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A novel method to detect hPG₈₀ (human circulating progastrin) in the blood

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hPG₈₀ (human circulating progastrin) is produced and released by cancer cells. We recently reported that hPG₈₀ is detected in the blood of patients with cancers from different origins, suggesting its potential utility for cancer detection. To accurately measure hPG₈₀ in the blood of patients, we developed the DxPG₈₀ test, a sandwich Enzyme-Linked Immunosorbent Assay (ELISA). This test quantifies hPG₈₀ in EDTA plasma samples. The analytical performances of the DxPG₈₀ test were evaluated using standard procedures and guidelines specific to ELISA technology. We showed high specificity for hPG₈₀ with no cross-reactivity with human glycine-extended gastrin (hG17-Gly), human carboxy-amidated gastrin (hG17-NH₂) or the CTFP (C-Terminus Flanking Peptide) and no interference with various endogenous or exogenous compounds. The test is linear between 0 and 50 pM hPG₈₀ (native or recombinant). We demonstrated a trueness of measurement, an accuracy and a variability of hPG₈₀ quantification with the DxPG₈₀ test below the 20% relative errors as recommended in the guidelines. The limit of detection of hPG₈₀ and the limit of quantification were calculated as 1 pM and 3.3 pM respectively. In conclusion, these results show the strong analytical performance of the DxPG₈₀ test to measure hPG₈₀ in blood samples.

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Introduction

Progastrin is a pro-protein able to generate several peptides upon maturation (Fig. 1A).^{1,2} The end product is carboxy-amidated gastrin (active gastrin also named hG17-NH₂), with known physiological functions such as the regulation of acid secretion or the control of proliferation of the antral mucosa.³ Aside from active gastrin, a number of other peptides have been identified, both in tissue extracts and in the plasma. Under the name of “active gastrins” are hG17-NH₂, hG34-NH₂ and hG71-NH₂ also known as component I.⁴ If the main maturation pathway generates hG17-NH₂ and hG34-NH₂, there is a minor pathway that generates the component I. In the main maturation pathway, it is glycine-extended gastrin (hG17-Gly) that is considered as the unique immediate precursor of hG17-NH₂, whereas in the minor maturation pathway, component I plays this role.⁴ The C-terminus flanking peptide (CTFP) has also been detected in the plasma at high concentration.⁵ And, although hG17-NH₂ is the effective functional product of progastrin maturation, other peptides have been attributed various functions, such as for the CTFP that is able to stimulate *in vitro* cell proliferation and migration.⁵

However, if the complexity of pro-proteins, due to their various maturation products, is well documented in physiology, their involvement in pathology further adds a degree to this complexity. This is true in particular for progastrin.

Indeed, it has been shown in the early 90's in colorectal carcinoma extracts that progastrin maturation is incomplete in tumor tissues.^{6–8} The unprocessed precursors, hG17-Gly and progastrin, accumulate in the tumor where they can regulate several features of the tumor and intervene on tumorigenesis such as the disruption of cell-cell junctions,⁹ cell proliferation,^{10,11} inhibition of apoptosis,^{12,13} regulation of cancer stem cells,^{14,15} and angiogenesis.¹⁶ But they have first to be released from the tumor cells to exert their functions, which has two major consequences: (1) they can be neutralized by specific antibodies, which has been done for both precursors,^{15,17} and (2) they are detectable in the plasma. Although we do not want to underestimate the potential role of hG17-Gly, the data accumulated on the role of progastrin during tumorigenesis clearly indicate its dominant role over hG17-Gly. In particular, the level of progastrin in the plasma of colorectal cancer patient is known to be increased unlike that of hG17-Gly.¹⁸ And for all the above reasons, we decided to focus on progastrin, that we named hPG₈₀ once secreted to avoid any confusion with progastrin as the physiological precursor of active gastrin.^{8,19}

Our goal was to generate a tool readily workable for physicians. We developed a kit (DxPG₈₀) that detects and quantifies

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Fig. 1 Overview of Gastrin maturation and antibody epitopes. (A) Processing of preprogastrin. Adapted from ref. 33. Numbers in red indicate the processing enzymes: 1 = signal peptidase, 2 = prohormone convertase 1/3, 3 = carboxypeptidase E, 4 = prohormone convertase 2, and 5 = peptidyl- α -amidating-monooxygenase. G34 for gastrin-34 and G17 for gastrin-17. (B) Amino acid sequence of progastrin. In blue the epitope sequence used to generate antibodies recognizing N-terminus of hPG₈₀. In red the epitope sequence used to generate antibodies recognizing C-terminus of hPG₈₀.

hPG₈₀ in human plasma. In the present study, we describe in details the analytical performance of the DxPG₈₀ test.

Materials and methods

Antibodies

The anti-hPG₈₀ antibodies were generated according to patents WO/2011/045080 and WO/2017/114973 and as described in Prieur *et al.*¹⁵ All antibodies were selected to bind hPG₈₀ but not other products using direct ELISA.¹⁵ Specifically, wells were coated with a solution containing one of the following peptides at 50 or 250 ng: hPG₈₀, Keyhole Limpet hemocyanin (KLH), hG17-Gly, hG17-NH₂, or the CTFP. Antibodies displayed no reaction to high quantities of KLH which was coupled to the antigenic peptide used to immunize the mice or rabbit. All antibodies displayed high specificity for binding to full length hPG₈₀ as compared to the gastrin-gene derived peptides hG17-Gly, hG17-NH₂, or the CTFP for which the antibodies showed no detectable binding.

Capture antibody

Briefly, a sequence residues 55 to 80 of hPG₈₀ coupled to KLH (KLH-Cys-Ahx-Ahx-QGPWLEEEEEEAYGWMDFGRRSAEDEN) was used to generate antibodies recognizing C-terminus of hPG₈₀ (Fig. 1B). This antigenic sequence corresponds to the COOH-terminal 26 amino acid residues shown to be sufficient for the growth promoting effect of hPG₈₀.²⁰ We generated 23 murine monoclonal antibodies (mAbs) (SysDiag).¹⁵ Using direct ELISA

and BIAcore, we showed that these mAbs exhibited high affinities for hPG₈₀, ranging K_d from 10^{-7} M to 10^{-12} M. Targeted epitopes were characterized using Alascan and SPOT techniques.¹⁵ We then selected the antibody with the highest affinity (*i.e.* $K_d = 6.9 \times 10^{-12}$ M).¹⁵ Antibodies produced by the hybridoma are purified using Akta purifier with a protein A column, eluted with a low pH buffer and dialysed in PBS1X. The capture antibody selected is coated in excess.

Detection antibody

Briefly, the N-terminus epitope corresponded to the sequence containing residues 1 to 14 of hPG₈₀ coupled to KLH (SWKPRSQQPDAPLG-Ahx-Cys-KLH). It was used to generate antibodies recognizing the N-terminus of hPG₈₀ (Fig. 1B). Polyclonal anti-hPG₈₀ antibodies (pAbs) were generated by immunizing rabbits, immunopurified by using affinity column coupled to the N-terminus peptide and then eluted with a low pH buffer and dialysed in PBS1X (Eurogentec).

Recombinant and native hPG₈₀

Recombinant hPG₈₀ (rhPG₈₀) was produced as described in McQueen *et al.*, with minor modifications.²¹ Briefly, BL21 DE3 Star bacterial cells (Invitrogen) were transformed with a vector containing the full-length human hPG₈₀ sequence (Fig. 1B) in a PGEX-GST-TEV backbone (GE Healthcare). Bacteria were grown in LB medium containing 0.5 mM IPTG for 3 hours at 37 °C. Bacterial pellets were broken using a French Press, and both soluble and non-soluble fractions were separated by

centrifugation. Thereafter, GST-tagged rhPG₈₀ was isolated using a glutathione affinity column and rhPG₈₀ was cleaved from GST with the Tobacco Etch Virus N1a (TEV) protease. Finally, rhPG₈₀ was dialyzed against the final buffer (10 mM Hepes, 0.5% BSA, pH 7.4). rhPG₈₀ was quantified using the absorbance at 280 nm and the specific absorbance calculated for the sequence of hPG₈₀ (2585 mAU at 1 g L⁻¹).

To ensure that the DxPG₈₀ test not only recognizes recombinant but also native hPG₈₀ (nhPG₈₀), including O-sulfated and phosphorylated forms,²² we stably overexpressed the *GAST* gene in HCT-116 cell line (human colon carcinoma cells) and showed that these cells secrete post-translationally-modified hPG₈₀.¹⁵ nhPG₈₀ was purified from HCT116-PG culture medium by gel-filtration. We showed that all the antibodies used in the DxPG₈₀ test were able to detect nhPG₈₀ as shown in.¹⁵ nhPG₈₀ was quantified using Bradford method and by sandwich ELISA using rhPG₈₀ to prepare the calibration samples (Fig. 2).

Specimen collection and storage

Human whole blood is collected using K2-EDTA or K3-EDTA tubes and centrifuged for 10 minutes at 1300×g at +4 °C using a refrigerated centrifuge to remove the cells and collect the plasma. Following centrifugation, the resulting supernatant is designated as plasma. It is important to immediately and carefully transfer the plasma into a clean polypropylene tube.

The plasma should be maintained between +2 and +8 °C if used immediately. If the plasma is not readily analysed, the plasma should be apportioned within maximum 2 hours into aliquots (minimum volume 0.5 mL) and stored at -20 °C (±5

°C) for a maximum of one (1) month, or stored at -80 °C (±10 °C) for long term storage.

Validation range preparation

The quality control sample (CTL) is a spiked sample used to monitor the performance of a bioanalytical method and to assess the integrity and validity of the results of the unknown samples analysed in an individual batch.

The calibration standard (CAL) is a matrix to which a known amount of analyte has been added or spiked. Calibration standards are used to construct calibration curves.

CAL and CTL samples are prepared using nhPG₈₀

Depending on the experiment, we used two different sets of CAL and CTL:

- Range 0–25 pM: 6 calibrators (CAL 0, 5, 10, 15, 20 and 25 pmol L⁻¹) and 3 external controls (CTL 5, 12.5 and 22.5 pmol L⁻¹).

- Range 0–45 pM: 6 calibrators (CAL 0, 5, 15, 25, 35, 45 pmol L⁻¹) and 3 external controls (CTL 5, 22.5 and 35 pmol L⁻¹).

The CAL and CTL are diluted in hPG₈₀-negative human EDTA plasma. The 1× CAL and 1× CTL were prepared by diluting 120-fold with hPG₈₀-negative human EDTA plasma.

Assay procedure

Samples, CAL and CTL are tested in duplicate. Add 50 µL of Sample Dilution Buffer to all the wells that will be used from the anti-hPG₈₀ antibody pre-coated 96 wells strips microplate included in the kit at room temperature. Transfer 50 µL of the 1× CAL, 1× CTL and samples with a multi-channel pipette (8 channels) to the pre-coated 96 wells strips microplate included in the kit at room temperature. Cover the plate with plastic paraffin film and incubate for 1 hour ± 5 min at +37 °C (±2 °C). At the end of the incubation step, discard all the liquid from the wells by inverting the plate. Proceed to a thorough washing step by adding 300 µL per well of 1× Wash solution using a multi-channel pipette. Discard the 1× Wash solution by inverting the plate and thoroughly pat dry the microtiter plate frame upside down on absorbent paper. Repeat the washing step 6 times. At the end of the washing steps, ensure the complete removal of the liquid from the wells. Add 100 µL of the 1× Conjugate (N-terminus pAb coupled to horse-raddish peroxidase, HRP) to each well using a multi-channel pipette. Cover the plate with plastic paraffin film and incubate 30 ± 3 min at +37 °C (±2 °C). At the end of the incubation step, discard all the liquid from the wells by inverting the plate. Proceed to a thorough washing step by adding 300 µL per well of 1× Wash solution using a multi-channel pipette. Discard the 1× Wash solution by inverting the plate and thoroughly pat dry the microtiter plate frame upside down on absorbent paper. Repeat the washing step 6 times. At the end of the washing steps, ensure the complete removal of the liquid from the wells. Add 100 µL of the substrate solution to each well using a multi-channel pipette. Incubate for 15 min at +37 °C (±2 °C) in the dark. Without removing the content of the wells, add 100 µL of the stop solution to each well using a multi-channel pipette in

Fig. 2 Comparison of detection between nhPG₈₀ and rhPG₈₀. Seven calibrators with different levels of rhPG₈₀ (circle) and nhPG₈₀ (square) from 0 to 25 pM were measured to generate calibration curves. The graph represents the mean of 3 independent experiments with standard deviations (SD). The linearity of the measurement is given by plotting the measured OD values at 450 nm against the analyte concentrations of the samples. The correlation of standard curve with linear regression model is expressed by the coefficient of determination R^2 . Linear regression $y = ax + b$ with "a" the slope and "b" the intercept is indicated for each calibration curve.

order to stop the reaction. Read and record the Optical Density (OD) at 450 nm. The OD can be corrected for TMB (3,3',5,5'-Tetramethylbenzidine) using a second reading at 620 nm.

Data analysis

Limits of the acceptance criteria were fixed according to EMEA/CHMP/EWP/192217/2009. The test is accepted when the acceptance criteria for the CAL and CTL have been met as described in Table 1. When at least one criterion is "Rejected", the test should be performed again. CAL and CTL concentrations were established as described in the section *Recombinant and native hPG₈₀*.

Standard curve calculation

A standard curve is generated for each set of specimens assayed. The mean OD values obtained from each CAL is calculated. The 6 CAL points are reported on a graph, where $\ll y \gg$ corresponds to the mean OD and $\ll x \gg$ corresponds to hPG₈₀ concentrations in pmol L⁻¹. CAL 0 is used as the anchor point. The linear regression $y = ax + b$ is calculated, where "a" is the slope and "b" is the intercept.

Calibrator acceptance criteria

CAL concentrations are calculated using the linear regression: $C = (\text{mean OD}_{\text{CAL}} - b)/a$.

A CAL is acceptable if the calculated value falls within or equal to the range indicated in the Table 1.

Negative control acceptance criteria

The assay negative control CAL 0 is acceptable if the mean OD (450 nm) falls within or equal to the range 0.12–0.19. This range was obtained by testing multiples hPG₈₀-negative human EDTA plasma.

Positive control acceptance criteria

CTL concentrations are calculated using the linear regression equation: $C = (\text{mean OD}_{\text{CTL}} - b)/a$.

Table 1 Calibrator and positive control acceptance criteria of DxPG₈₀ test. The acceptable range for each concentration of hPG₈₀ was calculated using the % CV accepted indicated in the table and according to EMEA/CHMP/EWP/192217/2009

	hPG ₈₀ concentration (pmol L ⁻¹)	Acceptable range of hPG ₈₀ concentration (pmol L ⁻¹)	% CV accepted
CAL	5	3.8–6.3	25
	10	8–12	20
	15	12–18	
	20	16–24	
	25	20–30	
CTL	5	3.8–6.3	25
	12.5	10–15	20
	22.5	18–27	

A CTL is acceptable if the calculated value falls within or equal to the range indicated in the Table 1. The plate results are acceptable if all three CTL are accepted.

Calculation of plasma sample hPG₈₀ concentration

Sample hPG₈₀ concentration is calculated using the linear regression equation:

$$C = \frac{\text{mean OD}_{\text{SAMPLE}} - b}{a}$$

Reagents

- Human carboxy-amidated Gastrin (hG17-NH₂), (Sigma, G9020).
- Human glycine-extended Gastrin (hG17-Gly), (Sigma, SCP0150).
- Human C-ter flanking peptide of gastrin (CTFP), (Auspep, CS).
- Human Recombinant Progastrin (rhPG₈₀), (Institut Pasteur, B60).
- Monoclonal anti-hGastrin antibody (Abcam, ab88282).
- Keyhole limpet hemocyanin (KLH), (Sigma, H7007).
- Carcinoembryonic antigen (CEA), (Lee-BioSolution, 151-11).
- Prostate specific antigen (PSA), (Lee-BioSolution, 497-11).
- Cancer antigen 125 (CA-125), (Lee-BioSolution, 151-25).
- Cancer antigen 15-3 (CA-15.3), (Lee-BioSolution, 151-53).
- Triglycerides (TG), (Sigma, 17811-1AMP).
- Cholesterol, (Sigma, C8667).
- Hemoglobin, (Sigma, H7379).
- Conjugated Bilirubin, (Lee-BioSolution, 910-12).
- SN-38 (7-ethyl-10-hydroxycamptothecin), (Tocris, 2684) is an active metabolite of CPT-11 (irinotecan) that inhibits DNA topoisomerase I (IC₅₀ values are 0.74 and 1.9 μM in P388 and Ehrlich cells respectively). Inhibits DNA and RNA synthesis (IC₅₀ values are 0.077 and 1.3 μM respectively) but does not affect protein synthesis.
- 5-FU (5-fluorouracil), (Sigma, F6627) is an agent that affects pyrimidine synthesis by inhibiting thymidylate synthetase, thus depleting intracellular dTTP pools. It is metabolized to ribonucleotides and deoxyribonucleotides, which can be incorporated into RNA and DNA.

Plasma samples

All cancer samples were from Tissue For Research Ltd (Spectrum Health System, Grand Rapids, Michigan, USA). All patients provided consent for research on their blood samples, in line with international regulations and ICH GCP (International Conference on Harmonization-Good Clinical Practice).

Results

(A) Specificity

(1) Peptides. The specificity of DxPG₈₀ test was assessed using the full length nhPG₈₀, various products of maturation of

Fig. 3 Cross-reactivity analysis of DxPG₈₀ test. (A) Direct ELISA with 50 ng of coated hG17-NH₂, hG17-Gly or mock using anti-gastrin (hG17) antibody. The graph represents the mean of 3 independent experiments with standard deviations (SD). (B) Different levels of hG17-NH₂, hG17-Gly, CTFP and rhPG₈₀ from 0 to 160 pM were measured at OD 450 nm to generate calibration curves. The graph represents the mean of 3 independent experiments with standard deviations (SD).

gastrin and rhPG₈₀ as positive control at concentrations ranging from 0 to 160 pM.

The following gastric peptides were tested:

- Human carboxy-amidated Gastrin (hG17-NH₂).
- Human glycine-extended Gastrin (hG17-Gly).
- Human C-ter flanking peptide of gastrin (CTFP).
- Human Recombinant Progastrin (rhPG₈₀).

The experiments were conducted using one lot of the DxPG₈₀ test. The concentration range of each analyte was measured on two different DxPG₈₀ test plates. Every point was measured on 4 replicates/plate. To validate hG17-NH₂ and hG17-Gly peptides, we performed a direct ELISA with an antibody that recognize all hG17. As shown Fig. 3A, both peptides are recognized by the anti-hG17 antibody.

The rhPG₈₀ is binding specifically to DxPG₈₀ test, whereas no binding was observed for hG17-NH₂, hG17-Gly, and CTFP (Fig. 3B). Based on these results, the experiment was considered valid and specificity was good.

(2) **Cross-reactants.** The potential cross-reactants described in Table 2 were tested.

hG17-NH₂, hG17-Gly and KLH cross-reactivity were assessed using a concentration 4 times higher than the concentration used during the non-binding test during antibodies production.

CEA (carcinoembryonic antigen), PSA (prostate specific antigen), CA-125 (cancer antigen 125), and CA15-3 (cancer antigen 15-3) are cancer antigens that are used for the screening or/and follow-up of different cancers.²³ Each marker was tested

at a concentration considered positive for the diagnosis of cancer. Each potential cross-reactant was prepared using a specific dilution buffer (vehicle).

Fixed concentrations of each potential cross-reactant and of its vehicle (as a control) were tested using the CAL panel. Each condition was tested in triplicates. The percentage of recovery was calculated for every potential cross-reactant by using as a control the vehicle used for the preparation of its stock solution.

There is no cross-reactivity when variation in the percentage of recovery is equal or does not exceed 20%, and there is no change in the interpretation of the result.

Based on the acceptance criteria, none of the substances tested are to be considered as cross-reactants (Table 2).

(3) **Interference.** Interfering factors are defined as substances that alter the measured concentration of the analyte in the sample. The potential interfering factors described in Table 3 were tested. The concentrations used in the study were extracted from NCCLS EP 7-A2 vol. 25 no. 27 (Clinical Laboratory Standard Institute) except for the 5-FU and SN-38 where the concentrations used were 60× higher than the concentrations used to treat cancer cell lines in culture. Each potential interfering factor was prepared using a specific dilution buffer (vehicle).

Fixed concentrations of each potential interfering factor and of its vehicle (as a control) were tested using the CAL panel. Each condition was tested in triplicates.

Table 2 % of recovery of Potential cross-reactants tested with DxPG₈₀ test % of recovery was calculated using the ratio (OD hPG₈₀ (+vehicle)/OD hPG₈₀ (+potential cross-reactant)) × 100

hPG80 (pmol L ⁻¹)	% of recovery						
	CEA 20 μg mL ⁻¹	PSA 10 mg mL ⁻¹	KLH 2 μg mL ⁻¹	CA-125 2000 U mL ⁻¹	CA15-3 100 U mL ⁻¹	hG17-NH ₂ 2 μg mL ⁻¹	hG17-Gly 2 μg mL ⁻¹
50	100	103	103	106	103	104	106
12.5	98	100	102	104	104	102	105
3.13	94	95	108	101	103	98	101
0.78	101	99	120	102	102	101	102
0	103	100	112	101	102	90	101

Table 3 % of recovery of potential interfering factors tested with DxPG₈₀ test % of recovery was calculated using the ratio (OD hPG₈₀ (+vehicle)/OD hPG₈₀ (+potential interfering factor)) × 100

% of recovery											
hPG ₈₀ (pmol L ⁻¹)	Vehicle			Endogenous			Conjugated bilirubin 0.5 µg mL ⁻¹	Exogenous chemotherapy		Exogenous anti-coagulant	
	DPBS1X	DMSO	CHCL3	TG 0.05 mg mL ⁻¹	Cholesterol 25 µg mL ⁻¹	Hemoglobin 2 mg mL ⁻¹		SN-38 60 µM	5-FU 3 mM	K2-EDTA 1.8 mg mL ⁻¹	Sodium heparin 17 IU mL ⁻¹
50	105	96	95	91	95	111	96	105	96	119	118
12.5	106	97	96	95	96	111	94	104	99	116	117
3.13	105	97	98	104	98	108	98	102	104	114	120
0.78	104	101	98	98	98	103	96	94	112	104	117
0	104	108	100	111	100	101	101	105	100	112	113

The percentage of recovery was calculated for every potential interfering factor using as a control the vehicle used for the preparation of its stock solution.

There is no interference when variation in the percentage of recovery is equal or does not exceed 20%, and there is no change in the interpretation of the result.

Based on the acceptance criteria, none of the eight substances tested showed interference according to our acceptance criteria (Table 3).

(B) Measuring range of assay

(1) Linearity of the measurement. The experiments were conducted using one lot of the DxPG₈₀ test. The linearity of the test was measured using nhPG₈₀ that was produced by the HCT116-PG cell line and diluted into hPG₈₀-negative human EDTA plasma. The coefficient of linearity (R^2) was calculated by plotting nhPG₈₀ concentrations, ranging from 0 to 50 pM, against their measured OD values at 450 nm in the DxPG₈₀ test.

As shown in Fig. 4A, DxPG₈₀ test is linear between nhPG₈₀ concentrations of 0 to 50 pM in human EDTA plasma.

(2) Hook effect. The hook effect is characterized by an unexpected decrease in the activity of the test when reaching

a high concentration of analyte. There is an hook effect when the activity (OD) is 20% lower than the expected nominal activity.

Hook effect was tested using nhPG₈₀ that was produced by the HCT116-PG cell line and diluted in hPG₈₀-negative human EDTA plasma. The concentrations of the nhPG₈₀ were ranging from 0 to 250 pM. The experiments were conducted using one lot of DxPG₈₀ test.

As shown in Fig. 4B, when testing DxPG₈₀ test with concentrations of nhPG₈₀ ranging from 0 to 250 pM, the signal begins to reach a plateau at a concentration above 60 pM.

Based on the data available we can conclude that no hook effect was observed with DxPG₈₀ test when measuring nhPG₈₀ ranging from 0 to 50 pM.

(C) Accuracy of measurement

(1) Trueness of measurement. The trueness of measurement of DxPG₈₀ test was tested with a standard reference of nhPG₈₀. In addition, rhPG₈₀ at known concentrations was also used to show binding to DxPG₈₀ test.

The experiments were conducted using two lots of kit.

- Titration of CTLs with nhPG₈₀ as calibrators.

Fig. 4 . Range assay measurement of DxPG₈₀ test. The linearity of the measurement is given by plotting the measured OD values at 450 nm against the analyte concentrations of the samples. The correlation of standard curve with linear regression model is expressed by the coefficient of determination R^2 . Linear regression $y = ax + b$ with "a" the slope and "b" the intercept is indicated for each calibration curve. (A) Linearity. Ten calibrators with different levels of nhPG₈₀ from 0 to 50 pM were measured to generate calibration curves. The graph represents the mean of 3 independent experiments with standard deviations (SD). (B) Hook effect. Fourteen calibrators with different levels of nhPG₈₀ from 0 to 250 pM were measured to generate calibration curves. The graph represents the mean of 3 independent experiments with standard deviations (SD).

Fig. 5 Trueness of measurement of DxPG₈₀ test. Eight calibrators with different levels of nhPG₈₀ from 0 to 25 pM were measured to generate calibration curves on lot 1 (circle) and lot 2 (square). The linearity of the measurement is given by plotting the measured OD values at 450 nm against the analyte concentrations of the samples. The correlation of standard curve with linear regression model is expressed by the coefficient of determination R^2 . Linear regression $y = ax + b$ with "a" the slope and "b" the intercept is indicated for each calibration curve. The graph represents the mean of 3 independent experiments with standard deviations (SD).

In this first experiment, we titrated three controls (CTL 2.5, 12.5 and 22.5 pmol L⁻¹) and we compared between the two different lots of DxPG₈₀ test, using nhPG₈₀ as calibrators (CAL 0; 1; 2.5; 5; 10; 15; 20 and 25 pmol L⁻¹).

CAL on the 2 lots of DxPG₈₀ test are shown in Fig. 5. As shown in Table 4, when we compare titration of the 3 controls (CTL) between the 2 lots of DxPG₈₀ test, we can notice that nhPG₈₀ relative errors are under 20% as recommended.

- Patients samples titration with nhPG₈₀ as calibrators.

In this second experiment, 21 patient plasmas were titrated and compared between the two lots of DxPG₈₀ test, using nhPG₈₀ as calibrators (CAL 0; 2.5; 1; 5; 10; 15; 20 and 25 pmol L⁻¹).

When we compare titration results obtained for 21 patient samples between 2 lots of DxPG₈₀ test, we can notice that nhPG₈₀ relative errors are under 20% for 19 of the 21 samples (Table 5).

In conclusion nhPG₈₀ can be used as analyte in calibrators for titration of controls or patients' samples.

(2) **Accuracy.** The Accuracy was defined as the mean of measurement calculated using the total mean concentration from all the experiments. The relative error was calculated following the COFRAC guidelines (SH GTA 04 Révision 01) as following:

$$\% = (\text{mean measured [nhPG}_{80}\text{]} - \text{nominal [nhPG}_{80}\text{]}) / \text{nominal [nhPG}_{80}\text{]} \times 100$$

The experiments were conducted using two lots of DxPG₈₀ kit.

Accuracy results for hPG₈₀ titration in controls using DxPG₈₀ test are shown in Table 6.

The Accuracy of the three controls 5, 12.5 and 22.5 pM is considered acceptable as each relative error is $\leq 20\%$ (and 25% for LLoQ, lower limit of quantification). With DxPG₈₀ test, the relative error is below 10% therefore in the acceptable range of the guideline.

(3) **Within-run variability.** The within-run variability is defined as being the mean coefficient of variation (% CV) value of each measured control (CTL) sample. It is considered acceptable when $\leq 20\%$ (and 25% for low nhPG₈₀ concentrations).

The % CV obtained on DxPG₈₀ test are shown in Table 7.

The mean within-run variability ranges from 6.0 to 8.8% and is hence found acceptable.

(4) **Inter-run variability.** The inter-run variability was defined as the % CV calculated using the total mean concentration from all the experiments.

The inter-run variability was evaluated on two lots of DxPG₈₀ kit, using the CAL panel.

For the inter-run variability, a total mean concentration was calculated for each CTL using mean concentrations from all the experiments used for the study. The inter-run variability is considered as acceptable when $\leq 20\%$ (and 25% for low nhPG₈₀ concentrations).

The % CV obtained on DxPG₈₀ test are shown in Table 7.

The mean inter-run variability ranges from 3.1 to 8.9% and is hence found acceptable.

(5) **Inter-operator variability.** The inter-operator variability was defined as the coefficient of variation (% CV) from all those mean nhPG₈₀ concentrations using the mean of calculated concentrations from the four operators experiments.

The inter-operator % CV was calculated over sixteen experiments performed by four different operators, on one lot of DxPG₈₀ test. Each operator measured the nhPG₈₀ from:

- $n = 2$ (duplicates) of all calibrators.

- $n = 16$ (replicates) of three controls (CTL 2.5, CTL 12.5 and CTL 22.5 pmol L⁻¹).

Mean hPG₈₀ concentrations were calculated for each CTL sample per plate. The inter-operator variability is considered

Table 4 Titration of CTLs with nhPG₈₀ as calibrators

	CTL	OD1	OD2	Mean	SD	% CV	Calculated concentration (pmol L ⁻¹)	R-Bias nhPG ₈₀ lot 1 vs. lot 2
lot 1	CTL 2.5	0.24	0.24	0.24	0.00	0.00	2.4	13.0
	CTL 12.5	0.68	0.73	0.70	0.04	5.54	13.1	0.7
	CTL 22.5	1.16	1.15	1.15	0.01	0.74	23.6	6.4
lot 2	CTL 2.5	0.32	0.31	0.32	0.00	1.57	2.7	
	CTL 12.5	0.89	0.86	0.87	0.02	2.52	13.1	
	CTL 22.5	1.35	1.38	1.36	0.01	0.99	25.7

Table 5 Patient's samples titration with rhPG₈₀ or nhPG₈₀ as calibrators. R-bias was calculated using the ratio ((mean concentration hPG₈₀ lot 1 - mean concentration hPG₈₀ lot 2)/mean concentration hPG₈₀ lot 2) × 100

Sample ID	Lot 1								Lot 2								R-Bias nPG lot 0001 vs lot 0002
	OD				nPG (pmol L ⁻¹)				OD				nPG (pmol L ⁻¹)				
	OD1	OD2	% Mean	CV	Conc DO1	Conc DO2	Mean Conc	% CV	OD1	OD2	% Mean	CV	Conc DO1	Conc DO2	Mean Conc	% CV	
Sample 1	0.29	0.307	0.30	4.03	2.74	3.16	2.95	10.04	0.29	0.29	0.29	0.25	2.72	2.70	2.71	0.51	8.99
Sample 2	0.307	0.307	0.31	0.00	3.16	3.16	3.16	0.00	0.31	0.32	0.31	1.13	3.19	3.29	3.24	2.15	2.41
Sample 3	0.137	0.137	0.14	0.00	0.00	0.00	0.00	0.00	0.14	0.14	0.14	0.50	0.00	0.00	0.00	0.00	0.00
Sample 4	0.266	0.269	0.27	0.79	2.38	2.44	2.41	1.68	0.29	0.29	0.28	2.50	2.76	2.56	2.66	5.23	9.40
Sample 5	0.332	0.339	0.34	1.48	3.64	3.77	3.70	2.54	0.33	0.33	0.33	0.64	3.56	3.62	3.59	1.16	3.08
Sample 6	0.275	0.287	0.28	3.02	2.55	2.78	2.67	6.06	0.31	0.32	0.31	1.58	3.17	3.31	3.24	3.01	17.70
Sample 7	0.314	0.332	0.32	3.94	3.29	3.64	3.47	6.99	0.37	0.37	0.37	0.96	4.27	4.37	4.32	1.61	19.79
Sample 8	0.337	0.318	0.33	4.10	3.73	3.37	3.55	7.20	0.33	0.35	0.34	4.63	3.48	3.92	3.70	8.27	4.05
Sample 9	0.391	0.399	0.40	1.43	4.76	4.91	4.84	2.23	0.40	0.41	0.40	3.15	4.86	5.21	5.03	4.97	4.01
Sample 10	0.315	0.328	0.32	2.86	3.31	3.56	3.44	5.09	0.37	0.38	0.37	2.47	4.29	4.55	4.42	4.09	22.22
Sample 11	0.354	0.348	0.35	1.21	4.76	3.94	4.35	2.02	0.38	0.38	0.38	0.37	4.51	4.55	4.53	0.61	11.67
Sample 12	0.71	0.692	0.70	1.82	10.83	10.49	10.66	2.27	0.73	0.71	0.72	2.35	11.51	11.04	11.27	2.96	5.40
Sample 13	0.495	0.486	0.49	1.30	6.74	6.57	6.65	1.82	0.55	0.53	0.54	2.73	7.99	7.57	7.78	3.75	14.48
Sample 14	0.524	0.543	0.53	2.52	7.29	7.65	7.47	3.42	0.57	0.60	0.59	3.14	8.34	8.85	8.60	4.21	13.07
Sample 15	0.481	0.465	0.47	2.39	6.47	6.17	6.32	3.41	0.48	0.50	0.49	2.92	6.43	6.83	6.63	4.19	4.68
Sample 16	0.304	0.299	0.30	1.17	3.1	3.01	3.06	2.20	0.31	0.32	0.32	1.56	3.25	3.37	3.32	2.93	7.88
Sample 17	0.651	0.674	0.66	2.45	9.71	10.15	9.93	3.12	0.69	0.70	0.70	1.42	10.66	10.94	10.80	1.80	8.06
Sample 18	0.339	0.356	0.35	3.46	3.77	4.09	3.93	5.82	0.49	0.46	0.47	4.48	6.71	6.12	6.41	6.50	38.70
Sample 19	0.605	0.599	0.60	0.70	8.83	8.72	8.78	0.92	0.66	0.66	0.66	0.00	10.15	10.15	10.15	0.00	13.53
Sample 20	1.163	1.202	1.18	2.33	19.46	20.20	19.83	2.65	1.22	1.28	1.25	3.17	21.08	22.18	21.63	3.60	8.34
Sample 21	0.464	0.445	0.45	2.96	6.15	5.79	5.97	4.29	0.52	0.52	0.52	0.55	7.22	7.30	7.26	0.77	17.78

Table 6 Accuracy of DxPG₈₀ test % relative error was calculated using the ratio ((mean concentration hPG₈₀ - nominal concentration hPG₈₀)/nominal concentration hPG₈₀) × 100

CTL (pmol L ⁻¹)	hPG ₈₀ Measured Mean (pmol L ⁻¹)	% relative error
5	4.5	-9.5
12.5	11.3	-9.3
22.5	22.2	-1.3

acceptable when ≤20% (and 25% for low nhPG₈₀ concentration).

The % CV obtained on DxPG₈₀ test are shown in Table 7.

The mean inter-operator variability ranges from 4.0 to 5.4% and is hence found acceptable.

(6) Analytical sensitivity. The limit of detection (LoD) and lower limit of quantification (LLoQ) were calculated based on the standard deviation (SD) of the measured concentrations of *n* = 74 blanks:

$$\bullet \text{ LoD} = 3 \times \text{SD.}$$

$$\bullet \text{ LLoQ} = 10 \times \text{SD.}$$

The experiments were conducted using three lots of DxPG₈₀ test.

hPG₈₀ concentrations in pmol L⁻¹ were calculated using the standard curve equation of the nhPG₈₀ calibrators prepared in hPG₈₀-negative human EDTA plasmas.

The analytical sensitivity obtained for the DxPG₈₀ is a LoD of 1 pM and a LLoQ of 3.3 pM. Of note, the calculation is slightly different from the LoD and LLoQ described in^{24,25} to follow the exact guideline EMEA/CHMP/EWP/192217/2009.

(7) Total error. The Total error is defined as the sum of the precision inter-run and of the absolute value of the accuracy. Total error is considered acceptable when ≤30% (and 40% for LLoQ).

The experiments were conducted using three lots of DxPG₈₀ test.

The Total errors obtained for the DxPG₈₀ kit are shown in Table 8.

Table 7 Within-run, inter-run, inter-operator variability of DxPG₈₀ test. The within-run variability was defined as being the mean total % CV value of each CTL calculated from 74 replicates on one run. The inter-run variability, a total mean concentration were calculated for each CTL using mean concentrations from different run used for the study. The inter-run variability was defined as the % CV calculated using the total mean concentration from these experiments

	Control panel	% CV
Within-run precision	CTL 5	8.8
	CTL 12.5	6.0
	CTL 22.5	6.6
Inter-run precision	CTL 5	3.1
	CTL 12.5	8.9
	CTL 22.5	6.9
Inter-operator precision	CTL 2.5	4.0
	CTL 12.5	5.4
	CTL 22.5	4.8

The total error ranges from 8.2 to 18.2% and is hence found acceptable.

Discussion

Before discussing the technology we developed to detect hPG₈₀ in the plasma, it is important to describe how progastrin and its processed peptides were first detected. Several laboratories played prominent roles over the years. They all generated antibodies able to recognize active gastrins, hG17-Gly or unmaturation progastrin. This allowed to switch from chromatography to radio-immunoassays and to ELISA technologies.^{7,26–28} Interestingly, Rehfeld has developed a technology able to quantify total peptide gene expression: the PIA for “Processing Independent Assay”, based on the detection of a sequence of 10 amino acid residues of the precursor protein that is neither modified nor cleaved during cellular processing.^{29,30} An antibody is raised against this sequence, and after trypsin digestion of the sample to be assayed, a radioimmunoassay is performed. However, this technology has some limitations, in particular in terms of analytical variance and labor-intensiveness of the measurements. The variability in clearance of the different precursors and bioactive end-products is also an issue.

However, due to the fact that hPG₈₀ is now recognized as a new cancer target, it was important to develop a test that could detect hPG₈₀ in the blood with 100% specificity. We thus choose to develop a sandwich ELISA, that fulfills this criteria.

Table 8 Total error of DxPG₈₀ test. % precision is the mean of % CV of each CTL of all experiments. % accuracy was calculated using the ratio ((mean concentration hPG₈₀ – nominal concentration hPG₈₀)/nominal concentration hPG₈₀) × 100% total error was calculated using % precision + % of accuracy

CTL panel	% precision	% accuracy	% total error
CTL 5	3.1	9.5	12.6
CTL 12.5	8.9	9.3	18.2
CTL 22.5	6.9	1.3	8.2

The challenge was to generate antibodies that were able to detect hPG₈₀ and not active gastrins, hG17-Gly or the CTFP. The capture antibody is a monoclonal antibody generated against the C-Terminus of hPG₈₀ and the detection antibody is a polyclonal antibody generated against the N-terminus.

This sandwich ELISA test thus ensures a high specific recognition of hPG₈₀. It has a good sensitivity (LoD = 1 pM), with a linearity from 0 to 50 pM. It recognizes recombinant hPG₈₀ and native hPG₈₀, which is very important as hPG₈₀ bears postmaturation modifications that may have induced differences in the recognition of hPG₈₀ present in human.^{31,32} The DxPG₈₀ test fulfills the EMEA/CHMP/EWP/192217/2009 guidelines for method validation. It is CE IVD marked and can therefore be used in the clinical environment by professionals.

The DxPG₈₀ kit has been used to detect hPG₈₀ in a number of cancer patients and in different situations. Before the development of this kit, only colorectal cancer patients were known to accumulate hPG₈₀ in their blood. Now, we know that 83% of the cancer patients have detectable levels of hPG₈₀ in the blood. Indeed You *et al.* showed that hPG₈₀ was present in the 11 tumor types tested.²⁵ hPG₈₀ was detected in the blood of patients ($n = 1546$) at significantly higher concentration than in healthy blood donors ($n = 557$) with a median hPG₈₀ of 4.88 pM versus 1.05 pM ($p < 0.0001$), respectively. The presence of hPG₈₀ in the blood reflects the variations in the tumor: (1) plasma levels correlate with mRNA expression (lung cancer; Spearman $r = 0.8$; $p = 0.0023$); (2) plasma levels significantly decrease upon surgery (peritoneal carcinomatosis decrease from 5.36 pM (before surgery) to 3.00 pM (post surgery), $p < 0.0001$ and upon remission (hepatocellular cancer, decrease from 11.54 pM to 1.99 pM ($p < 0.0001$); (3) the level of hPG₈₀ at diagnostic is a prognostic factor in metastatic renal cell carcinoma (mRCC) patients: Furthermore, mRCC patients with high hPG₈₀ levels (> 4.5 pM) had significantly lower OS (overall survival) compared to patients with low hPG₈₀ levels (< 4.5 pM) (12 versus 31.2 months, respectively; $p = 0.0031$); (4) efficacy of treatments correlates with hPG₈₀ level kinetic variations and recurrence is associated with an increase in hPG₈₀ level (hepatocellular cancer).²⁵ All these data re-inforce the value of hPG₈₀ as a new cancer target and prone to the usefulness of the detection of hPG₈₀ in the blood.

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Author contributions

AP, DJ principle investigators. AP, DJ wrote the manuscript. BV and MC provided edits to the first draft in manuscript writing. AP, DJ, MC, MF, VS, JS, EB, FB, EO, LT and PL analysed the data. MC, MF, VS, JS, EB, FB, EO, LT and PL performed the hPG₈₀ assays. SP and ST provided quality management. MB contributed to the recruitment of patients. All authors have read and agreed to the published version of the manuscript.

Conflicts of interest

DJ is a co-founder of the company and a senior scientific advisor. AP is a co-founder of the company and the Chief Scientific Officer of the company. All other authors: no conflict of interest.

References

- 1 J. F. Rehfeld, *Scand. J. Clin. Lab. Invest.*, 2008, **68**, 353–361.
- 2 A. Varro, S. Voronina and G. J. Dockray, *Journal*, 1995, **95**, 1642–1649.
- 3 L. R. Johnson, *Scand. J. Clin. Lab. Invest., Suppl.*, 1982, **74**, 89–92.
- 4 J. F. Rehfeld and A. H. Johnsen, *Eur. J. Biochem.*, 1994, **223**, 765–773.
- 5 K. A. Smith, O. Patel, S. Lachal, I. Jennings, B. Kemp, J. Burgess, G. S. Baldwin and A. Shulkes, *Journal*, 2006, **131**, 1463–1474.
- 6 L. Bardram, *Gastroenterology*, 1990, **98**, 1420–1426.
- 7 G. D. Ciccotosto, A. McLeish, K. J. Hardy and A. Shulkes, *Gastroenterology*, 1995, **109**, 1142–1153.
- 8 W. W. Van Solinge, F. C. Nielsen, L. Friis-Hansen, U. G. Falkmer and J. F. Rehfeld, *Gastroenterology*, 1993, **104**, 1099–1107.
- 9 F. Hollande, D. J. Lee, A. Choquet, S. Roche and G. S. Baldwin, *Journal*, 2003, **116**, 1187–1197.
- 10 C. Seva, C. J. Dickinson and T. Yamada, *Science*, 1994, **265**, 410–412.
- 11 P. Singh, A. Owlia, A. Varro, B. Dai, S. Rajaraman and T. Wood, *Cancer Res.*, 1996, **56**, 4111–4115.
- 12 J. Pannequin, N. Delaunay, M. Buchert, F. Surrel, J. F. Bourgaux, J. Ryan, S. Boireau, J. Coelho, A. Pèlerin, P. Singh, A. Shulkes, M. Yim, G. S. Baldwin, C. Pignodel, G. Lambeau, P. Jay, D. Joubert and F. Hollande, *Journal*, 2007, **133**, 1554–1568.
- 13 H. Wu, A. Owlia and P. Singh, *Am. J. Physiol.: Gastrointest. Liver Physiol.*, 2003, **285**, G1097–G1110.
- 14 J. Giraud, L. M. Failla, J. M. Pascussi, E. L. Lagerqvist, J. Ollier, P. Finetti, F. Bertucci, C. Ya, I. Gasmi, J. F. Bourgaux, M. Prudhomme, T. Mazard, I. Ait-Arsa, L. Houhou, D. Birnbaum, A. Pelegrin, C. Vincent, J. G. Ryall, D. Joubert, J. Pannequin and F. Hollande, *Cancer Res.*, 2016, **76**, 3618–3628.
- 15 A. Prieur, M. Cappellini, G. Habif, M. P. Lefranc, T. Mazard, E. Morency, J. M. Pascussi, M. Flaceliere, N. Cahuzac, B. Vire, B. Dubuc, A. Durochat, P. Liaud, J. Ollier, C. Pfeiffer, S. Poupeau, V. Saywell, C. Planque, E. Assenat, F. Bibeau, J. F. Bourgaux, P. Pujol, A. Sezeur, M. Ychou and D. Joubert, *Clin. Cancer Res.*, 2017, **23**, 5267–5280.
- 16 S. Najib, A. Kowalski-Chauvel, C. Do, S. Roche, E. Cohen-Jonathan-Moyal and C. Seva, *Oncogene*, 2015, **34**, 3120–3130.
- 17 S. Khajeh, M. R. Tohidkia, A. Aghanejad, T. Mehdipour, F. Fathi and Y. Omid, *Artif. Cells, Nanomed., Biotechnol.*, 2018, **46**, 1082–1090.
- 18 R. K. Siddheshwar, J. C. Gray and S. B. Kelly, *Gut*, 2001, **48**, 47–52.
- 19 T. J. Koh and D. Chen, *Regul. Pept.*, 2000, **93**, 37–44.
- 20 P. D. Ottewill, A. Varro, G. J. Dockray, C. M. Kirton, A. J. Watson, T. C. Wang, R. Dimaline and D. M. Pritchard, *Am. J. Physiol.: Gastrointest. Liver Physiol.*, 2005, **288**, G541–G549.
- 21 K. McQueen, S. Kovac, P. K. Ho, K. Rorison, J. Pannequin, G. Neumann, A. Shulkes and G. S. Baldwin, *J. Protein Chem.*, 2002, **21**, 465–471.
- 22 J. R. Bundgaard, J. Vuust and J. F. Rehfeld, *J. Biol. Chem.*, 1997, **272**, 21700–21705.
- 23 S. Holdenrieder, L. Pagliaro, D. Morgenstern and F. Dayyani, *BioMed Res. Int.*, 2016, **2016**, 9795269.
- 24 M. Kohli, W. Tan, B. Vire, P. Liaud, M. Blairvacq, F. Berthier, D. Rouison, G. Garnier, L. Payen, T. Cousin, D. Joubert and A. Prieur, *Cancers*, 2021, **13**.
- 25 B. You, F. Mercier, E. Assenat, C. Langlois-Jacques, O. Glehen, J. Soule, L. Payen, V. Kepenekian, M. Dupuy, F. Belouin, E. Morency, V. Saywell, M. Flaceliere, P. Elies, P. Liaud, T. Mazard, D. Maucourt-Boulch, W. Tan, B. Vire, L. Villeneuve, M. Ychou, M. Kohli, D. Joubert and A. Prieur, *EBioMedicine*, 2019, **51**, 102574.
- 26 L. Bardram, L. Hilsted and R. JF, *Journal*, 1990, **1**–5.
- 27 M. L. Kochman, J. DelValle, C. J. Dickinson and C. R. Boland, *Biochem. Biophys. Res. Commun.*, 1992, **189**, 1165–1169.
- 28 J. Nemeth, B. Taylor, S. Pauwels, A. Varro and G. J. Dockray, *Gut*, 1993, **34**, 90–95.
- 29 J. F. Rehfeld and J. P. Goetze, *Peptides*, 2021, **135**, 170427.
- 30 N. R. Jorgensen, J. F. Rehfeld, L. Bardram and L. Hilsted, *Scand. J. Gastroenterol.*, 1998, **33**, 379–385.
- 31 A. Varro, H. Desmond, S. Pauwels, H. Gregory, J. Young and G. J. Dockray, *Biochem. J.*, 1988, **256**, 951–957.
- 32 S. J. Brand, J. Klarlund, T. W. Schwartz and J. F. Rehfeld, *J. Biol. Chem.*, 1984, **259**, 13246–13252.
- 33 J. Copps, R. F. Murphy and S. Lovas, *Protein Pept. Lett.*, 2009, **16**, 1504–1518.