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Proneuropeptide Y and neuropeptide Y metabolites in healthy volunteers and patients with a pheochromocytoma or paraganglioma

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ABSTRACT

Neuropeptide Y (NPY1-36) is a vasoconstrictor peptide co-secreted with catecholamines by sympathetic nerves, the adrenal medulla, and neoplasms such as pheochromocytomas and paragangliomas (PPGLs). It is produced by the intracellular cleavage of proNPY and metabolized into multiple fragments with distinct biological activities. NPY immunoassays for PPGL have a diagnostic sensitivity ranging from 33 to 100%, depending on the antibody used.

We have validated a multiplex micro-UHPLC-MS/MS assay for the specific and sensitive quantification of proNPY, NPY1-39, NPY1-37, NPY1-36, NPY2-36, NPY3-36, NPY1-35, NPY3-35, and the C-flanking peptide of NPY (CPON) (collectively termed NPYs), and determined the NPYs reference intervals and concentrations in 32 PPGL patients before, during, and after surgery.

Depending on the peptide measured, NPYs were above the upper reference limit (URL) in 20% to 67% of patients, whereas plasma free metanephrine and normetanephrine, the gold standard for PPGL, were above the URL in 40% and 87% of patients, respectively. Age, sex, tachycardia, and tumor localization were not correlated with NPYs. Plasma free metanephrines performed better than NPYs in the detection of PPGL, but NPYs may be a substitute for an early diagnosis of PPGL for patients that suffer from severe kidney impairment or receiving treatments that interfere with catecholamine reuptake.

1. Introduction

Pheochromocytomas and paragangliomas (PPGLs) are catecholamines secreting tumors arising from chromaffin cells in the adrenal medulla and outside the adrenal medulla, respectively. Norepinephrine, epinephrine, and dopamine are intratumorally converted into metanephrines (MNs), which are established as the gold standard for the biochemical diagnosis of PPGLs [1]. Neuropeptide Y (NPY1-36) is co-released with catecholamines from sympathetic neurons and PPGLs [2]. Fig. 1 depicts the synthesis and metabolism of NPY1-36. NPY1-36 is generated from a 69 amino acid precursor, proNPY, which undergoes intravesicular cleavage by the serine proteases PC1/3 and, to a lower extent, PC2 (PC, prohormone convertase) [3] at the C-terminal side of the dibasic site (Lys^{38}-Arg^{39}), resulting in the generation of NPY1-39 and

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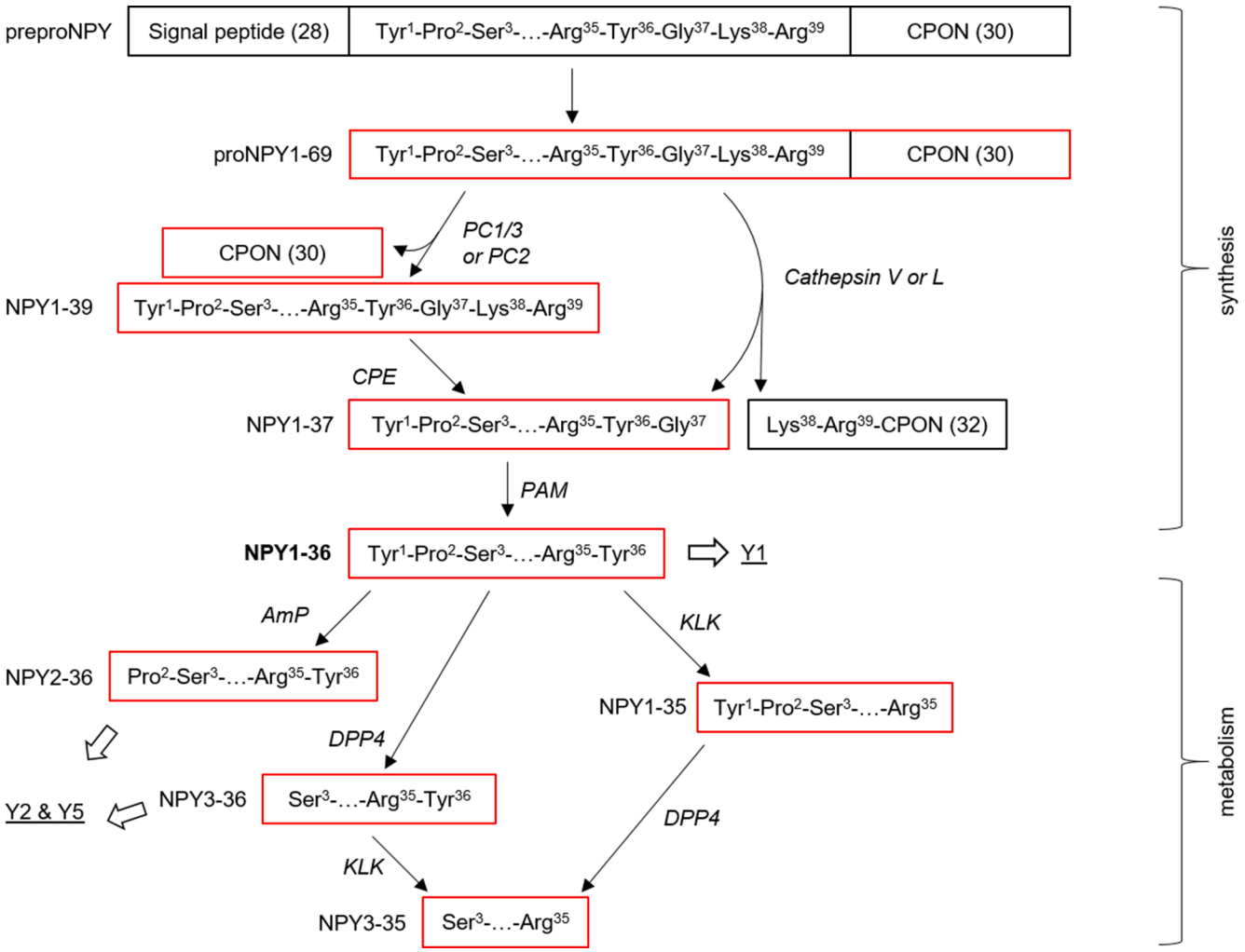

Fig. 1. NPY synthesis and metabolism with known proteases and activities on NPY receptors Y1, Y2 and Y5. PC: prohormone convertase, CPE: carboxypeptidase-like enzyme, PAM: peptidyl-glycine-α-amidating monooxygenase, AmP: aminopeptidase P, KLK: plasma kallikrein, DPP4: dipeptidylpeptidase 4. Numbers in brackets indicate the number of amino acids for each peptide, and large arrows indicate the target receptor. Peptides that are quantified by the assay are framed in red.

CPON (the C-flanking peptide of NPY). NPY1-39 is further processed by a carboxypeptidase-like enzyme (CPE) into NPY1-37. Alternatively, the cysteine proteases cathepsin L [4] and cathepsin V [5] may cleave proNPY at the N-terminal side of the Lys^{38}-Arg^{39} to directly produce NPY1-37 and Lys-Arg-CPON (KR-CPON). NPY1-37 is subsequently processed by PAM (peptidyl-glycine-α-amidating monooxygenase) to yield the active amidated NPY1-36. NPY1-36 is a substrate for DPP4 (dipeptidylpeptidase IV, EC 3.4.14.5), which removes the N-terminal dipeptide, Tyr^1-Pro^2, and thereby converts NPY1-36 into NPY3-36. NPY1-36 can also be degraded by AmP (aminopeptidase P, EC 3.4.11.9) to generate NPY2-36 or by plasma kallikrein to generate NPY1-35, which is also a substrate of DPP4, yielding NPY3-35 [6].

Pioneering work from Adrian *et al.* showed that pheochromocytoma biopsies are strongly stained with an anti-NPY antibody, and that plasma NPY immunoreactive (NPY-IR) concentrations were very high in all patients, with a mean of 460 pmol/L, while concentrations in healthy volunteers were 55 pmol/L [7]. Another study used a collection of five polyclonal antibodies with distinct epitope specificity to better characterize the peptides that were detected and confirmed that NPY-IR was present in the majority (23 out of 26; 89%) of the pheochromocytomas tumors investigated [8]. Of note, the normal range for NPY-IR was below 7 pmol/L, while other authors reported even lower concentrations of 0.25–5 pM when bioactive amidated NPY was measured [9,10]. The promising diagnostic value of NPY for pheochromocytomas was however tempered using a more specific sandwich assay with better characterized antibodies that recognized the bioactive amidated peptide, and the plasma NPY concentration was found to be higher than the upper reference limit (URL) in only 7 out of 21 (33%) patients with a benign pheochromocytoma and 8 out of 12 (67%) patients with a malignant pheochromocytoma [10]. The discrepancies observed between RIA and sandwich ELISA are probably due to the distinct epitopes recognized by the anti-NPY polyclonal antibodies along the proNPY sequence. Moreover, to date, reference intervals for proNPY, NPY1-39, NPY1-37, and CPON are unavailable.

The present study aimed at defining how NPYs perform as a biomarker for PPGL diagnosis. For this, first, we aimed at improving an existing validated micro-UHPLC-MS/MS assay for NPY and its metabolites, to enable the multiplex measurement of proNPY and the precursors of NPY1-36. Second, we aimed at establishing NPYs reference intervals in plasma. Third, we aimed at investigating in 32 PPGL patients before, during, and after the removal of the tumor, whether the diagnostic performance of one or several of these peptides may prove to be better than the gold standard plasma free MNs to detect PPGL. We also studied whether a correlation existed between NPYs or MNs and symptoms, tumor localization, familial disease, or tumor size.

2. Material and methods

2.1. Chemicals and reagents

Human proNPY was obtained from the Protein and Peptide Chemistry Facility (University of Lausanne, Switzerland), NPY1-39, and NPY1-37 were kindly provided by Prof. David Woldbye (University of Copenhagen, Denmark), and CPON was obtained from the American Peptide Company. The proNPY and CPON used as internal standards (IS), prepared with ^{13}C,^{15}N labeling on 5 and 3 leucine residues, respectively, were provided by the Protein and Peptide Chemistry Facility (University of Lausanne, Switzerland). NPY1-39 and NPY1-37 IS were prepared with ^{13}C,^{15}N labeling on 4 alanine residues by Genecust (France). All stock solutions were prepared taking into account the peptide purity and content to obtain net peptide concentrations in the working solutions. CPON01 antibody was produced by the Protein Production and Structure Core Facility (Swiss Federal Institute of Technology (EPFL), Switzerland) from an in-house hybridoma [11]. Other chemicals and reagents were used as described in a previous study [12].

2.2. Patients and sample collection

This Swiss and US multicenter study included two different populations. First, 122 healthy volunteers were included for reference intervals determination. They were recruited at Vanderbilt University Medical Center (Nashville, TN), Hôpital de l'Enfance (Lausanne, Switzerland), and Lausanne University Hospital (Lausanne, Switzerland) and were presented in a previous study [12]. These participants were aged 0.1–61 years (median 24 (sd 19) years, 57% males), with an equilibrated representation of all ages, to ensure the proper determination of reference intervals. Second, 32 patients with PPGL aged 18–84 years (median 51 (sd 18) years, 50% males) were recruited at Lausanne University Hospital, Inselspital (Bern, Switzerland), Luzerner Kantonsspital (Lucerne, Switzerland), Kantonsspital St. Gallen (St. Gallen, Switzerland), and Zürich University Hospital (Zürich, Switzerland). Among them, two had a paraganglioma. The PPGL-related mutations status was known for 10 participants and distributed as follows: 1 patient with Multiple Endocrine Neoplasia Syndrome type 2A (MEN2A), 1 patient with MYC-associated factor X (MAX), 2 patients with Succinate Dehydrogenase Subunit B (SDHB), 2 patients with von Hippel-Lindau (VHL) Syndrome, and 4 without known mutation. The study was approved by the Vanderbilt University Institutional Review Board and the ethical committee of the Canton de Vaud (CER-VD study 2017–00366). All subjects (or their legal representative for children) provided informed written consent before sample collection.

Participants were fasting and coffee, tea, cigarettes, and alcohol were prohibited during the 12 h preceding the sample collection. Samples for reference intervals were collected in the morning. Samples from the 32 PPGL patients were collected 3 h before anesthesia induction and 24 h after surgery; for 16 of them, 9 additional samples were collected during surgery at 0, 5, 7, 10, 20, 40, 60, 90, and 120 min after clamping of the main vessels. Blood samples were collected in ice-cooled 2.6 to 4.5 mL Li-heparin tubes containing a mixture of protease inhibitors [13]. Within 30 min of collection, the samples were centrifuged at 4 °C, 2500 RCF for 10 min, and the plasma was aliquoted and stored at − 80 °C until needed.

2.3. Calibration curve and quality control (QC) samples

The NPYs were quantified in all samples by micro-UHPLC-MS/MS using the method previously published for NPY1-36 and its metabolites [12], modified to enable the quantification of its precursors, namely proNPY, NPY1-39, NPY1-37, and CPON, in the same chromatographic run. Therefore, in addition to NPY1-36 and its metabolites (NPY2-36, NPY3-36, NPY1-35, and NPY3-35), calibrants were spiked with 1000 to 5 pM of proNPY, 10 to 0.05 pM of NPY1-39 and NPY1-37, and 100 to 0.5 pM of CPON (Supporting Information Table 1). Quality control (QC) samples were prepared independently from calibrants by spiking NPYs in charcoal-stripped plasma at three concentrations (low, medium, high), as follows: 800 to 40 pM of proNPY, 40 to 2 pM of NPY1-39, 8 to 0.4 pM of NPY1-37, and 80 to 4 pM of CPON. The final concentrations take into account the residual concentrations in the charcoal-stripped plasma used (Supporting Information Table 2).

2.4. Sample preparation

The sample preparation was adapted from Vocat *et al.* [12], with two modifications to enable the measurement of the precursors. First, Dynabeads M−280 Tosylactivated coated with NPY02, and CPON01 antibodies were mixed [11]. CPON01 recognizes the 16–30 region of CPON, specifically targeting both the CPON and the proNPY, the latter being recognized by both antibodies. Second, 25000 pM ^{13}C,^{15}N-labeled proNPY, 2500 pM ^{13}C,^{15}N-labeled CPON, 500 pM of ^{13}C,^{15}N-labeled NPY1-39, and 50 pM of ^{13}C,^{15}N-labeled NPY1-37 were added to the IS solution.

In summary, the peptides were extracted from 500 μL of plasma supplemented by 10 μL of IS solution to reach final concentrations of IS corresponding to half of the concentration of the highest calibrants. The peptides were first extracted by immunoenrichment using monoclonal antibodies coupled to magnetic beads, and then cleaned by solid-phase extraction. The extracts were then dried and reconstituted in 20 μL of injection solution, of which 10 μL were injected onto the micro-UHPLC-MS/MS system. The whole procedure in detailed in [12].

2.5. Micro-UHPLC-MS/MS analyses

The micro-UHPLC-MS/MS analyses were performed as previously described [12], with the inclusion of the MS parameters for peptides deriving from proNPY (Supporting Information Table 3).

2.6. Method validation for the measurement of NPY precursors by micro-UHPLC-MS/MS

Method validation followed the current bioanalytical method validation guidelines of the US Food and Drug Administration (FDA) [14] and the European Medicines Agency (EMA) [15].

The slope, y-intercept, and r^2 were measured to assess the linearity, using calibrants from three independent runs. Intra-assay precision was evaluated on quintuplicates of the three QC samples, while inter-assay precision was studied on quintuplicates of the three QC samples on three separate runs. Accuracy is not reported here, as blank unmodified matrix is not available, thus lowering the interest of bias calculation, especially on low concentrations. The matrix effect and recovery were assessed as described by Matuszewski *et al.* [16] using six plasmas at two concentration levels. They were determined based on peak areas, on the whole process, *i.e.* immunoenrichment, SPE, and final drying. The lower limit of quantification (LLOQ) was defined as the lowest concentration that provided a CV $< 30\%$ and a bias $< 20\%$ for quintuplicates.

Carryover was estimated in three independent runs by injecting the highest calibrant, followed by two blanks, and was expressed as the ratio of the area measured for the blank to the area measured for the lowest calibrant. The second blank was taken into account for the estimation since the procedure of this assay includes the injection of a blank between every sample.

2.7. Stability study

The freeze and thaw stability and the short-term stability of the NPY precursors in plasma were studied using triplicates of native plasma containing NPY precursors at a concentration equivalent to calibrant B, with or without the protease inhibitors. The freeze and thaw stability

Table 1
Reference intervals for NPYs (pM). The values for NPY and its metabolites are reproduced and slightly modified from a previous work [12]. Age groups are not constantly distributed as the optimal partitioning differ between the peptides (see section 2.9.), and medians of age may differ as a few values were removed by the Tukey tests. The * shows values that could not be calculated because of limited data above the LLOQ.

Analyte	Age group	Median (sd)	n	2.5th percentile (95% CI)	25th percentile	50th percentile	75th percentile	97.5th percentile (95% CI)
proNPY	0 to < 1 years	0.75 (0.30)	18	* (* to 14.3)	34.4	47.2	55.2	100 (75.5 to 130.3)
	1 to 70 years	28 (18)	104	12.6 (10.7 to 14.8)	22.0	29.9	37.9	59.2 (53.1 to 65.6)
CPON	0 to < 10 years	3.3 (2.8)	51	* (* to 7.7)	32.2	39.9	58.1	86.3 (74.0 to 97.9)
	10 to 70 years	37 (12)	65	1.07 (* to 2.43)	5.05	6.96	9.51	13.3 (11.6 to 14.8)
NPY1-39	0 to < 10 years	3.3 (2.8)	51	*	4.01	8.36	13.2	19.7 (17.1 to 22.1)
	10 to 70 years	*	*	*	*	*	*	*
NPY1-37	0 to < 8 years	2.9 (2.5)	42	*	0.46	0.64	0.87	1.45 (1.17 to 1.68)
	8 to 70 years	33 (13)	69	*	0.08	0.19	0.26	0.42 (0.34 to 0.49)
NPY1-36	0 to < 10 years	3.3 (2.8)	49	* (* to 0.32)	0.86	1.56	2.37	3.87 (3.38 to 4.34)
	10 to 70 years	35 (12)	99	* (* to 0.07)	0.18	0.32	0.50	0.88 (0.78 to 0.99)
NPY3-36	0 to < 10 years	3.2 (2.7)	54	0.17 (0.10 to 0.32)	0.86	1.66	2.88	6.66 (5.32 to 8.18)
	10 to 70 years	33 (12)	96	0.23 (0.17 to 0.30)	0.60	0.89	1.23	1.88 (1.72 to 2.03)
NPY2-36	0 to < 10 years	3.3 (2.7)	53	*	0.07	0.13	0.20	0.34 (0.29 to 0.38)
	10 to 70 years	*	87	*	*	*	*	*
NPY1-35	0 to < 8 years	2.4 (2.5)	43	*	0.12	0.25	0.37	0.66 (0.56 to 0.73)
	8 to 70 years	*	98	*	*	*	*	*
NPY3-35	0 to < 8 years	2.1 (2.5)	46	0.14 (* to 0.32)	0.90	1.53	2.28	3.89 (3.24 to 4.52)
	8 to 70 years	33 (13)	102	0.09 (* to 0.18)	0.53	0.83	1.15	1.73 (1.59 to 1.84)

was tested for three cycles, with > 24 h at −80 °C and > 15 min at + 22 °C. The short-term stability was tested for 3 h at + 37 °C. All samples were extracted during the same run, and concentrations were compared with reference samples stored at −80 °C.

The long-term stability of NPY precursors in plasma was monitored using the three QC samples prepared in charcoal-stripped plasma stored at −80 °C, quantified 10 times over a period of six months using three different calibration curves.

2.8. Metanephrines (MNs) measurements

MNs were quantified in the samples from PPGL patients by UHPLC-MS/MS [17]. The reference intervals of MNs for the control groups were derived from a previous study [18].

2.9. Reference intervals for NPY precursors

Reference intervals were determined using the algorithm used in the CALIPER study [19], in accordance with CLSI C28-A3 guidelines, as detailed previously [12]. In summary: (1) partitioning was performed by visual inspection; (2) outliers were removed by applying Tukey test twice; (3) partitions were tested by Harris and Boyd's test to verify that they should be considered separately; and (4) the 2.5th, 25th, 50th, 75th, and 97.5th percentiles were calculated by the Analyse-it add-on for Microsoft Excel, version 5.01 (Analyse-it Software, Leeds, UK) with bi-weight quantile estimator and Box-Cox normalization. The 90% confidence intervals (CI) were calculated for the 2.5th and 97.5th centiles.

2.10. PK parameters and statistical analyses

The half-life ($t_{1/2}$) values of biomarkers were calculated from the log-linear slope term of the curves for all kinetics where the concentrations were above the 97.5 percentile and followed a logarithmic decay, and for patients that were not administered catecholamines during resection of the tumor.

Pre-op, post-op, and control concentrations were compared with the Kruskal-Wallis test using Prism 9.1.0 for Windows (GraphPad, San Diego, CA, USA). Correlations between values were considered true when $r > 0.5$ and $p < 0.05$ and were calculated with Prism.

3. Results

3.1. Validation of the assay for NPY precursors by micro-UHPLC-MS/MS

Linearity was assessed for all four precursors ($r^2 > 0.99$) over the calibration curve ranges. The mean slope, intercept, and r^2 measured in three separate runs are shown in Supporting Information Fig. 1. The intra-assay precision ranged from 3% to 19% for the three QC concentrations, and the inter-assay precision ranged from 8% to 27% (Supporting Information Table 4). Recovery ranged from 10% to 51% with high CV values, and the matrix effect ranged from 38% to 197% (Supporting Information Table 5). The LLOQ were determined at 8.72, 0.61, 0.71, and 0.16 pM for proNPY, CPON, NPY1-39, and NPY1-37, respectively, and at 0.116, 0.12, 0.16, 0.12, and 0.07 pM for NPY1-36, NPY2-36, NPY3-36, NPY1-35, and NPY3-35, respectively, based on a CV < 30% and a bias < 20%. The carryover measured in three separated runs was up to 2% for CPON and below detectable levels for the other precursors.

The freeze–thaw stability was evaluated after three cycles to represent the maximum number of freeze–thaw cycles observed for a given sample in usual laboratory conditions. The freeze–thaw stability and the short-term stability in plasma at 37 °C showed excellent recoveries (81% to 105%) when protease inhibitors were added, with the exception of NPY1-39 with 206% after 3 h (Supporting Information Fig. 2). Conversely, without protease inhibitors, proNPY and NPY1-37 were degraded (recoveries of 39% and 12%, respectively), whereas NPY1-39 concentrations were increased (1154%), and CPON concentrations were unchanged (106%). Finally, we noticed that the IS areas exhibited a high variability for NPY1-37 and NPY1-39 when protease inhibitors were used.

The long-term stability, measured on QC samples prepared in charcoal-stripped plasma and stored at −80 °C and analyzed 10 times over a period of six months, showed limited degradation over time. A CV of 28%, 24%, 37%, and 30% was obtained for proNPY, CPON, NPY1-39, and NPY1-37, respectively, for the low QC samples, while values for the high QC samples were 20%, 13%, 19%, and 7%, respectively. No particular trend was observed over time (Supporting Information Fig. 3).

3.2. Reference intervals for the NPY precursors

The NPYs concentrations measured in the 122 healthy volunteers' samples are detailed in Supporting Information Table 6. Circulating levels were above the LLOQ in 100%, 100%, 51%, and 79% of the studied samples for proNPY, CPON, NPY1-39, and NPY1-37,

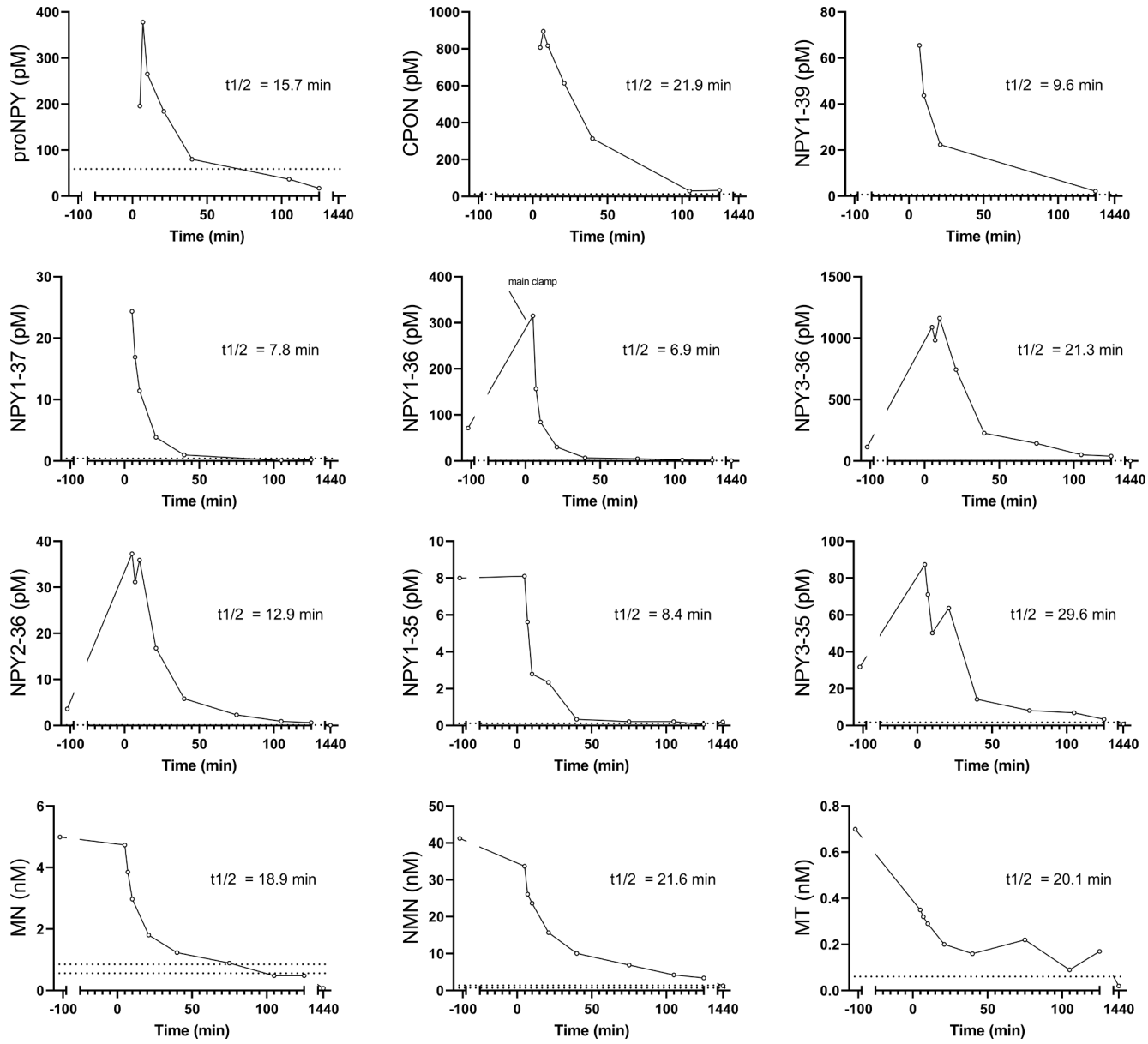

Fig. 2. Concentrations of NPYs and metanephrines (MNs) during resection of a 138 g tumor from the left adrenal gland of a 42-year-old patient without related mutation, selected as an example. Time 0 corresponds to the clamp of the main vessels. The dotted lines for NPYs show the 97.5th percentile, and the two dotted lines for MNs show the upper reference limits in healthy and hypertensive controls [18]. Half-life values were calculated for this patient, and differ from those in Table 2 that are mean values calculated for all participants.

respectively. For the reference interval calculation, values under the LLOQ were set at a concentration equal to half the LLOQ.

Concentrations were visually age-dependent, as is clearly shown by the scatter plots in Supporting Information Fig. 4, with higher concentrations of NPYs in younger subjects compared to adults, with the exception of proNPY. The proNPY and CPON concentrations in adults were far higher than those of other peptides, whereas NPY1-39 was not detected, and NPY1-37 was half the level of NPY1-36. The low number of values and the asymmetric age repartition prevented the proper statistical study of any age *vs.* concentration correlation.

For all NPY precursors, the reference intervals (presented in Table 1) were best determined by separating participants into two groups. The groups were not identically distributed as the optimal partitioning differ between the peptides. The reference intervals for NPY1-39 were not calculated for adults because 61 out of the 71 samples presented concentrations below the LLOQ, at 0.71 pM. Although the Tukey test for the pediatric cohort for proNPY suggested that the three highest values should be removed, they were included in the calculation because they were derived from the three younger participants and would have been included as an additional group if the number of participants aged below 10 had been higher, instead of being rejected as outliers.

Correlations between NPYs concentrations and gender were studied for all groups, except where one gender represented<25% of all participants. Concentrations were not gender-dependent according to the Harris and Boyd's test, except for CPON in adults. A wider distribution of CPON was observed for men than for women (SD = 3.62 and 2.27, respectively, $p = 0.17$). Since the number of values for men was too low to build reference intervals, and since the difference of SD did not exceed 50%, one single reference interval was determined for both genders for CPON.

3.3. NPYs and PPGL

Thirty-two PPGL patients were included in this study. Plasma NPYs

Table 2
Mean (SD) half-life values for all NPYs and metanephrines (MNs) measured after tumor resection.

Analyte	Half-life (min)
proNPY	27.9 (10.7)
CPON	22.0 (3.2)
NPY1-39	11.1 (2.2)
NPY1-37	29.1 (21.4)
NPY1-36	10.1 (2.9)
NPY3-36	27.3 (6.3)
NPY 2-36	22.7 (5.6)
NPY1-35	12.9 (6.4)
NPY3-35	23.2 (5.6)
MN [1]	20.8 (5.5)
NMN [1]	23.1 (4.7)
MT [1]	15.3 (6.8)

[1] MN: metanephrine, NMN: normetanephrine; MT: 3-methoxytyramine.

and free MNs were measured 3 h before anesthesia induction and 24 h after surgery for all patients, and 8 to 10 additional measurements were performed on samples collected within 120 min of clamping the tumor to measure the decay of NPYs for 16 patients. As an example, Fig. 2 shows the concentrations measured after the removal of a tumor weighing 138 g in a 42-year-old patient.

Half-lives were determined for all NPYs and MNs (Table 2), using each curve that presented a sufficient number of time points above the upper reference intervals to prevent bias due to the contribution of endogenous levels. The half-lives of NPYs ranged between 10.1 min for NPY1-36 and 27.9 min for proNPY. Interestingly, half-life values were 2- to 3-fold longer for NPY3-36 and NPY3-35 than for their respective precursors NPY1-36 and NPY1-35 (27.3 and 23.2 min *vs.* 10.1 and 12.9 min, respectively). The half-lives of MNs ranged between 15.3 and 23.1 min.

The concentrations of NPYs and MNs measured before the induction of anesthesia (usually 3 h before the main clamp, hereafter referred to as "pre-op") and those measured 24 h later ("post-op"), as well as the values measured in healthy volunteers to build the reference intervals ("controls"), are presented in Fig. 3 (and Supporting Information Fig. 5 for data with individual connecting lines). The pre-op concentrations of NPYs and MNs were higher than the controls ($p < 0.05$), with the exception of proNPY and NPY3-35. After tumor removal, NPY1-37, NPY1-36, NPY3-35, MN, and methoxytyramine were significantly lower than the pre-op concentrations. Interestingly, post-op concentrations were significantly different from the controls for all NPYs except NPY1-37 and NPY1-36 and for normetanephrine (NMN).

The ratios of the medians for the pre-op *vs.* post-op groups are shown in Fig. 4. Overall, the medians of all NPYs and MNs measured pre-op were either higher or similar to those post-op. The precursors proNPY and NPY1-39 and CPON were unchanged after surgery (ratios of 1.1 to 1.2, $p > 0.05$). Conversely, NPY1-37 and NPY1-36 were significantly higher (ratios of 5.3, $p = 0.003$ and 3.8, $p = 0.001$, respectively). All metabolites of NPY1-36 were higher (ratios of 1.8 to 3.3), but this was significant only for NPY3-35 ($p = 0.002$). The three MNs were also higher (ratios of 3.1 to 10.7, all $p < 0.001$).

NPYs were above the URL in 20% (for proNPY) to 67% (for NPY3-36) of the patients' pre-op samples (Table 3), while MNs were above the URL in 40% to 87% of the samples. Amongst the four patients with normal NMN, one had elevated MN, whereas the other three had concentrations of the three MNs in the normal range. One displayed CPON and NPY3-36 values above the URL, with values that were 2.6- and 1.4-fold higher than the 97.5th percentile; the second patient displayed NPY1-37 concentrations that were 1.2-fold higher than the 97.5th percentile; and the third patient had NPYs concentrations within the reference intervals. The combinations or ratios of NPYs did not improve the performance of NPYs (data not shown).

Correlations between pre-op concentrations of all NPYs and MNs were studied, as well as with the presence of tachycardia or hypertension, age, sex, tumor localization, weight, and genetic mutation. Pre-op concentrations of NPYs and MNs did not correlate except NMN with NPY1-37 and proNPY ($r = 0.55$, $p = 0.01$ and $r = 0.61$, $p = 0.004$, respectively, see Supporting Information Table 7). Tumor weight correlated with NMN ($r = 0.66$, $p = 0.002$) and with MT ($r = 0.52$ and $p = 0.02$) concentrations, but not with NPYs (Supporting Information Table 8). PPGL patients with hypertension had a significantly higher NMN than PPGL patients without hypertension ($p = 0.038$, Supporting Information Fig. 6), and female patients had a higher MN than male patients ($p = 0.049$, Supporting Information Fig. 7). No significant correlation or difference was highlighted in these PPGL patients for any of the biomarkers with regard to age, tachycardia, or tumor localization (Supporting Information Table 8 and Supporting Information Figs. 8 and 9). No association was highlighted between concentrations of NPYs and MNs and known genetic mutations related to PPGL, maybe because of the low number of patients that were included (see Supporting Information Fig. 10 for a graphical representation). In addition, NPYs and MNs were not different in paraganglioma and pheochromocytoma patients.

4. Discussion

4.1. Validation of the assay for NPY precursors by micro-UHPLC-MS/MS

For the first time, we report herein an LC-MS/MS assay for the measurement of nine peptides derived from proNPY in plasma (Fig. 1). The assay is fully validated and meets the analytical criteria required for the measurement of NPY precursors and metabolites in clinical studies. In particular, this LC-MS/MS assay is specific and sensitive enough to measure circulating concentrations of proNPY, CPON, NPY1-37, NPY1-36, NPY3-36, and NPY3-35 at rest. The stability of the peptides was ensured by a dedicated mixture of protease inhibitors to prevent any *ex vivo* proteolytic degradation. Nevertheless, NPY1-39 concentrations were increased (+206% after 3 h at 37 °C), probably because of the degradation of its precursor, which circulates at > 100-fold higher concentrations. This limitation of the method was not explored further, as it involves only NPY1-39, which circulates mostly at concentrations below the LLOQ, but it highlights the need to work on ice at all times, to prevent uncontrolled *ex vivo* degradation. Besides, even though recovery of NPY1-39 was very low, its extraction was not further optimized since even an improvement to 100% would not allow NPY1-39 detection in samples. Validation data showed that peptide measurement was affected by matrix effects that were efficiently corrected by the IS.

4.2. Reference intervals for the NPY precursors

Hence, using this assay, reference intervals were determined for proNPY, CPON, and NPY1-37, in addition to those established for NPY1-36 and its metabolites [12]. Reference intervals were not determined for NPY1-39 in adults since concentrations were above the LLOQ in only 14% of the subjects aged ≥ 10 years.

We found that proNPY is not fully processed in neuroendocrine vesicles since it is secreted unprocessed in the bloodstream. In healthy adults, proNPY concentrations are > 100-fold higher than those of NPY1-39, NPY1-37, and NPY1-36. Since the metabolic clearance is similar for all these peptides, including proNPY (half-lives between 10 and 30 min), these differences clearly suggest a strongly regulated intracellular processing of proNPY by prohormone converting enzymes along the *trans*-Golgi network to regulate NPY1-36 bioactive release. These data contrast with another study showing an almost complete proteolytic processing of proNPY to mature NPY in 16 human pheochromocytoma tumors (median, 93%; range, 72–100%) using HPLC followed by RIA [20]. However, these data must be carefully evaluated since they are based on tumoral biopsies and not on blood specimens and were obtained using immunoassays with a limited specificity.

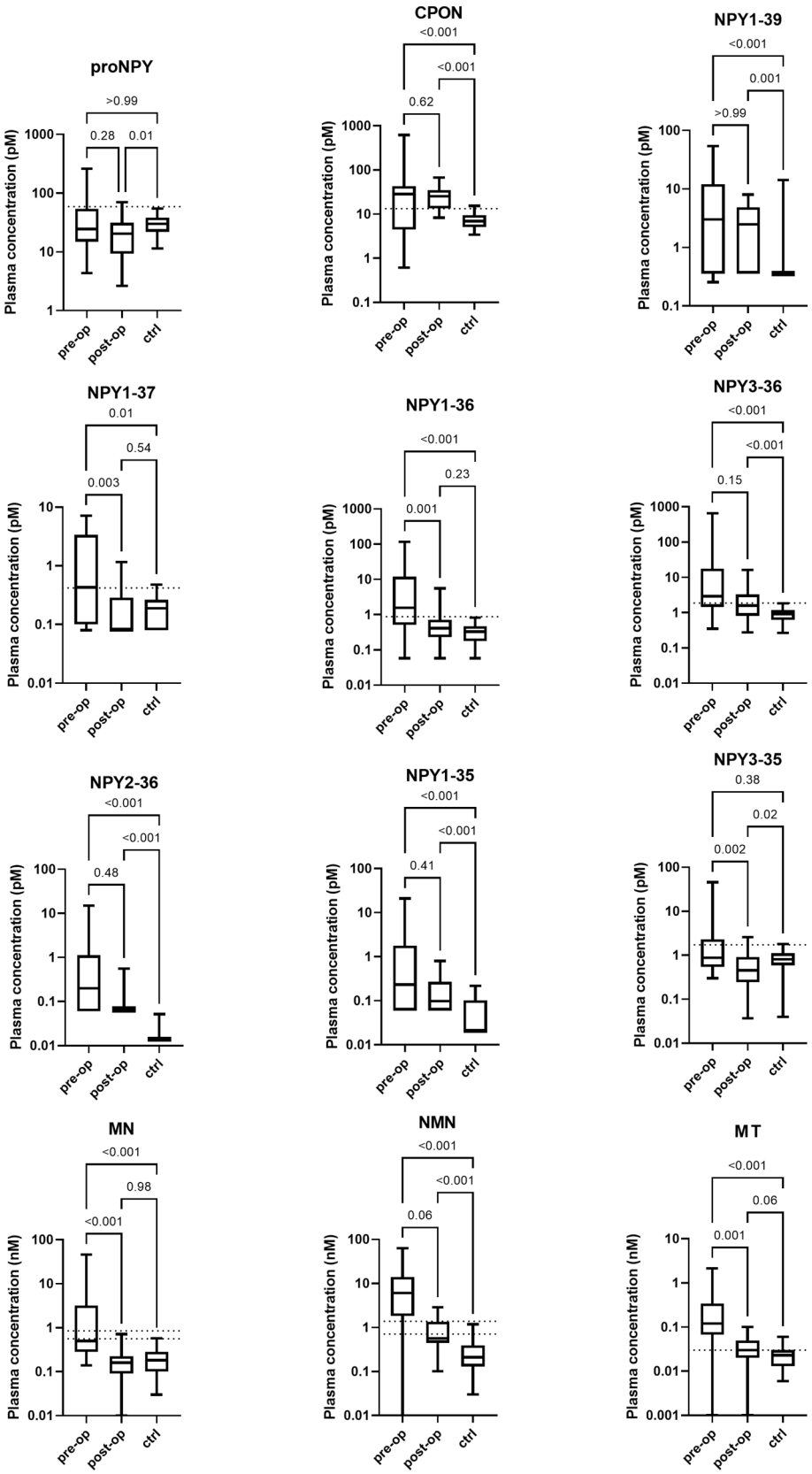

Fig. 3. Plasma concentrations of NPYs and metanephrines (MNs) in pre-op, post-op, and healthy volunteer groups (n = 32). Pre-op corresponds to 3 h before the main clamp of the vessels, with the patient still awake, and post-op corresponds to 24 h after operation. The center line of each box represents the median, and the whiskers show the 2.5th and 97.5th percentiles. The dotted lines for NPYs show the 97.5th percentile, and the two dotted lines for MNs show the upper reference limits in healthy and hypertensive controls [18]. The same plots with individual connecting lines are presented in Supporting.

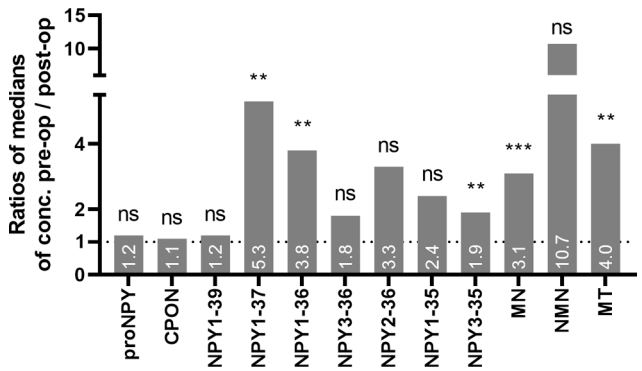

Fig. 4. Ratios of median plasma concentrations of NPYs and metanephrines (MNs) between the pre-op and post-op groups. The p-values are the same as displayed in Fig. 3; ns stands for $p > 0.05$, * for $p \leq 0.05$, ** for $p \leq 0.01$, and *** for $p \leq 0.001$.

In adults, NPY1-39 concentrations were below the LLOQ (0.71 pM) in 86% of the healthy participants, while the median was found to be 8.36 pM for participants aged < 10 years. Conversely, proNPY concentrations were similar in both age groups, suggesting that the difference in NPY1-39 concentrations in younger and older subjects results from distinct levels of PC1/3 expression with age; alternatively, an increase in cathepsin activity with age may favor NPY1-37 and KR-CPON production. Indeed, PC1/3 gene expression is markedly lower in aged rats compared to young rats [21], while reduced levels of cathepsin S were observed in the skin of aged *vs*. young individuals [22]. An increase in cathepsin activity would result in a shift in the major pathway, from proNPY → CPON + NPY1-39 via PC1/3 in young individuals to proNPY → KR-CPON + NPY1-37 via cathepsins in adults (see Fig. 1 for the graphical description). To assess this hypothesis, the presence of KR-CPON presence was determined by LC-MS/MS in plasma and pheochromocytoma tissue samples. Despite an estimated limit of quantification of 10 pM in plasma using synthetic peptide, no endogenous KR-CPON was detected in any plasma or tissue sample, while NPY1-37 and CPON were detected in the same samples at concentrations up to 4033 pM and 6286 pM, respectively, in tissues (data not shown). This suggests that cathepsins L and V are not involved in proNPY processing, despite a higher catalytic activity than PC1/3 [23], or that KR-CPON has a very low stability, which precludes its detection. Further investigations are needed to fully understand the metabolic pathway from proNPY to its fragments and to explain the NPY1-39 concentrations observed in adults compared to young people.

4.3. NPYs and PPGL

Our study confirmed that plasma NPYs concentrations increased during tumor manipulation and returned to near normal range rapidly after operation [24,25]. The half-lives of plasma NPYs ranged between 10 and 30 min after PPGL surgical resection but have to be carefully considered as they probably include an important secretion component induced by the stress and pain occurring during surgery. For example, the NPY1-36 half-life determined here was two-fold longer (10.1 min) than that measured in healthy volunteers exercising at heavy intensity (4.6 min) [26]. The relatively long-lasting concentrations observed with NPY3-36 and NPY3-35 compared to NPY1-36 or catecholamine are due to the fast degradation of NPY1-36 into fragments that are still measured by immunoassays [27]. We previously studied the kinetics of the elimination of plasma NPY during the surgical removal of seven pheochromocytoma tumors [24] using a specific two-site sandwich ELISA for amidated NPY (*i.e.*, NPY1-36 + NPY2-36 + NPY3-36) [10]. The half-life value estimated by non-linear regression was 12.3 ± 7.8 min for NPY [24], reflecting the mix of NPY1-36 (10.1 min in the present study) and NPY3-36 (27.3 min). The half-lives for MNs reported here were similar to those published by Eisenhofer *et al.* [28], but shorter than those previously reported in two patients after pheochromocytoma resection (82.5 min (mean of two values) for MN and 95 min for NMN) [29].

Because the blood collection time-points differed between patients, data from kinetics such as the one presented in Fig. 2 were not suitable for a statistical study of NPY concentrations. To determine the diagnostic value of NPY species, we compared pre-op, post-op, and control concentrations. The concentrations of all NPYs (except proNPY and NPY3-35) measured in the pre-surgery group were higher than those measured in the healthy volunteer group, demonstrating the production of these peptides by the tumor. The absence of a reduction in the proNPY concentration after surgery suggests a low secretion by the tumor compared with the endogenous basal neurogenic secretion (median circulating concentration in healthy volunteers = 30 pM, compared with 15 pM and 54 pM for the 25th and 75th centiles in PPGL patient samples), suggesting that a significant proportion of proNPY escaped the regulatory pathway [23]. Conversely, NPY1-37 and NPY1-36 concentrations decreased dramatically after tumor resection, confirming the secretion of these metabolites by the tumor. The concentrations of the metabolites of NPY1-36 only decreased slightly after tumor resection, probably because they are processed by circulating enzymes such as DPP4, plasma kallikrein, and aminopeptidase P [6], which may not be significantly expressed within the tumor. Of note, the age of the control and PPGL groups differ, which is a limitation of our study (e.g. for NPY1-36: median (sd) of age: 33 (12) and 51 (18), respectively). Indeed, an age-dependent decline in adrenal hormone secretion was demonstrated, partly because of adrenal degeneration during aging [30]. NPYs concentrations are however not significantly modified with age in adults, as described by the partitioning and as visually shown in Supporting Information Fig. 4.

We did not notice any correlation between NPY1-36 or other NPY fragment concentrations and the hypertensive state of these patients, whereas NMN, which derives from norepinephrine, was associated with hypertension. This confirms our previous observation relating to a minor contribution of NPY1-36 to the hemodynamics in subjects undergoing physical exercise [26].

NPYs were significantly increased in the group of patients with pheochromocytoma but not in all patients; values were increased in 20% (proNPY) to 67% (NPY3-36) of patients, and combining more NPYs did not improve the sensitivity of the diagnostic test. Our data do not show a correlation between genetic diseases associated with a PPGL and NPY species, and the diagnostic performance of NPYs for the detection of a PPGL is, therefore, inferior to MNs, whose sensitivity is already excellent (≥95%) [18].

Three patients (out of 30) were negative for the combination of MN, NMN, and MT. Of them, one had values of CPON and NPY3-36 above the

Table 3
Number of pre-op values above the upper reference limits (URL) for each individual marker and for all NPYs or metanephrines (MNs). No reference limits were established for NPY1-39, NPY2-36, or NPY1-35. Of note, 32 patients were included in the study, but the total of the values mentioned in the table is lower as it was impossible to collect plasma during certain periods of surgery.

	proNPY	CPON	NPY1-37	NPY1-36	NPY3-36	NPY3-35	NPYs	MN [1]	NMN [1]	MT [1]	MNs [1]
Number of values above URL	4/20	13/20	10/20	19/30	20/30	8/27	24/30	12/30	26/30	23/30	27/30
% of values above URL	20%	65%	50%	63%	67%	30%	80%	40%	87%	77%	90%

[1] MN: metanephrine, NMN: normetanephrine; MT: 3-methoxytyramine; MNs: metanephrines, *i.e.*, MN, NMN, and MT.

97.5th percentile, the second had slightly increased NPY1-37, and the third had no increased NPYs at all. Despite these valuable results, our data do not support the idea of selectively measuring each NPY to help improve the sensitivity of MNs to detect a PPGL.

The measurement of MNs in plasma may be falsely raised in patients suffering from kidney impairment [31], in patients who have received catecholamines in ICU or in patients treated by a polycyclic antidepressant that interferes with catecholamine reuptake, such as venlafaxine [32,33]. In such situations, the determination of NPYs that are not affected would present an interesting alternative, despite its inferior sensitivity.

5. Conclusion

We have validated the first LC-MS/MS quantitative assay of the precursor of NPY and its metabolites, together with NPY1-36 and its post-translational products. The wide variation in NPY1-39 concentrations observed between young participants and adults suggests that proNPY is mainly processed into NPY1-39 and CPON by PC1/3 in young individuals and into NPY1-37 and KR-CPON by cathepsins L and/or V in adults. In PPGL patients, the relative concentrations of NPY species deriving from the mature peptide are no different from healthy individuals, indicating that NPY species are not produced intratumorally by specific enzymes, which would have resulted in specific post-translationally modified NPYs. Finally, we conclude that measuring proNPY, NPY and their metabolites does not improve the sensitivity of plasma free MNs for the diagnosis of PPGL. However, NPYs may be of interest where a PPGL is suspected in patients suffering from kidney impairment or receiving treatments that interfere with catecholamine reuptake, which preclude the quantification of MNs.

CRediT authorship contribution statement

Philippe J. Eugster: Conceptualization, Data curation, Formal analysis, Funding acquisition, Methodology, Project administration, Visualization, Writing – review & editing. **Jonathan Maurer:** Data curation, Funding acquisition, Investigation, Methodology, Validation, Visualization, Writing – review & editing. **Céline Vocat:** Data curation, Investigation, Methodology, Validation. **Karim Abid:** Resources. **Maurice Matter:** Resources. **Grégoire Wuerzner:** Resources. **Roman Trepp:** Resources. **Stefan Fischli:** Resources. **Christoph Henzen:** Resources. **Walter Kolb:** Resources. **Stefan Bilz:** Resources. **Sarah Sigrist:** Resources. **Felix Beuschlein:** Resources. **Svenja Nölting:** Resources. **Astrid Reul:** Resources. **Ina Schütze:** Resources. **Scott A. Hubers:** Resources. **Nancy J. Brown:** Resources. **Eric Grouzmann:** Conceptualization, Funding acquisition, Supervision, Writing – review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.cca.2022.07.018.

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