members with NF1, MEN type 2A or 2B, VHL, hereditary paraganglioma/pheochromocytoma syndromes (especially SDH-gene-associated disorders) or adrenal incidentaloma.

Low-risk patients: patients with treatment-resistant hypertension, unexplained heart failure, paroxysmal headaches, palpitations, sweating, and panic attacks associated with hypertension, hypertensive crisis developing during surgery or general anaesthesia, hypertension triggered by beta-blockers or by monoamine oxidase inhibitors or by micturition/changes in abdominal pressure, orthostatic hypotension in a hypertensive patient or new-onset diabetes mellitus in a young, lean individual with hypertension (18)

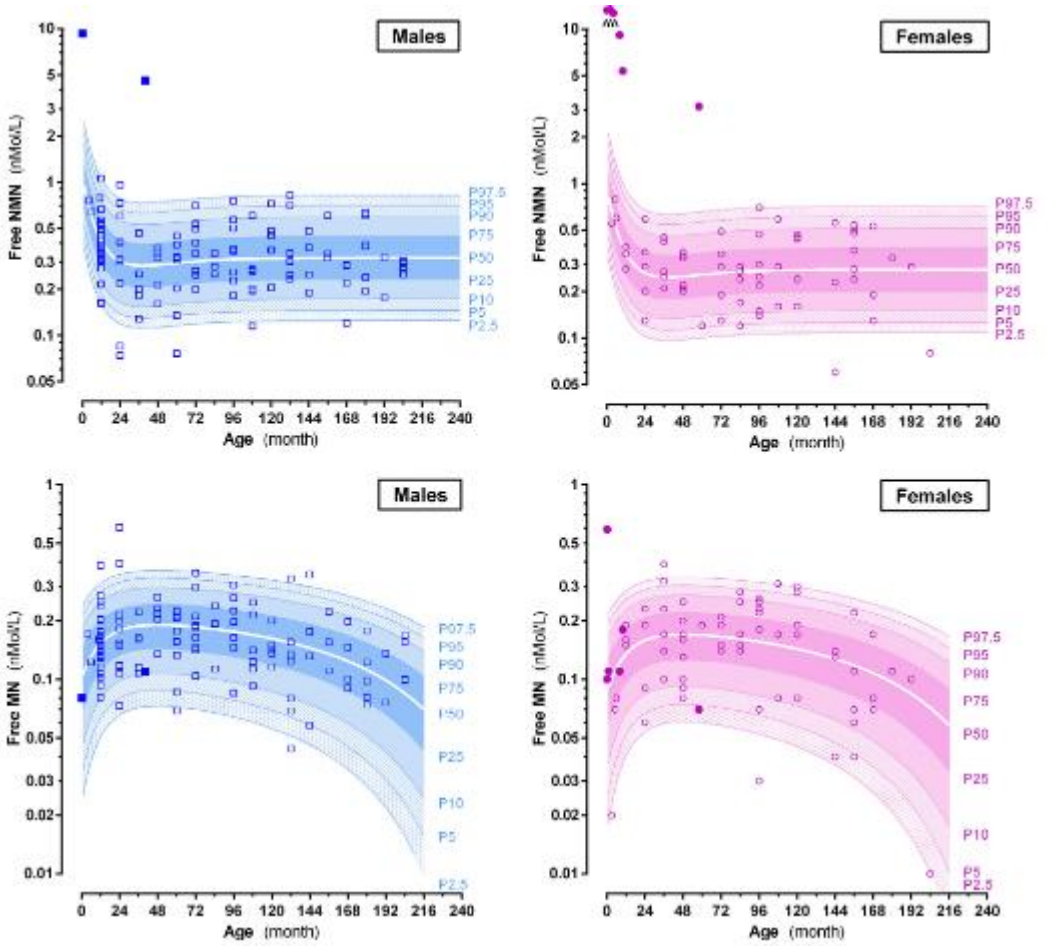


Figure 3: Concentrations of plasma free metanephrines in a control group and in patients with neuroblastoma. The open symbols are controls, and the closed symbols are patients diagnosed with neuroblastoma (27)

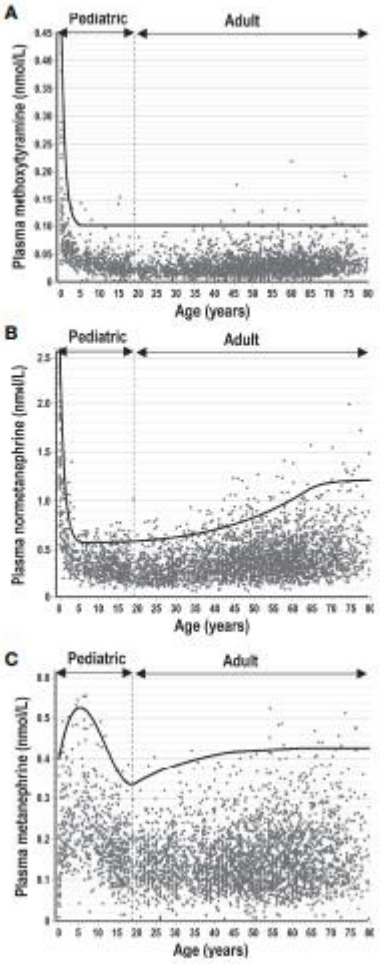


Figure 4: Plasma concentrations of 3MT, NMN and MN in 3706 individuals without chromaffin tumors. The solid lines indicate the upper limit of reference intervals (12)

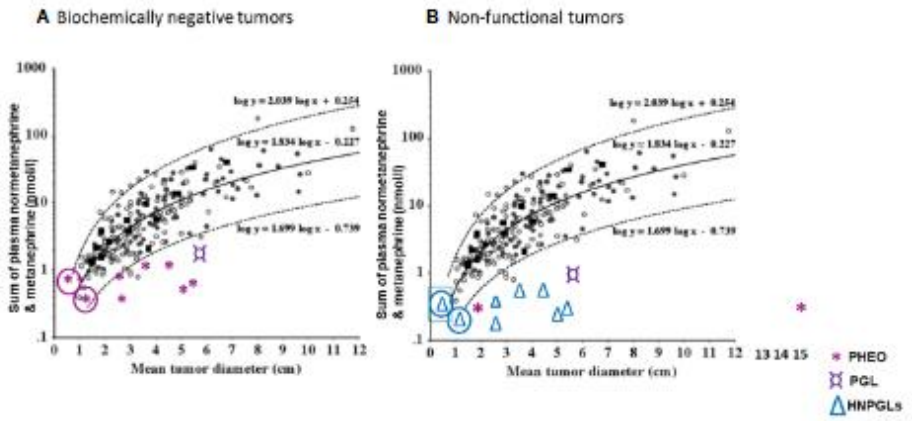


Figure 5: Correlation between tumor size and the sum of plasma metanephrines (20)

Table 1: Overview of interfering substances on the sympathoadrenal system (3)

Drug category	Pharmacodynamic actions	Main impact
Stimulants		
Nicotine	• Activation of nicotinic cholinergic receptors	Increased adrenal epinephrine secretion
Caffeine	• Mobilization of intracellular calcium stores	Increased adrenal epinephrine secretion
Sympathomimetics		
Amphetamine Methamphetamine	• Increased release of monoamines from vesicular stores of sympathetic nerves • Inhibition of monoamine oxidase • Blockade of neuronal cell membrane norepinephrine (NE) transporters (NET)	Increased NE concentrations in the neuronal cytoplasm Reversed transport of NE by NET from cytoplasm to extracellular space Increased NE escape from reuptake
Ephedrine Pseudoephedrine	• Activation of alpha and beta-adrenergic receptors • Inhibits function of vesicular monoamine transporters • Inhibits NE reuptake (indirectly)	Increased NE release Increased NE release from secretory vesicles
Norepinephrine reuptake blockers		
Tricyclic antidepressants Venlafaxine/Duloxetine Cocaine	• Blockade of neuronal cell membrane NE transporters • Centrally mediated sympathoinhibition	Decreased sympathetic nerve firing and secretion of NE from sympathetic nerves, but opposing increased escape of NE from reuptake after neuronal secretion
Alpha$_2$ adrenoreceptor antagonists		
Phenoxybenzamine Mirtazapine Yohimbine	• Antagonism of alpha2-adrenoreceptors at central sympathoinhibitory sites and on sympathetic neurons	Increased sympathetic nerves firing secretion of NE from sympathetic nerves
Monoamine oxidase (MAO) inhibitors	• Blockade of the deamination of the O-methylated catecholamine metabolites	Increased plasma and urinary metanephrines with normal catecholamines
Atypical antipsychotics		
Quetiapine, Clozapine, Risperidone	• Inhibition of dopaminergic, adrenergic, and serotoninergic receptors • Antagonism to α_2-adrenoreceptors	Increased secretion of NE from sympathetic nerves

Table 2: Frequency of the different underlying aetiologies in adrenal incidentalomas (11)

Etiology	Prevalence of the different entities among adrenal incidentalomas
Adrenocortical adenoma or macronodular bilateral adrenal hyperplasia	80%-85%
• Nonfunctioning	40%-70%
• Mild autonomous cortisol secretion	20%-50%
• Primary aldosteronism	2%-5%
• Overt Cushing's syndrome	1%-4%
Other benign mass	
• Myelolipoma	3%-6%
• Cyst and pseudocyst	1%
• Ganglioneuroma	1%
• Schwannoma	<1%
• Hemorrhage	<1%
Pheochromocytoma	1%-5%
Adrenocortical carcinoma	0.4%-4%
Other malignant mass (mostly adrenal metastases)	3%-7%

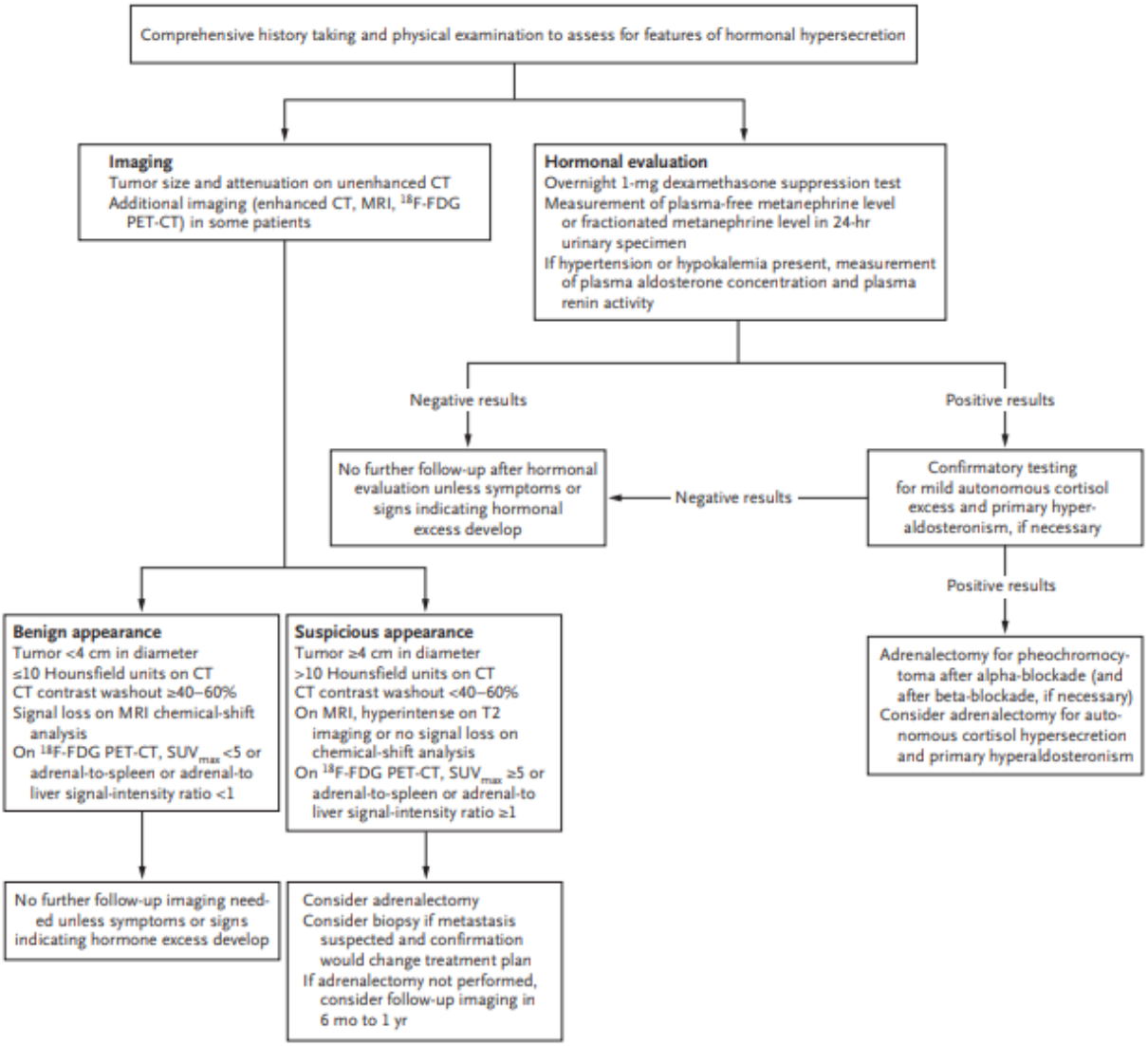


Figure 6: Proposed algorithm by Kebebew (*49)*

Table 3: Overview of the biochemical tests

Metabolite	Sample type	Indication	Measuring technique	Sensitivity	Specificity	Reference	Cost in Belgium
VMA plus HVA	urine	Neuroblastoma	n.a.	73-92%	96-100%	Peitzsch et al. (15)	546556-546560, B 1000 546571-546582, B 1000
*Eight-metabolite panel**	urine (free)	Neuroblastoma	UPLC-MS/MS	94%	75%	Matser et al. (SIOPEN Catecholamine Working Group) (5)	546556-546560, B 1000 546571-546582, B 1000 546512-546523, B 1400 546534-546545, B 1400
3MT plus NMN	plasma	Neuroblastoma	LC-MS/MS	97.9%	95.1%	Peitzsch et al. (15)	546011-546022, B 1400°
MN plus NMN	urine (free)	PPGL	LC-MS/MS	92.9%	94.%	Eisenhofer et al. (10)	546534-546545, B 1400
MN, NMN plus 3MT	urine (free)	PPGL	LC-MS/MS	93.4%	94.2%	Eisenhofer et al. (10)	546534-546545, B 1400
MN plus NMN	plasma	PPGL	LC-MS/MS	96.6%	94.6%	Eisenhofer et al. (10)	546011-546022, B 1400°
MN, NMN plus 3MT	plasma	PPGL	LC-MS/MS	97.9%	94.2%	Eisenhofer et al. (10)	546011-546022, B 1400°
Chromogranin A	plasma	PPGL**	Radioimmunoassay	83%	n.a.	Eisenhofer et al. (3)	Niet-ZIV prijs 22 euro
MN, NMN plus chromogranin A	plasma	PPGL	LC-MS/MS, chemiluminometric assay	87%	73-89%	Algeciras-Schimnich et al. (22)	546011-546022, B 1400° Niet-ZIV prijs 22 euro

* VMA, HVA, E, NE, DA, free MN, free NMN, free 3MT
** Other indications: neuroendocrine tumors (e.g. gastroenteropancreatic neuroendocrine tumors)
° Terugbetaling is al voorzien voor catecholamines, maar nog niet voor metanefrines. Dit volgt spoedig.