Title: Semi-quantitative $^{123}$I-metaiodobenzylguanidine scintigraphy to distinguish pheochromocytoma and paraganglioma from physiological adrenal uptake and its correlation with genotype-dependent expression of catecholamine transporters

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ABSTRACT

$^{123}$I-metaiodobenzylguanidine ($^{123}$I-MIBG) scintigraphy plays an important role in the diagnostic evaluation of patients with pheochromocytoma and paraganglioma (PPGL). MIBG targets cell membrane and vesicular catecholamine transporters of chromaffin cells and facilitates localization of the primary tumor and metastatic lesions. Its specificity for the diagnosis of adrenomedullary chromaffin cell tumors can be jeopardized by physiological uptake by the normal adrenal medulla. The aim of this study was to distinguish between PPGLs and normal adrenal glands by evaluating semi-quantitative $^{123}$I-MIBG uptake and to examine genotype-specific differences in correlation with expression of catecholamine transporter systems.

Methods: Sixty-two PPGLs collected from 57 patients with hereditary mutations in SDHA (n=1), SDHB (n=2), SDHD (n=4), VHL (n=2), RET (n=12), NF1 (n=2), MAX (n=1) and with sporadic PPGLs (n=33) were investigated. Pre-operative planar and single-photon emission computed tomographic (SPECT) images were semi-quantitatively analyzed using uptake measurements. Tumor-to-liver (T/L) and normal-adrenal-to-liver (NA/L) ratios were calculated and correlated with clinical characteristics including genotype, tumor size and plasma metanephrines concentrations. The expression of norepinephrine transporter (NET) and vesicular monoamine transporter (VMAT-1) was evaluated immunohistochemically in paraffin-embedded tumor tissues.

Results: Mean T/L ratios of PPGL lesions were significantly higher than NA/L ratios (p<0.001). Cut-off values to distinguish between physiological and pathological adrenal uptake were established at 0.7 (100% sensitivity, 10.3% specificity) and 4.3 (100% specificity, 66.1% sensitivity). No statistically significant differences in $^{123}$I-MIBG uptake were found across PPGLs of different genotypes. Mean NET expression in hereditary cluster 2 (RET, NF1, MAX) and apparently sporadic tumors was significantly higher than for hereditary cluster 1 (SDHx, VHL) PPGLs (p=0.011 and p=0.006, respectively). Mean VMAT-1 expression in hereditary cluster 1 PPGLs was significantly higher than for cluster 2 tumors (p=0.010). $^{123}$I-MIBG uptake significantly correlated with maximum tumor diameter (p=0.002). MIBG uptake, however, did not correlate with either NET or VMAT-1 expression.
Conclusion: Liver normalized semi-quantitative $^{123}$I-MIBG uptake may be helpful to distinguish between pheochromocytoma and physiological adrenal uptake. Genotype-specific differences in expression of NET and VMAT-1 do not translate into differences in $^{123}$I-MIBG uptake.

Key words: pheochromocytoma, paraganglioma, $^{123}$I-metaiodobenzylguanidine, scintigraphy, norepinephrine transporter, vesicular monoamine transporter 1
INTRODUCTION

Meta-iodobenzylguanidine (MIBG) is a structural and functional analog of norepinephrine that selectively accumulates in noradrenergic neurosecretory granules of sympathetic neurons, ganglia and chromaffin cells (1). Active cellular uptake of MIBG is mediated by the norepinephrine transporter (NET). Intracellularly, MIBG is stored in neurosecretory granules through the action of vesicular monoamine transporters (VMAT) (2, 3). MIBG has proven to be very useful for scintigraphic imaging of pheochromocytomas and paragangliomas (PPGLs) (4, 5). These rare and often benign catecholamine-producing tumors arise from the adrenal medulla and extra-adrenal sympathetic chromaffin tissues (5). PPGLs can occur sporadically or in the context of hereditary syndromes (~34%) (6). The major susceptibility genes for PPGLs include those encoding succinate dehydrogenase (SDH) complex subunits and cofactor 2 (SDHA/B/C/D/AF2, abbreviated SDHx), von Hippel-Lindau (VHL), RET, neurofibromin 1 (NF1), MYC-associated factor X (MAX) and transmembrane protein 127 (TMEM127) and more recently discovered fumarate hydratase (FH) and hypoxia-inducible factor-2α (HIF2A) (7, 8). Furthermore, somatic mutations of mainly NF1 and VHL can be detected in up to 30% of sporadic PPGLs (8). Hereditary PPGLs have been divided into two clusters based on their transcriptional signature (9, 10). Cluster 1 includes SDHx-, VHL-, FH- and HIF2A-related tumors and is characterized by a hypoxia/angiogenesis signature. Cluster 2 includes NF1-, RET-, MAX- and TMEM127-related tumors and is associated with the activation of MAP kinase/AKT/mTOR pathways.

At present, scintigraphy with 123I-MIBG is the most common used functional imaging technique for localizing PPGLs due to its high specificity and worldwide availability (4, 5). Its whole-body exploration makes it particularly useful for evaluation of multifocality, metastatic and recurrent disease. 123I-MIBG is chosen over 131I-MIBG because of higher diagnostics sensitivity, shorter half-life, favorable dosimetry and improved imaging quality (11, 12). Besides conventional planar imaging, single photon emission computed tomography (SPECT) imaging provides three-dimensional information which can be fused with computerized tomography (SPECT/CT) for anatomic correlation (13). Overall, specificities of
\[^{123}\text{I}-\text{MIBG}\text{ scintigraphy range between 70-100\% for pheochromocytomas and 84-100\% for paragangliomas, while sensitivities ranges between 85-88\% and 56-76\% respectively (14-17). Physiological uptake of \[^{123}\text{I}-\text{MIBG}\text{ by normal adrenal glands can give false-positive results or obscure small lesions (18-20). Sensitivity appears to be lower for extra-adrenal, metastatic, recurrent and certain hereditary PPGLs (14, 16, 21-24). Metastatic lesions might be missed due to cell dedifferentiation and consequently loss of NET and/or VMAT expression. It has been demonstrated that sensitivity was especially low in }SDHB-\text{ and }VHL-\text{related PPGLs (22, 25, 26). Theoretically, the amount of uptake and consequently tumor visualization and susceptibility for }^{131}\text{I-MIBG therapy might be dependent of the underlying genotype.}

The aim of this study was to semi-quantitatively evaluate \[^{123}\text{I}-\text{MIBG}\text{ uptake in PPGLs and to determine if this can be used to differentiate between pathological and physiological adrenal uptake and to distinguish between PPGLs with different underlying genotypes. Therefore, planar and single-photon emission tomographic (SPECT) images were analyzed using automated measurements. Results were correlated with patient clinical characteristics. Additionally, the immunohistochemical expression of NET and VMAT-1 was directly correlated with in vivo }^{123}\text{I-MIBG uptake in PPGLs of different genotypes.}

MATERIALS AND METHODS

Patient Population

This study retrospectively included 57 patients (23 males, 34 females, aged [mean, range] 44.2, 8-71 years) who consecutively underwent pre-operative diagnostic \[^{123}\text{I}-\text{MIBG}\text{ scintigraphy between October 2002 and February 2014. All patients were suspected of PPGL and referred to the Radboud University Medical Centre. In all cases, the diagnosis of PPGL was confirmed histologically. Fifty-four patients had 58 non-metastatic PPGLs (49 adrenal, 9 extra-adrenal). Four patients had metastatic PPGL, including one with paravertebral thoracic lymph node, two with para-aortic lymph node and one with retrocaval lymph node metastasis. The presence of germline mutations and large deletions in }SDHA/B/C/D/AF2, RET, VHL, TMEM127\text{ and }MAX\text{ was investigated using standard procedures in all patients. Twenty-four patients had...}
an underlying mutation (1 with SDHA mutation, 2 with SDHB mutation, 4 with SDHD mutation, 2 with VHL mutation, 12 with RET-related MEN-2, 1 with MAX mutation and 2 with NF1 mutation). In the remaining patients no genetic alterations were detected in the above mentioned genes. Their tumors were classified as apparently sporadic. Patients’ clinical characteristics and genotype are listed in Table 1 and Supplementary Table 1. All patients underwent biochemical testing prior to and after surgery. Plasma concentrations of metanephrines (sum of normetanephrine and metanephrine) were assayed using high performance liquid chromatography (HPLC)(27). Tumor sizes were retrieved from pathology reports and for each PPGL the maximum tumor diameter in cm was recorded. Data were collected under conditions of regular clinical care, with the approval of ethics committee obtained for the retrospective use of those data, for scientific purposes.

**Imaging Procedures**

Patients received thyroid blockade with a saturated solution of potassium iodide before ¹²³I-MIBG scintigraphy (100 mg/day starting one day before tracer injection and continuing for two days). Whole-body planar and tomographic (SPECT) images were obtained 24 hours after intravenous administration of 302±67 MBq ¹²³I-MIBG (Figure 1). Medication with potential interference with ¹²³I-MIBG uptake (28, 29) was not discontinued in eleven patients for blood pressure safety reasons (Supplementary Table 1).

Of 62 ¹²³I-MIBG scans, 39 were performed before November 2011, using an ECAM (Siemens Healthcare) dual-head gamma camera. After November 2011, a Symbia-T16 (Siemens Healthcare) dual-head gamma camera was used (22 scans) and SPECT was combined with low dose CT for anatomic co-registration. One scan was performed using an Infinia (GE Healthcare) dual-head gamma camera. Both Siemens scanners were equipped with a medium energy low penetration (MELP) parallel-hole collimator and calibrated in accordance with the European Association of Nuclear Medicine (EANM) guidelines for ¹²³I-MIBG scintigraphy (28). The acquisition matrix size was 128 x 128. The whole body scans were performed from feet to head 8cm/min continuous bed movement. SPECT imaging was performed with 64 projections per camera head (128 views, sampling angle 2.8°) and an acquisition time of 30 (ECAM) or 24
(Symbia T-16) seconds per projection using body-contour orbit (step-and-shoot). During image acquisition, energy windows were centered at 159 keV (photo peak window) and at 135 keV (lower scatter window) with a window width of 15%. CT imaging for SPECT/CT was performed without contrast, and the X-ray tube peak voltage (kVp) was set to 110 kV whilst the X-ray tube current was modulated using CARE Dose4D, with a reference tube current of 50 mAs.

SPECT images were reconstructed using an iterative ordered subset expectation (OSEM) algorithm as implemented in ReSPECT 2.5 (Scivis wissenschaftliche Bildverarbeitung) with 6 iterations and 32 subsets. Scatter correction was performed using a background subtraction method. In this approach, a body contour detection is used to estimate contribution of background scatter, which is subsequently subtracted from the image. Furthermore, homogeneous attenuation correction is performed during image reconstruction using a semi automatic body contour detection (Chang’s attenuation correction) using a linear attenuation coefficient ($\mu$) of 0.14 cm$^{-1}$ as measured attenuation correction is not implemented in this version of ReSPECT. Post-reconstruction filtering was performed with a 3D Gaussian filter kernel with a full width half maximum (FWHM) of 40 mm. The reconstruction matrix size was 128x128 voxels with an isotropic voxel size of 4.80 mm in all three orthogonal directions.

**Image Interpretation**

Scintigraphic images were retrospectively analyzed and $^{123}$I-MIBG uptake was assessed in adrenal and extra-adrenal located lesions on SPECT and, if available, planar images. For the non-metastatic cases, lesions were identified as PPGL in case of histological evidence and normalization of plasma metanephrines after surgery. In case of right adrenal or extra-adrenal PPGL, $^{123}$I-MIBG uptake was also assessed in the normal left adrenal gland. Normal right adrenal glands were not evaluated because of the difficulty separating them from physiological uptake by the liver.

SPECT images were reviewed using two different software systems; Inveon Research Workplace (IRW) 4.1 (Preclinical Solutions, Siemens Medical Solutions USA) and Hermes 4.6-A (Hermes Medical
Solutions AB). Planar images were only reviewed with the latter. Uptake of $^{123}$I-MIBG in PPGLs was quantified in relation to hepatic uptake. On SPECT images, lesions were segmented using a relative threshold region growing algorithm. The seed point for the algorithm was the maximum intensity voxel and the segmentation threshold was 70% of the maximum activity concentration within the lesion to eliminate areas with tumor necrosis. A 125-cm$^3$ cubic or spheric volume of interest (VOI) was positioned centrally in the patient’s upper right liver lobe. In case of liver involvement, unaffected regions were visually identified. Tumor-to-liver (T/L) ratios were calculated as the mean counts/pixel in the segmented tumor VOI over the mean counts/pixel in the liver VOI. Likewise, $^{123}$I-MIBG uptake in normal left adrenals was quantified resulting in normal adrenal-to-liver (NA/L) ratios. On posterior planar images, PPGLs were visually delineated and a region of interest (ROI) was manually drawn around the tumor. A circular ROI of 5-cm diameter was positioned centrally in the right upper liver lobe and T/L ratios were calculated.

**Immunohistochemistry for NET and VMAT-1 Protein Expression**

Tumor tissue sections (4 µm) were deparaffinized, rehydrated and washed with 50mM phosphate buffered saline (PBS). Immunohistochemistry (IHC) for NET was performed using an automated immunostainer (Ventana Medical Systems) as previously reported (30). The primary antibody directed against NET (clone 05-2; Mab Technologies, dilution: 1:500) was diluted in Dako REALTM antibody diluent (Dako). The SuperSensitive IHC detection system from BioGenex was employed to visualize the antibody binding and the immunoreaction was developed in the diamino-benzidine (DAB) supplied with the kit (Vector lab). Sections were counterstained with Mayer’s Haemalum, dehydrated and covered with glass coverslips. Sections of normal adrenal gland was used as positive control and included in each run. Sections incubated without the primary antibody were included in each batch as a negative control.

IHC for VMAT-1 was performed manually according to the following protocol. Endogenous peroxidase activity was blocked with 30% hydrogen peroxide ($\text{H}_2\text{O}_2$) in PBS for 30 minutes. Antigen retrieval was performed by boiling the sections in 10 mM sodium citrate, pH 6.0 in a microwave for 2’20”
at 800 W followed by 10 minutes at 160 W. Sections were cooled at room temperature for 90 minutes. Prior to incubation with the primary antibody, endogenous avidin and biotin was blocked (Sp-2001, Vector Laboratories). Non-specific interactions were blocked using normal goat serum (20% in PBS/1%BSA) for 10 minutes. Slides were incubated with the primary antibody VMAT-1 (H-V001, Phoenix Pharmaceuticals) at a 1:500 dilution overnight at 4°C. The sections were then washed with PBS, followed by incubation with a secondary biotinylated goat-anti-rabbit antibody (Vector Laboratories) at a 1:200 dilution for 30 minutes at room temperature. Slides were subsequently incubated with avidin-biotin reagent (PK6100 Vectastain Elite ABC Kit, Vector Laboratories) for 30 minutes. The sections were washed with PBS and incubated with 3,3-diaminobenzidine (DAB) (BS04-110, Immunologic) for 3 minutes. After rinsing with running tap water for 5 minutes, the sections were counterstained with hematoxylin for 1’30”, washed and dehydrated in ethanol series (50%, 70%, 100%) followed by xylene and mounted with Permount (ThermoFisher Scientific).

All staining results were assessed by an experienced pathologist (BK) who was blinded to patients’ clinical data. The presence of VMAT-1 and NET tumor cell cytoplasmic staining was graded by the percentage of tumor cells with positive staining. The intensity of staining was categorized as none (0), weak staining (1+), medium staining (2+), and intense staining (3+), ignoring staining of sustentacular cells, which was frequently noted for both proteins. Areas containing adrenal cortex and endothelial cells were used as controls for comparison between different sections. The IHC staining score represents the expression of the proteins and was calculated by multiplication of both grades (% positive X intensity).

**Statistical Analysis**

Normally distributed variables are expressed as mean ± standard deviations (SD). Variables not obeying the normal distribution are described by median and interquartile range (IQR). Calculated uptake ratios from SPECT images reviewed in IRW were used for data analysis. Results were validated against analysis of planar and SPECT images in Hermes by using Spearman rank correlation ($\rho$) and by calculating Bland-Altman (BA) limits of agreement($\delta$). Cut-off values for pathological $^{123}$I-MIBG uptake were determined
using receiver operating characteristic curve (ROC) analysis and the area under the curve (AUC) was calculated. Non-parametric tests (Mann-Whitney U) were used to compare difference in $^{123}$I-MIBG uptake between PPGLs with different location and normal adrenal glands. For comparisons of $^{123}$I-MIBG uptake across different genotypes, calculated uptake ratios were analyzed using independent samples Kruskal-Wallis test with Dunn’s post test. To test for differences in immunohistochemical staining scores for NET and VMAT-1 among different hereditary clusters, Mann Whitney U test was used. Spearman’s rank correlations were used to estimate relation between $^{123}$I-MIBG uptake, NET and VMAT-1 protein expression, tumor size and concentrations of plasma metanephrines. Overall, a two-sided p-value below 0.05 was considered statistically significant. Statistical analyses were performed using SPSS 20.0 statistical software (SPSS Inc, Chicago, IL, USA).

RESULTS

Agreement between Different Methods for Assessing $^{123}$I-MIBG Uptake in PPGLs

Calculated T/L ratios of SPECT images in Hermes and IRW were highly correlated ($\rho=0.995$, $p<0.001$, Supplementary Figure 1A) with narrow BA limits of agreement (-0.92 to 1.15, Supplementary Figure 1B). In the BA plot the mean-difference line overlapped the line of equality, indicating that Hermes provided similar T/L ratios as IRW (Supplementary Figure 1B). Furthermore, T/L ratios of planar images, reviewed in Hermes, significantly correlated with T/L ratios of SPECT images reviewed in Hermes ($\rho=0.582$, $p<0.001$) and IRW ($\rho=0.588$, $p<0.001$).

$^{123}$I-MIBG Uptake in PPGLs vs. Normal Adrenal Glands

Median T/L ratios of PPGLs were 5.5 (IQR: 3.1-9.5) and significantly higher than NA/L ratios of normal left adrenal glands (median 1.5, IQR: 0.9-2.3, $p<0.001$, Figure 2). T/L ratios of extra-adrenal PPGLs were significantly higher than ratios of adrenal PPGLs (median 8.4, IQR: 6.0-11.1 vs. 5.4, IQR: 2.9-7.3, $p<0.05$, Figure 2). Tumor necrosis was visually observed in three adrenal and four extra-adrenal located PPGLs.
and T/L ratios were similar to non-necrotic tumors (data not shown). The median T/L ratio of the 11 patients using potentially interfering medication was 3.7 (IQR: 2.0-6.7), whereas that of the 46 patients without those drugs was 5.8 (IQR: 3.1-9.6, p=0.164).

A ROC was constructed from T/L ratios of (extra-)adrenal PPGLs and NA/L ratios of normal left adrenal glands (AUC 0.916, 95% CI: 0.86-0.97, Figure 3). To provide 100% sensitivity, the upper reference for physiological adrenal 123I-MIBG uptake was established at 0.7, resulting in a specificity of 10.3%. To provide 100% specificity, the upper reference for physiological adrenal 123I-MIBG uptake was established at 4.3 (the maximum value for PPGL-negative normal adrenals), resulting in a sensitivity of 66.1%.

Distribution of 123I-MIBG Uptake across Different Genotypes

The distribution of T/L ratios in PPGLs across hereditary and apparently sporadic tumors is shown in Figure 4. The median T/L ratio for hereditary cluster 1 tumors (SDHx, VHL) was 6.2 (IQR: 5.0-8.1), for hereditary cluster 2 (RET, NF1, MAX) 5.4 (IQR: 3.8-8.2) and apparently sporadic tumors 5.8 (2.5-10.9). No statistical differences in 123I-MIBG uptake were found between these groups.

The maximum tumor diameter ranged from 0.6 to 14.0 cm (median 4.1, IQR: 2.5-6.2). 123I-MIBG uptake was positively correlated with maximum tumor diameter in both adrenal and extra-adrenal PPGLs (ρ=0.380, p=0.002). The five smallest PPGLs, each measuring < 2.0 cm, all showed uptake of 123I-MIBG with T/L ratios higher than 1.0 (Supplementary Table 1). Furthermore, no statistical significant correlation was found between 123I-MIBG uptake and concentrations of plasma metanephrines (ρ=0.229, p=0.076).

Evaluation of NET and VMAT-1 Protein Expression in PPGLs

Of 62 PPGLs, 61 samples were available for NET staining. Tumor cells in all samples showed a positive cytoplasmic staining for NET and a clear variability in staining intensity among samples was observed (Figure 5). Figure 6A shows NET immunohistochemical staining intensity according to genotype. Negative to occasionally weak cytoplasmic staining was encountered in VHL- and MAX- related PPGLs.
The expression of NET in hereditary cluster 2 (RET, NF1, MAX) and apparently sporadic tumors was higher than for hereditary cluster 1 (SDHx, VHL) PPGLs (108.1±18.7, 94.4±10.9 vs. 41.3±18.4, p=<0.05, p=<0.01, Figure 6A). No statistically significant differences in NET staining were found between cluster 2 and apparently sporadic PPGLs (p=0.354, Figure 6A).

PPGLs showed cytoplasmic immunoreactivity with the anti-VMAT-1 antibody and occasionally also sustentacular cells stained positive (Figure 5). VHL-, RET- and MAX-related tumors showed an overall similar VMAT-1 expression, which was usually scored as medium. A highly heterogeneous expression of VMAT-1 was encountered in sporadic PPGLs (Figure 6B). VMAT-1 expression in hereditary cluster 1 (SDHx, VHL) PPGLs was higher than in hereditary cluster 2 (RET, NF1, MAX) tumors (193.8±19.1 vs. 135.8±12.3, p<0.01). No significant differences were observed between hereditary and sporadic samples (160.6±11.9 vs. 168.7±12.5, p=0.197, Figure 6B). Neither NET nor VMAT-1 protein expression correlated with ¹²³I-MIBG uptake (p=-0.103, p=0.203; p=0.046, p=0.723, respectively).

DISCUSSION

Our study provides the first semi-quantitative analysis of ¹²³I-MIBG scintigraphy in patients with PPGL. Uptake of ¹²³I-MIBG in PPGLs was significantly higher than in normal adrenal glands and cut-off values were established to distinguish between pathological and physiological uptake. The uptake of ¹²³I-MIBG significantly correlated with maximum tumor diameter. No significant differences in ¹²³I-MIBG uptake were found between PPGLs of various genotypic clusters. NET expression was significantly higher in cluster 2 (RET, NF1, MAX) and in apparently sporadic PPGLs than in cluster 1 (SDHx, VHL) PPGLs. VMAT-1, expression was significantly higher in cluster 1 than cluster 2 tumors. MIBG uptake, however, did not correlate with either NET or VMAT-1 expression.

Traditionally, ¹²³I-MIBG scintigraphy is considered as non-quantitative due to a relatively low tumor-to-background ratio and limited spatial resolution. Scintigraphic image interpretation can be difficult in case of small lesions and physiological uptake of ¹²³I-MIBG by normal adrenal glands, which is observed
in 50-80% of cases. Cecchin et al. (32) proposed a scoring system in which $^{123}$I-MIBG uptake in adrenal glands was visually compared with hepatic uptake. Scintigraphic images were classified as positive in case of adrenal uptake more intense than in the liver or non-homogeneous uptake in an enlarged adrenal gland or an extra-adrenal focus. They demonstrated that the scoring system was highly specific and sensitive (91.5% and 100%, respectively) for detecting PPGL and is useful to discriminate normal adrenal $^{123}$I-MIBG uptake from pheochromocytoma. Others have adopted this approach (19, 23, 33). However, a major disadvantage is that visual interpretation remains subjective and is highly observer-dependent. Thus, assessments of $^{123}$I-MIBG uptake may facilitated by using semi-quantitative measurement methods. Our present study demonstrates that semi-quantitative analysis of $^{123}$I-MIBG images can be used for uptake measurements and facilitates the discrimination between adrenal PPGL and physiological adrenal uptake. Nevertheless, there was considerable overlap between tumoral uptake and physiological uptake by normal adrenals and this might still lead to false negative results, as also observed by Cecchin et al (32). Also, T/L ratios provide a semi-quantitative uptake parameter. Actual quantification would require quantitative SPECT using accurately calibrated SPECT reconstructions.

Mechanisms of uptake and storage of $^{123}$I-MIBG in sympathetic chromaffin cells is similar to that of (nor)epinephrine. At the cellular membrane level, uptake is mediated by NET (solute carrier family 6 member 2, SLC6A2), a monoamine transporter, which is responsible for the regulation of extracellular norepinephrine levels by active re-uptake mechanisms following their release from neuronal or endocrine stores. NET is typically expressed in noradrenergic neurons and sympathetic nerves, but also prominent in chromaffin cells of the adrenal medulla and PPGLs. After entering the cell, similar to norepinephrine, $^{123}$I-MIBG translocates from the cytosol into neurosecretory storage vesicles. This is mediated by VMAT. There are two VMAT isoforms, VMAT-1 and VMAT-2, encoded by two different genes and displaying different cellular distributions (34). VMAT-1 is mainly expressed in neuroendocrine cells including chromaffin cells, whereas VMAT-2 is expressed in peripheral and central neurons. Fottner et al. have shown that VMAT-1 expression is essential for positive $^{23}$I-MIBG scintigraphy of PPGLs (35).
Differences in the expression of catecholamine transporters have been reported between RET- and VHL-related tumors (36, 37). Huyhn et al. (36) showed that RET-related PPGLs expressed more NET mRNA and protein than VHL-related tumors. In contrast, a higher expression of VMAT-1 was found in VHL-related tumors, although this was not confirmed at the protein level. Saveanu et al. (37) observed a lower NET mRNA expression in VHL-related PPGLs compared to sporadic PPGLs, whereas expression of NET in RET-related PPGLs was higher. We observed a cytoplasmic staining for NET and VMAT-1 as previously reported by Huyhn et al. (36). Our study confirms genotype-dependent differences in the expression of NET and VMAT-1.

The various PPGL genotypes are increasingly recognized as important determinants of functional imaging results. For example, several studies demonstrated a poor overall sensitivity of $^{123}$I-MIBG scintigraphy in SDHB-related PPGLs (less than 50%) (24, 25). Similar to $^{123}$I-MIBG, several positron emission tomography (PET) tracers specifically target catecholamine synthesis, storage and secretion pathways including $^{18}$F-fluorodihydroxyphenylalanine ($^{18}$F-FDOPA) and $^{6}$F-fluorodopamine ($^{18}$F-FDA). Kaji et al. showed that $^{18}$F-FDOPA PET is superior to $^{123}$I-MIBG scintigraphy in the context of VHL syndrome (26). We have previously shown that $^{18}$F-fluorodeoxyglucose ($^{18}$F-FDG) PET can distinguish between PPGLs with different underlying genotypes and sensitivity of $^{18}$F-FDG PET is higher in SDHB/D-related PPGLs than non-SDHB/D-related metastatic PPGLs (92 vs. 37%) (38). Sensitivity of $^{123}$I-MIBG scintigraphy appears to be lower in VHL- and SDHB-related PPGLs (22, 25, 26). Based on these studies and the differences in catecholamine transporter expression between genotypes, we hypothesized that a genotype-dependent imaging phenotype also exists for $^{123}$I-MIBG scintigraphy. With the current study we show that this is not the case and that $^{123}$I-MIBG uptake appears to be independent of the underlying genotype. Furthermore, we did not find significant correlations between $^{123}$I-MIBG uptake and transporter (NET, VMAT-1) expression. Based on our results, we argue that, as opposed to certain PET-tracers, $^{123}$I-MIBG scintigraphy cannot be used for the prediction of underlying genotypes. The observation of relatively low T/L ratios and low NET expression in VHL might suggest that $^{123}$I-MIBG
scintigraphy is of limited use in VHL-related PPGLs. This, however, remains to be confirmed in larger series.

The lack of correlations between $^{123}$I-MIBG avidity and NET and VMAT-1 staining might suggest that $^{123}$I-MIBG accumulation is not primarily determined by the expression of these transporter systems. However, $^{123}$I-MIBG uptake may be determined by NET and VMAT-1 transporter activity rather than transporter quantity. Also, differences in the recruitment of NET from the cytoplasm towards the cell and vesicular membranes may play a role. In addition to transmembrane transport, $^{123}$I-MIBG accumulation is also determined by catecholamine storage capacity as reflected by the number of neurosecretory granules (39). Other mechanisms besides uptake via NET and VMAT-1 includes passive diffusion, fractional blood content, non-specific uptake by other transporters and uptake by non-tumor cells such as macrophages and endothelial cells. Another potential explanation for the lack of correlation is the fact that we used the IHC staining score in a single tumor tissue section to determine the expression of NET and VMAT-1 in these tumors. This may not be representative of the protein expression of the entire tumor.

Besides genetic factors, we investigated other possible determinants of $^{123}$I-MIBG uptake including tumor location, tumor size and plasma metanephrines concentrations. Several studies have shown that the sensitivity of $^{123}$I-MIBG scintigraphy is lower in extra-adrenal PPGLs (33, 40). Saveanu et al. (37) reported a higher NET expression in adrenal versus extra-adrenal PPGLs. This has also been shown to be the case for VMAT-1 expression (35). In contrast, we found no differences in NET or VMAT-1 expression between tumor locations and, surprisingly, even a slightly higher $^{123}$I-MIBG uptake in extra-adrenal PPGL. In addition, we found a significant and positive correlation between maximum tumor diameter and $^{123}$I-MIBG uptake regardless tumor location. This is in line with previous results (20, 23). MIBG uptake did not correlate with plasma metanephrines. Others also failed to identify any correlation between $^{123}$I-MIBG uptake and hormonal levels (20, 33, 39).

This study has several limitations. First, a relatively low number of hereditary PPGLs was available for comparison of genotype-specific $^{123}$I-MIBG imaging results. Additional studies with a larger sample size
are desired. It would be interesting to also take into account somatic mutations in apparently sporadic PPGLs. Second, semi-quantitative T/L ratios may be helpful to distinguish between pathological and physiological adrenal uptake, but do not necessarily provide a ‘definite answer’. There is a considerable overlap between tumors and normal adrenals. The T/L ratio should therefore be merely regarded as an additional ‘aid’ for the evaluation of MIBG images in the individual clinical context. Third, the use of the left adrenal gland for assessments of physiological $^{123}$I-MIBG uptake in hereditary cases may have some limitations. This adrenal might be affected by adrenal hyperplasia or a subclinical tumor, especially in patients with germline mutations who are at risk for bilateral disease. Although we observed slightly higher NA/L ratios in hereditary PPGLs (data not shown), the ratios largely overlapped with ratios of sporadic PPGLs. This, however, deserves further investigations in a larger study sample.

CONCLUSION
Liver normalized semi-quantitative $^{123}$I-MIBG uptake may be helpful to distinguish between pheochromocytoma and physiological adrenal uptake. The presence of an underlying hereditary syndrome in individual patients cannot be predicted by semi-quantitative uptake of $^{123}$I-MIBG. Genotype-specific differences in expression of NET and VMAT-1 do not translate in differences in $^{123}$I-MIBG uptake.

Disclosure: Authors have nothing to disclose.
REFERENCES


FIGURE LEGENDS

FIGURE 1. $^{123}$I-metaiodobenzylguanidine (MIBG) scintigraphy scans of a 50-year-old male patient with a $RET$ mutation and pheochromocytoma on the left side (indicated by arrow). (A) Planar-whole body scan (B) Single photon emission tomography (SPECT) scan (C) Computed tomography (CT) scan.
FIGURE 2. $^{123}$I-metaiodobenzylguanidine single photon emission tomography (SPECT) uptake expressed as tumor-to-liver (T/L) ratio in PPGLs according to tumor location. Uptake in normal adrenals is expressed as normal adrenal-to-liver (NA/L) ratio. All ratios are corrected for injected dose and decay. The horizontal bar represents the mean and error bars the standard deviation. *p<0.01, **p<0.01 (Mann Whitney U test).
FIGURE 3. Receiver operating characteristic curve for $^{123}$I-metaiodobenzylguanidine uptake values. This curve was constructed from the tumor-to-liver (T/L) ratios of PPGL lesions and normal adrenal-to-liver (NA/L) ratios of normal adrenals in patients with PPGL. The diagonal line represents the line of no-discrimination.
FIGURE 4. $^{123}$I-metaiodobenzylguanidine uptake expressed as tumor-to-liver (T/L) ratio in PPGLs across different genotypes. Single photon emission tomography (SPECT) images analyzed in IRW. All T/L ratios are corrected for injected dose and decay.
**FIGURE 5.** Immunohistochemical staining of PPGLs for norepinephrine transporter (NET) and vesicular monoamine transporter type 1 (VMAT-1). Representative images of *SDHD, VHL, RET, NF1, MAX* and sporadic tumors with magnifications as indicated. Areas with brown color (DAB polymer) are representative of positive staining.
FIGURE 6. Comparison of staining for (A) norepinephrine transporter (NET), (B) vesicular monoamine transporter type 1 (VMAT-1) among different PPGL genotypes. Graphs represent immunohistochemical staining scores, calculated as percentage area stained positive times staining intensity. The horizontal bar represents the mean. Statistical comparisons were performed based on conventional clustering according to predefined transcriptional profiles. *p<0.05, **p<0.01 (Mann Whitney U test).
TABLES

**TABLE 1. Patient characteristics**

<table>
<thead>
<tr>
<th>Genotype</th>
<th>No. of patients</th>
<th>Sex (M/F)</th>
<th>Age (yrs)</th>
<th>Tumor location (A/EA)</th>
<th>Maximum tumor diameter (cm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sporadic</td>
<td>33</td>
<td>10/23</td>
<td>45.0 ± 14.8</td>
<td>30/4</td>
<td>5.2 ± 2.9</td>
</tr>
<tr>
<td>SDHA</td>
<td>1</td>
<td>0/1</td>
<td>19.0</td>
<td>1/0</td>
<td>8.0</td>
</tr>
<tr>
<td>SDHB</td>
<td>2</td>
<td>1/1</td>
<td>30.0 ± 22.6</td>
<td>0/2</td>
<td>5.7 ± 1.0</td>
</tr>
<tr>
<td>SDHD</td>
<td>4</td>
<td>2/2</td>
<td>44.0 ± 16.2</td>
<td>1/4</td>
<td>3.4 ± 1.2</td>
</tr>
<tr>
<td>VHL</td>
<td>2</td>
<td>1/1</td>
<td>24.0 ± 13.9</td>
<td>1/2</td>
<td>2.2 ± 0.6</td>
</tr>
<tr>
<td>MEN-2</td>
<td>12</td>
<td>8/4</td>
<td>49.0 ± 12.1</td>
<td>14/0</td>
<td>3.8 ± 2.4</td>
</tr>
<tr>
<td>NF1</td>
<td>2</td>
<td>0/2</td>
<td>48.0 ± 15.6</td>
<td>1/1</td>
<td>5.3 ± 2.4</td>
</tr>
<tr>
<td>MAX</td>
<td>1</td>
<td>1/0</td>
<td>57.0</td>
<td>1/0</td>
<td>1.8</td>
</tr>
</tbody>
</table>

Study included a total of 57 patients with 62 PPGLs. Data are expressed as mean ± SD.

*Abbreviations: M=male, F=female, yrs = years, A=adrenal, EA=extra-adrenal.*
Semi-quantitative $^{123}\text{I}$-metaiodobenzylguanidine scintigraphy to distinguish pheochromocytoma and paraganglioma from physiological adrenal uptake and its correlation with genotype-dependent expression of catecholamine transporters

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