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Chapter 5

Plasma chromogranin A levels are increased in a small portion of patients
with hereditary head and neck paragangliomas

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Abstract

**Context:** The majority of patients with head and neck paragangliomas (HNPGL) have biochemically silent tumors. Chromogranin A (CgA) is a tumor marker for neuroendocrine tumors.

**Objective:** To assess the role of CgA as a tumor marker in patients with hereditary HNPGL.

**Patients and Methods:** We included 95 consecutive patients with hereditary HNPGL for screening of plasma CgA levels and catecholamine excess by measurement of 24-hour urinary excretion of (nor)metanephrine, (nor)epinephrine, VMA, dopamine and 3-methoxytyramine. In all patients with catecholamine excess, abdominal/intrathoracic paragangliomas were excluded by $^{123}$I-MIBG scintigraphy, MRI and/or CT.

**Results:** Plasma CgA levels were increased in only 15 of 95 patients (16%). Thirty-three of the 95 patients (35%) had increased urinary excretion rates of catecholamines. Six of these 33 patients (18%) had increased plasma CgA levels. Nine of the 62 patients (15%) with a biochemically silent tumor (i.e. no increased urinary excretion of catecholamines or their metabolites) had increased CgA levels.

Increased plasma CgA levels were positively correlated with urinary excretion rates of norepinephrine ($r=0.68$, $p=0.005$) and normetanephrine ($r=0.68$, $p=0.005$). There was a positive correlation between maximal HNPGL diameter and plasma CgA levels in the 57 patients with a single HNPGL ($r=0.57$, $p=0.001$).

**Conclusions:** Plasma CgA levels are increased in only a small portion of patients with hereditary HNPGL and have limited additional value to the combination of radiological and routine biochemical assessment of HNPGL patients. Increased plasma CgA levels are associated with increased noradrenergic activity and tumor size in patients with a single HNPGL.
**Introduction**

Head and neck paragangliomas (HNPGL) are rare neuroendocrine tumors derived from parasympathetic ganglia (1). Paragangliomas can occur as a consequence of a mutation in genes of the succinate dehydrogenase (SDH) family (2-4). These SDH genes (SDHA, SDHB, SDHC and SDHD) encode the four subunits of complex II of the mitochondrial electron transport chain. SDH contributes to the energy metabolism as a component of the tricarboxylic acid cycle, converting succinate to fumarate, and by serving as a source of electrons for mitochondrial respiration, as complex II of the electron transport chain (5). Except for the SDHA gene, mutations of SDHB, SDHC and SDHD genes are associated with familial paraganglioma syndromes (2;6). In the Netherlands, a large proportion of hereditary paragangliomas are caused by mutations in the SDHD gene, but mutations in SDHB, SDHC and SDHAF2 are also found (5;7-10).

Although HNPGL have the ability to produce and secrete catecholamines (11;12), we recently demonstrated that only 29% of patients with HNPGL have evidence of increased urinary excretion of catecholamines and/or their metabolites (13). Consequently, the majority of these patients have biochemical silent tumors and the clinical characteristics of these HNPGL can be evaluated only by imaging techniques.

Chromogranin A (CgA) is a secretory protein from neuroendocrine cells that mediates chromaffin granule biogenesis, necessary for catecholamine storage (14;15). CgA is secreted from neurosecretory vesicles, along with catecholamines (16). In accordance with these biological concepts, plasma CgA is a useful tumor marker in patients with pheochromocytoma (17-23). Lloyd et al. demonstrated the presence of chromogranin in head and neck paraganglia, indicating the presence of secretory granules(24). At present the clinical relevance of the measurement of plasma CgA levels in patients with hereditary HNPGL is unclear. Therefore, the aim of the present study was to assess the prevalence of increased CgA levels in patients with hereditary HNPGL and to identify a possible role of CgA in patients with biochemically silent tumors.

**Patients and Methods**

We evaluated in a cross sectional study the clinical, biochemical and radiological data of 95 consecutive patients with hereditary HNPGL. These patients were selected from all HNPGL patients who were followed in the outpatient clinic of the Leiden University Medical Center, a tertiary referral center for patients with paragangliomas. Inclusion criteria for participation in the study were the presence of HNPGL, biochemical screening of catecholamine excretion in two 24-h urinary samples, plasma sample for CgA analysis and genetic screening for SDH mutations. Exclusion criteria were the use of proton pump inhibitors (25), no radiological assessment in case of increased catecholamine excretion, the presence of paragangliomas in the thorax or abdomen and because renal failure is associated with increased plasma CgA levels, patients with a GFR of less than 60 mL/min were also excluded from the study (26).
All patients were investigated according to structured protocols, which were standard care. These included questions focused on tumor and catecholamine related signs and symptoms, measurement of blood pressure in the supine position, and after 5 minutes of upright position, in order to screen for orthostatic hypotension. Repetitive head and neck MRI was performed with intervals of at least 2 years. Biochemical screening included the measurement of catecholamine excretion in two 24-h urinary samples: (nor)metanephrines, (nor)epinephrine, vanillylmandelic acid (VMA), dopamine and 3-methoxytyramine. Urine was collected during 24 hours in duplicate under strict dietary regulations (patients abstained from pineapple, avocado, bananas, kiwi, nuts, plums, coffee, tea and other caffeine containing beverages from 2 days preceding and during urine collection) and after withdrawal of medication for at least one week or after changing antihypertensive medication to doxazosin. In order to ascertain adequacy of collection, urinary creatinine excretion was measured as well. Plasma samples for CgA analysis were stored at -80 °C until analysis.

In case of excessive catecholamine excretion (i.e., any value above the upper reference limit in two urine samples), radiological assessment by MIBG-scans and MRI and/or CT scans of thorax and abdomen was performed to identify the source of excessive catecholamine production.

The study was an evaluation of routine patient care. According to the requirements of the Dutch law, it was not necessary to obtain permission from the institutional ethical commission. Prior to germ line mutation testing, informed consent was obtained from each patient.

**Assays**

Epinephrine, norepinephrine and dopamine excretion rates in 24-h urine collections were quantified by reversed high pressure liquid chromatography (HPLC) by an electrochemical detector. Inter- and intra-assay coefficients of variations CVs for epinephrine were 4.3-9.0% ranging from high to low levels. For norepinephrine these data are 2.7-3.6% and for dopamine 3.1-4.8%. Vanillylmandelic acid (VMA) excretion in urine was measured using HPLC with fluorometric detection with inter- and intra-assay CVs of 2.4-9.1%. (Nor)metanephrine and 3MT were determined by stable isotope mass fragmentography. The coefficients of variations of the 3-O-methylated catecholamine metabolites (metanephrine, normetanephrine and 3MT) ranged from 1.7 to 4.2% (27). Plasma chromogranin A level was determined by solid phase two-site immunoradiometric assay (CIS Bio International, Gif-sur-Yvette, France).

Reference ranges were obtained in healthy volunteers. These values were for urinary excretion: norepinephrine 0.06-0.47 µmol/24h, epinephrine <0.16 µmol/24h, dopamine 0.46-3.40 µmol/24h, VMA <30 µmol/24h, metanephrine 33-90 µmol/mol creatinine, normetanephrine 64-260 µmol/mol creatinine and 3MT 45-197 µmol/mol creatinine (28), plasma chromogranin < 98 ng/mL. SDH mutation analysis was performed by restriction digestion as described by Taschner et al. (7;29).
Data analysis

SPSS for windows version 16.0 (SPSS inc., Chicago, IL) was used for data analysis. Results are expressed as means±standard error (SE), unless specified otherwise. Independent sample t-tests and chi-square tests were used to compare patients with and without increased plasma levels of CgA. The average value of catecholamine excretion rates of two urine samples was used for calculation of p values. Pearson’s correlation test was used to study dependence between variables. A p-value <0.05 was considered to represent a significant difference.

Results

Clinical and biochemical data of patients with increased versus normal CgA levels
Plasma CgA levels were increased in 15 of the 95 patients with hereditary HNPGL (16%). Tumor related signs and symptoms (e.g., palpitations, diaphoresis, headache, flushes, dizziness, tinnitus, hearing loss, hoarseness, nausea, vomiting) were not different between patients with increased plasma CgA levels and patients with normal CgA levels (p=0.26). Six of these 15 patients (40%) with increased CgA levels had increased 24 hour urinary excretion rates of catecholamines: 2 patients had an elevated urinary excretion rate of norepinephrine, 2 patients of dopamine, 4 patients of VMA, 2 patients of normetanephrine, 3 of metanephrine and 5 patients of 3-methoxytyramine (Table 2). Although none of these patients had increased urinary excretion rates of epinephrine, urinary excretion rates of epinephrine and VMA were significantly higher in the 15 patients with increased plasma CgA levels compared to the patients with normal plasma CgA levels (p=0.026).

Twenty-seven of the 80 patients with normal CgA levels (34%) had increased urinary excretion rates of catecholamines: 4 patients had elevated excretion rates of norepinephrine, 5 of dopamine, 7 of VMA, 1 of metanephrine, 1 of normetanephrine and 19 of 3MT. The number of patients with increased urinary catecholamine excretion rates was not significantly different between the patients with increased CgA levels versus the patients with normal CgA levels (40% vs. 34%, p=0.77).

Biochemical data of patients with increased versus normal rates of urinary catecholamine excretion
Thirty-three of the 95 patients (35%) had increased urinary excretion of catecholamines. Six of these 33 patients (18%) had increased plasma CgA levels. Nine of the sixty-two patients (15 %) with a biochemically silent tumor (i.e., no increased urinary excretion of catecholamines or their metabolites) had increased CgA levels. Of the 9 patients with increased CgA levels and a biochemical silent tumor, 7 (78%) had glomus caroticum tumors, 5 (56%) glomus vagale tumors, 2 (22%) and 1 (11%) had glomus jugulotympanicum tumors. Mean maximal tumor diameter was 4.4±0.8 cm (range 2-9.7 cm). The number of HNPGL varied from 1 to 4 tumors, the median number of HNPGL was 2. Eight of these patients had SDHD mutations, and 1 patient a SDHB mutation.
Table 1: Clinical characteristics of 95 patients with hereditary HNPGL, with and without increased plasma chromogranin A levels.

<table>
<thead>
<tr>
<th>Patient characteristics</th>
<th>Plasma chromogranin A</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Normal (range)</td>
<td>Elevated (range)</td>
</tr>
<tr>
<td>Age (yrs) m (range)</td>
<td>46 (14-69)</td>
<td>52 (32-78)</td>
</tr>
<tr>
<td>Gender N (%)</td>
<td>Men 42 (53%)</td>
<td>11 (73%)</td>
</tr>
<tr>
<td></td>
<td>Women 38 (48%)</td>
<td>4 (27%)</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>25 ± 0.5</td>
<td>23 ± 1</td>
</tr>
<tr>
<td>Systolic BP (mm Hg)</td>
<td>133 ± 2</td>
<td>136 ± 6</td>
</tr>
<tr>
<td>Diastolic BP (mmHg)</td>
<td>79 ± 1</td>
<td>83 ± 3</td>
</tr>
<tr>
<td>Heart rate (beats/min)</td>
<td>72 ± 1</td>
<td>76 ± 3</td>
</tr>
<tr>
<td>Symptoms/signs</td>
<td>49 (61%)</td>
<td>7 (47%)</td>
</tr>
<tr>
<td>Type of glomustumor</td>
<td>Caroticum 63 (79%)</td>
<td>11 (73%)</td>
</tr>
<tr>
<td></td>
<td>Jugulare 7 (9%)</td>
<td>3 (20%)</td>
</tr>
<tr>
<td></td>
<td>Vagale 34 (43%)</td>
<td>10 (67%)</td>
</tr>
<tr>
<td></td>
<td>Jugulotympanicum 12 (15%)</td>
<td>1 (7%)</td>
</tr>
<tr>
<td></td>
<td>Tympanicum 6 (8%)</td>
<td>0</td>
</tr>
<tr>
<td>Number of HNPGL</td>
<td>1 32 (40%)</td>
<td>4 (27%)</td>
</tr>
<tr>
<td></td>
<td>2 24 (30%)</td>
<td>6 (40%)</td>
</tr>
<tr>
<td></td>
<td>3 15 (19%)</td>
<td>4 (27%)</td>
</tr>
<tr>
<td></td>
<td>&gt; 3 9 (11%)</td>
<td>1 (7%)</td>
</tr>
<tr>
<td>SDHx</td>
<td>SDHD 73 (91%)</td>
<td>13 (87%)</td>
</tr>
<tr>
<td></td>
<td>SDHB 6 (8%)</td>
<td>2 (13%)</td>
</tr>
<tr>
<td></td>
<td>SDHC 1 (1%)</td>
<td>0</td>
</tr>
</tbody>
</table>

Biochemical and genetic correlations with CgA levels in HNPGL patients
In the 15 patients with an increased CgA level, CgA levels correlated positively with urinary excretion of norepinephrine (r=0.68, p=0.005), normetanephrine (r=0.68, p=0.005) and VMA (r=0.67, p=0.006). In the patients with normal plasma CgA levels, there was a positive correlation between CgA levels and the urinary excretion rate of VMA (r=0.24, p=0.03).
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The prevalence of SDHB and SDHD mutations was not different between patients with increased CgA levels and patients with normal CgA levels.

Table 2: Urinary catecholamine excretion rates in 95 HNPGL patients, with and without increased plasma chromogranine A levels.

<table>
<thead>
<tr>
<th>Catecholamines</th>
<th>Plasma chromogranin A level</th>
<th>normal</th>
<th>elevated</th>
<th>normal</th>
<th>elevated</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>N tested pos</td>
<td>N tested pos</td>
<td>Mean ± SE</td>
<td>Mean ± SE</td>
<td>p-value</td>
<td></td>
</tr>
<tr>
<td>Epinephrine (µmol/24 h)</td>
<td>0</td>
<td>0</td>
<td>0.02 ± 0.0</td>
<td>0.04 ± 0.0</td>
<td>0.01</td>
<td></td>
</tr>
<tr>
<td>Norepinephrine (µmol/24 h)</td>
<td>4 (5%)</td>
<td>2 (13%)</td>
<td>0.37 ± 0.1</td>
<td>0.63 ± 0.2</td>
<td>0.32</td>
<td></td>
</tr>
<tr>
<td>Dopamine (µmol/24 h)</td>
<td>5 (6%)</td>
<td>2 (13%)</td>
<td>1.9 ± 0.1</td>
<td>2.4 ± 0.4</td>
<td>0.24</td>
<td></td>
</tr>
<tr>
<td>VMA (µmol/24 h)</td>
<td>7 (9%)</td>
<td>4 (27%)</td>
<td>22.1 ± 0.8</td>
<td>29.6 ± 2.7</td>
<td>0.02</td>
<td></td>
</tr>
<tr>
<td>Metanephrine (µmol/molcre)</td>
<td>1 (1%)</td>
<td>3 (20%)</td>
<td>51.8 ± 2.5</td>
<td>70.0 ± 8.3</td>
<td>0.05</td>
<td></td>
</tr>
<tr>
<td>Normetanephrine (µmol/molcre)</td>
<td>1 (1%)</td>
<td>2 (13%)</td>
<td>148 ± 6.5</td>
<td>274 ± 91</td>
<td>0.19</td>
<td></td>
</tr>
<tr>
<td>3M-tyramine (µmol/molcre)</td>
<td>19 (24%)</td>
<td>5 (33%)</td>
<td>266 ± 55</td>
<td>761 ± 426</td>
<td>0.27</td>
<td></td>
</tr>
<tr>
<td>Chromogranin A (µg/ L)</td>
<td>80</td>
<td>15</td>
<td>58.5 ± 2.1</td>
<td>167 ± 19</td>
<td>0.00</td>
<td></td>
</tr>
</tbody>
</table>

A patient is considered to be positive for the urinary excretion of a specific catecholamine or metabolite if the value is above the reference limit in two consecutive urine samples.

Radiological assessment
Radiological studies of the head and neck region were performed in all 95 patients. Radiological evaluation of the head and neck region was performed by MRI in 94% of
patients, and by CT-scanning in 6% of the patients. We refer to Table 1 for further details of the type and number of HNPGL.

There was no significant difference in the number and type of HNPGL between the patients with normal and increased CgA levels. Most patients had carotid body tumors (78%).

Most patients with HNPGL (n=59) had multiple HNPGL, which prohibited the identification of a culprit lesion of CgA secretion. We identified 36 patients with only a single HNPGL. In these 36 patients there was a positive correlation between maximal tumor diameter and plasma CgA concentrations (r=0.57, p=0.001).

**Table 3:** Plasma CgA levels and urinary catecholamine excretion rates in 95 HNPGL patients with normal and increased urinary catecholamine excretion.

<table>
<thead>
<tr>
<th>Urinary catecholamine excretion</th>
<th>normal</th>
<th>elevated</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Epinephrine</strong> (µmol/24 h)</td>
<td>0</td>
<td>0.02 ± 0.0</td>
<td></td>
</tr>
<tr>
<td><strong>Norepinephrine</strong> (µmol/24 h)</td>
<td>6</td>
<td>0.36 ± 0.1</td>
<td>1.3 ± 0.5</td>
</tr>
<tr>
<td><strong>Dopamine</strong> (µmol/24 h)</td>
<td>7</td>
<td>1.8 ± 0.1</td>
<td>4.8 ± 0.5</td>
</tr>
<tr>
<td><strong>VMA</strong> (µmol/24 h)</td>
<td>11</td>
<td>21.3 ± 0.7</td>
<td>38.3 ± 2.7</td>
</tr>
<tr>
<td><strong>Metanephrine</strong> (µmol/molcre)</td>
<td>4</td>
<td>51.5 ± 2.1</td>
<td>125 ± 12</td>
</tr>
<tr>
<td><strong>Normetanephrine</strong> (µmol/molcre)</td>
<td>3</td>
<td>146 ± 4.7</td>
<td>828 ± 334</td>
</tr>
<tr>
<td><strong>3M-tyramine</strong> (µmol/molcre)</td>
<td>24</td>
<td>102 ± 5.0</td>
<td>1062 ± 280</td>
</tr>
<tr>
<td><strong>Plasma CgA levels</strong> (ug/L)</td>
<td>15</td>
<td>58.5 ± 2.1</td>
<td>167 ± 19</td>
</tr>
</tbody>
</table>

A patient is considered to be positive for the urinary excretion of a specific catecholamine or metabolite if the value is above the reference limit in two consecutive urine samples.
Additional radiological analysis

In the 33 patients with increased urinary excretion of catecholamines and/or their metabolites, abdominal/thoracic paragangliomas were excluded by MRI in 5 patients, by MRI and MIBG in 13 patients, by CT and MIBG in 1 patient, by MIBG and abdominal MRI in 11 patients, by MIBG alone in 2 patients. In 1 patient abdominal paragangliomas were excluded by MRI.

Discussion

The present study was designed to assess the prevalence of increased CgA levels in patients with HNPGL and the clinical relevance of CgA measurements in these patients. Plasma CgA levels correlated positively with the urinary excretion of norepinephrine, normetanephrine and VMA. Moreover, in patients with a single paraganglioma there was a positive correlation between maximal paraganglioma diameter and plasma CgA levels. Therefore, increased plasma CgA concentrations are associated with increased urinary excretion of catecholamines and their metabolites and tumor size in patients with HNPGL. However, only 16% of all HNPGL patients, and only 15% of the patients with biochemically inactive HNPGL had increased plasma CgA concentrations. Therefore, the practical implications of CgA measurements are limited in HNPGL patients.

CgA has been proposed as a tumor marker in patients with pheochromocytomas. The sensitivity to detect these tumors ranges from 83-90% (18;19;22;23;30). However, the sensitivity in patients with paragangliomas is much lower compared to pheochromocytomas, because we observed that only 16% of patients with hereditary HNPGL without evidence of pheochromocytomas had increased plasma CgA levels.

There is a positive correlation between tumor load and plasma CgA concentration (22;31). As patients with head and neck paragangliomas have relatively small tumors, the concentrations of CgA in our patients are relatively low compared to patients with other neuro-endocrine tumors (23). Some of our patients were only identified with HNPGL after a family member was found to have a SDHx mutation. Because family members of an affected individual are screened for HNPGL, these tumors are found in relatively early stages. Therefore, the prevalence of increased CgA levels in patients with HNPGL detected by screening is probably very low. This is in line with the findings of Neumann et al., who reported a lower sensitivity of CgA for the detection of pheochromocytoma in patients with hereditary disease compared to patients with sporadic pheochromocytomas (32). The difference in sensitivity can be explained by the fact that these pheochromocytomas were found by screening at earlier stage, when the tumor was smaller.

Nobels et al. observed a positive correlation between tumor load and CgA secretion (23). We found no correlation between the number of HNPGL and plasma levels of CgA. This is probably caused by the relatively small size of the HNPGL. Because most patients have more than one tumor, it is difficult to identify a relationship between the size of the tumor and plasma CgA level. We identified 36 patients with only a single HNPGL and in these patients plasma CgA level was positively related with the diameter of the HNPGL, in accordance with the notion put forward by Nobels et al.
Chromogranin A (CgA) mediates chromaffin granule biogenesis, is necessary for catecholamine storage and is secreted from neurosecretory vesicles, along with catecholamines (14;15). We found a significant correlation between plasma CgA level and urinary excretion rates of noradrenaline and normetanephrine, but not with urinary dopamine and 3-methoxytyramine excretion. This indicates that increased plasma CgA levels are associated with increased noradrenergic activity, but not with increased dopaminergic activity. This raises the possibility that there are differences between the secretion of norepinephrine versus dopamine by HNPG in relation to that of CgA. However, at present the precise role of CgA in the monoamine sorting process in the chromaffin cells is still unclear and additional studies are required to elucidate the role of CgA in the sorting and transport of dopamine into granule vesicles.

The patients with increased plasma CgA levels had higher excretion rates of epinephrine and metanephrine compared to the patients with normal plasma CgA levels. Epinephrine is released together with CgA after sympatho-adrenal stimulation. The question erases whether sympatho-adrenal stimulation might be the cause of the higher mean epinephrine and metanephrine excretion in the patients with increased CgA levels. It is known from earlier reports that metanephrine levels increase significantly after physical activity, changes in posture, caffeine or food intake (33;34). Because our patients collected urine under strict dietary regulations, the influence of food and caffeine intake was kept to a minimum. We cannot prevent the influence of physical activity and changes in body posture during urine collection, but we expect these circumstances to occur similarly in both patient groups. Metanephrine levels are also influenced by age and gender (33;35;36). Metanephrine levels are significantly higher in men compared to women, furthermore metanephrine levels increase with age (33). However, there were no differences in age and gender between both groups. Therefore, we cannot simply explain the relation between CgA and catecholamine levels by confounding parameters other than HNPG.

It has been reported previously that plasma CgA levels are particularly elevated in patients with malignant pheochromocytoma (37). Our cohort consisted of only patients with benign HNPGL as part of genetic syndromes caused by SDHB or SDHD mutations. We found no differences in the prevalence of mutations in the SDHD and SDHB genes between HNPG patients with increased versus normal CgA levels. Additional studies are required to evaluate the role of CgA as a tumor marker in patients with malignant HNPG.

In patients with pheochromocytoma, postoperative CgA levels are a good index of the curative outcome of surgery (18;21). Because patients with HNPGL have much smaller tumors and therefore lower plasma CgA levels, it remains questionable whether plasma CgA levels are useful as tumor marker in tracking treatment. Therefore, the role of CgA as a potential tumor marker for follow-up of patients with HNPG remains to be elucidated.

In conclusion, the measurement of CgA in patients with HNPGL has limited additional value to the combination of radiological and routine biochemical assessment of HNPGL patients.
Chapter 5

References

Chromogranin A secretion in HNPGL


