Blood measurement of neuroendocrine gene transcripts defines the effectiveness of operative resection and ablation strategies

Irvin M. Modlin, MD, PhD, DSc,[a] Andrea Frilling, MD, PhD,[b] Ronald R. Salem, MD,[c] Daniele Alaimo, MS,[d] Panagiotis Drymousis, MD, PhD,[b] Harpreet S. Wasan, MD, FRCP,[b] Stephen Callahan, MS,[d] Omar Faiz, MBBS, FRCS, MS,[e] Lei Weng, MBBS,[b] Nancy Teixeira, MSN,[d] Lisa Bodei, MD, PhD,[d] Ignat Drozdov, PhD,[d] *and* **Mark Kidd, PhD,[d]** *New Haven and Branford, CT, and London, UK*

Background. *Surgery is the only curative treatment for gastroenteropancreatic neuroendocrine tumors (GEP-NETs), but the prediction of residual disease/recurrence is limited in the absence of optimal biomarkers. We examined whether a blood-based multianalyte neuroendocrine gene transcript assay (NETest) would define tumor cytoreduction and therapeutic efficacy.*

Methods. *The NETest is a polymerase chain reaction–based analysis of 51 genes. Disease activity is scaled 0–100%; minimal <14%, low 14–47%, and high >47%. A total of 35 GEP-NETs in 2 groups were evaluated. I: after surgery (R0,* n *= 15; residual,* n *= 12); II: nonsurgery (*n *= 8: embolization with gel-foam alone [bland:* n *= 3]), transarterial chemoembolization (*n *= 2), and radiofrequency embolization (*n *= 3). Measurement (quantitative real-time-polymerase chain reaction) and chromogranin A (CgA; enzyme-linked immunosorbent assay) were undertaken preoperatively and 1 month after treatment.*

Results. *NETest score was increased in 35 (100%) preoperatively; 14 (40%) had increased CgA ($\chi^2 = 30$,* P *$< 2 \times 10^{-8}$). Resection reduced NETest from 80 ± 5% to 29% ± 5, (*P *< .0001). CgA decrease was insignificant (14.3 ± 1.6U/L to 12.2 ± 1.7U/L). NETest decreases correlated with diminished tumor volume ($R^2 = 0.29$,* P *= .03). Cytoreduction significantly reduced NETest from 82 ± 3% to 41% ± 6,* P *< .0001). CgA was not decreased (21.4 ± 5.5U/L to 18.4 ± 10.1U/L). Four (36%) of 11 R0s with increased NETest at 1 month developed positive imaging (sensitivity 100%, specificity 20%). One hundred percent (ablated group) were transcript- and image-positive.*

Conclusion. *Blood NET transcripts delineate surgical resection/cytoreduction and facilitate identification of residual disease. (Surgery 2016;159:336-47.)*

From Surgery, Emeritus Prof,[a] and Department of Surgery,[c] Yale University School of Medicine, New Haven, CT; Department of Surgery and Cancer,[b] Imperial College London, London, UK; Wren Laboratories,[d] Branford, CT; and Department of Colorectal Surgery,[e] St Mark's Hospital, London, UK

Supported by Clifton Life Sciences and a grant from the Dr Heinz-Horst Deichmann Foundation (project no. P31294), the National Institute for Health Research (NIHR) Biomedical Research Centre (Imperial College Healthcare NHS Trust), and Imperial College London.

Presented at the Annual Meeting of the American Association of Endocrine Surgeons, May 17–19, 2015, Nashville, TN.

Accepted for publication June 4, 2015.

Reprint requests: Irvin M. Modlin, MD, PhD, DSc, Yale University School of Medicine, 333 Cedar Street, New Haven, CT 06510. E-mail: imodlin@optonline.net.

0039-6060/$ - see front matter

http://dx.doi.org/10.1016/j.surg.2015.06.056

Neuroendocrine tumors (NETs) are diverse tumors considered previously as "carcinoids."[1] The lesions are ubiquitous in location but are especially common within the gastrointestinal tract.[2] There is a general consensus that operative resection is a critical element of therapy and remains the only curative treatment option.[3] Curative surgery, however, often is not feasible because most gastroenteropancreatic (GEP)-NETs exhibit metastatic disease at diagnosis. Additional therapeutic strategies include pharmacotherapeutics, eg, somatostatin analogs, which diminish symptoms that may extend progression-free survival in low-grade disease.[4] Similarly, a variety of targeted agents, including everolimus, sunitinib, and temozolamide, have been used

with variable efficacy. Imaging currently is the mainstay of therapeutic assessment. The more recent introduction of positron emission tomography/computed tomography (PET/CT) with somatostatin analogues, DOTATOC, DOTATATE, and DOTANOC, has improved detection rates, with pooled sensitivities of 93–96% and specificities of 85–100% (area under the receiver operating characteristic curve: 0.96–0.98).[5] ^{68}Ga-PET is able to modify the overall therapeutic strategy in 55–60% of cases.[6] In particular, operative management is modified in 20% of cases; however, standardization and metrics are still considered to have not attained optimal parameters.

The determination of a patient's survival after surgery reflects the primary site, the tumor grade, disease stage, and location of metastatic disease, as well as the magnitude of postresection tumor burden. Despite apparent complete resection of hepatic metastases, early detection of covert residual disease represents a major clinical problem and is a key determinant in defining the timing of further therapeutic intervention and determination of long-term prognosis. For both operative interventions as well as ablation approaches, strategies for early detection of disease recurrence remain relatively limited in their sensitivity and specificity.[1]

Operative resection is associated with improved and prolonged disease control. Retrospective studies, despite limitations, demonstrate enhanced outcomes compared with individuals who did not undergo surgical resection.[7] Although tumors with metastatic spread have overall poorer outcomes, surgery often is undertaken to obviate local mechanical events such as bleeding, bowel obstruction, or vascular encasement. R0 and R1 resections are the norm, and outcomes are predicated on a number of factors, including residual tumor burden.[8]

NET recurrence usually is identified by a combination of biochemical as well as radiologic and nuclear medicine techniques. Imagery strategies used are both anatomical and functional; however, all have significant limitations in their capacity of tumor resolution: 2 millimeters for computed tomography/magnetic resonance imaging, 4–6 mm for positron emission tomography (PET including ^{68}Ga-PET) and ~10 mm for Somatostatin Receptor Scintigraphy.[9] Similarly, current biomarkers (eg, chromogranin A [CgA], pancreastatin, neurokinin A) used for the detection of NET have substantial limitations in terms of sensitivity, specificity, and reproducibility.[10]

We have reported previously the utility of a PCR-based tool to quantitate (score) the circulating GEP-NET molecular signature with high sensitivity and specificity (>95%).[11,12] This multianalyte-derived signature encompassing 51 genes identifies all GEP-NETs and significantly out-performs monoanalyte-based assays for the detection of NET.[11,12] Gene expression is captured in a 0–8 score derived from 4 different prediction algorithms that can be mathematically scaled to disease activity (0–100%) by the use of expression of transcripts that capture the hallmarks of neoplasia.[13] Disease activity scores of 0–14% are associated with minimal activity, 14–47% low activity and >47%, high activity.[14] Activity levels correlate with clinical status, eg, stable or progressive disease.[14]

Currently, alteration in tumor size generally is regarded as indicative of disease progression or regression. The clinical difficulty, however, is the absence of sensitive or specific enough imaging to define this change. An alternative strategy would therefore entail the development of blood-based measurements of tumor function. We hypothesized that alteration in the NET circulating blood signature would reflect operative resection or ablation of liver metastases. Our aims were to evaluate the effect of surgery and ablation/chemoembolization on the NET signature and specifically examine (1) whether tumor resection decreased the blood NET signature, (2) whether this decrease reflected the extent of resection, (3) whether R0 resection reduced circulating NET transcript levels to normal, and (4) whether increased blood NET transcript levels after R0 resection predicted clinical recurrence.

PATIENTS AND METHODS

Patients with GEP-NET (n = 35) [M/F 14:21; median age: 58 years, range: 33–80; stomach n = 1, pancreas n = 8, gall bladder: n = 1, small intestine: n = 21, appendix n = 2, rectum n = 2; G1 = 27, G2 = 7, G3 = 1] were included (Table I). Surgery was performed in 27 (1) to remove primary tumor, including loco-regional lymph nodes (n = 21); (2) for debulking (n = 4); and (3) for suspicion of NET (small intestine: n = 1; appendix: n = 1). Tumor volume pre- and postsurgery was assessed with imaging, and operative measurement and pathological data were used to quantitate tumor volumes. Nonoperative strategies were undertaken in 8 subjects and included embolization with gel-foam alone (bland: n = 3), trans-arterial chemoembolization (n = 2), and radiofrequency ablation (n = 3) for hepatic metastases. Ablation/embolization was applied to liver lesions.

Table I. Gene panel included in the NETest

Biomarker or housekeeping gene		
Symbol	*Name*	*NCBI chromosome location*
AKAP8L	A kinase (PRKA) anchor protein 8-like	Chr.19: 15490859 – 15529833
ALG9	asparagine-linked glycosylation 9, alpha-1,2-mannosyltransferase homolog	Chr. 11 - 111652919 - 111742305
APLP2	amyloid beta (A4) precursor-like protein 2	Chr. 11 - 129939716 - 130014706
ARAF1	v-raf murine sarcoma 3611 viral oncogene homolog	Chr. X - 47420578 - 47431320
ATP6V1H	ATPase, H+ transporting, lysosomal 50/57kDa, V1, Subunit H	Chr.8: 54628115 – 54755850
BNIP3L	BCL2/adenovirus E1B 19kDa interacting protein 3-like	Chr.8: 26240523 – 26270644
BRAF	v-raf murine sarcoma viral oncogene homolog B1	Chr. 7 - 140433812 - 140624564
C21ORF7	chromosome 21 open reading frame 7	Chr.21: 30452873 – 30548204
CD59	CD59 molecule, complement regulatory protein	Chr. 11 - 33724556 - 33758025
COMMD9	COMM domain containing 9	Chr.11: 36293842 – 36310999
CTGF	connective tissue growth factor	Chr. 6 - 132269316 - 132272518
ENPP4	ectonucleotide pyrophosphatase/phosphodiesterase 4	Chr.6: 46097701 – 46114436
FAM131A	family with sequence similarity 131, member A, transcript variant 2	Chr.3: 184053717 – 184064063
FLJ10357	Rho guanine nucleotide exchange factor (GEF) 40 (ARHGEF40)	Chr.14: 21538527 – 21558036
FZD7	frizzled homolog 7 (Drosophila)	Chr. 2 - 202899310 - 202903160
GLT8D1	glycosyltransferase 8 domain containing 1, transcript variant 3	Chr.3: 52728504 – 52740048
HDAC9	histone deacetylase 9, transcript variant 6	Chr.7: 18535369 – 19036993
HSF2	heat shock transcription factor 2, transcript variant 1	Chr.6: 122720696 – 122754264
Ki-67	antigen identified by monoclonal antibody Ki-67	Chr. 10 - 129894923 - 129924655
KRAS	v-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog	Chr. 12 - 25358180 - 25403854
LEO1	Leo1, Paf1/RNA polymerase II complex component homolog (S. cerevisiae)	Chr.15: 52230222 – 52263958
MORF4L2	mortality factor 4 like 2, transcript variant 1	Chr.X: 102930426 – 102943086
NAP1L1	nucleosome assembly protein 1-like 1	Chr. 12 - 76438672 - 76478738
NOL3	nucleolar protein 3 (apoptosis repressor with CARD domain), transcript variant 3	Chr.16: 67204405 – 67209643
NUDT3	nudix (nucleoside diphosphate linked moiety X)-type motif 3	Chr.6: 34255997 – 34360441
OAZ2	ornithine decarboxylase antizyme 2	Chr.15: 64979773 – 64995462
PANK2	pantothenate kinase 2	Chr.20: 3869486 – 3904502
PHF21A	PHD finger protein 21A, transcript variant 1	Chr.11: 45950870 – 46142985
PKD1	polycystic kidney disease 1 (autosomal dominant), transcript variant 2	Chr.16: 2138711 – 2185899
PLD3	phospholipase D family, member 3, transcript variant 1	Chr.19: 40854332 – 40884390
PQB1	polyglutamine binding protein 1, transcript variant 2	Chr.X: 48755195 – 48760422
PNMA2	paraneoplastic antigen MA2	Chr. 8 - 26362196 - 26371483
RAF1	v-raf-1 murine leukemia viral oncogene homolog 1	Chr. 3 - 12625100 - 12705700
RNF41	ring finger protein 41, transcript variant 4	Chr.12: 56598285 – 56615735
RSF1	remodeling and spacing factor 1	Chr.11: 77377274 – 77531880
RTN2	reticulon 2, transcript variant 1	Chr.19: 45988550 – 46000313
SMARCD3	SWI/SNF related, matrix associated, actin dependent regulator of chromatin, subfamily d, member 3, transcript variant 3	Chr.7: 150936059 – 150974231
SPATA7	spermatogenesis associated 7, transcript variant 2	Chr.14: 88851988 – 88904804
SST1	somatostatin receptor 1	Chr.14: 38677204 – 38682268
SST3	somatostatin receptor 3	Chr.22: 37602245 – 37608353
SST4	somatostatin receptor 4	Chr.20: 23016057 – 23017314
SST5	somatostatin receptor 5, transcript variant 1	Chr.16: 1122756 – 1131454
TECPR2	tectonin beta-propeller repeat containing 2, transcript variant 2	Chr.14: 102829300 – 102968818
TPH1	tryptophan hydroxylase 1	Chr. 11 - 18042538 - 18062309
TRMT112	tRNA methyltransferase 11–2 homolog (S. cerevisiae)	Chr.11: 64084163 – 64085033
VMAT1	solute carrier family 18 (vesicular monoamine), member 1	Chr. 8 - 20002366 - 20040717
VMAT2	solute carrier family 18 (vesicular monoamine), member 2	Chr. 10 - 119000716 - 119037095

(*continued*)

Table I. (*continued*)

Biomarker or housekeeping gene		
Symbol	*Name*	*NCBI chromosome location*
VPS13C	vacuolar protein sorting 13 homolog C (S. cerevisiae), transcript variant 2B	Chr.15: 62144588 – 62352647
WDFY3	WD repeat and FYVE domain containing 3	Chr.4: 85590690 – 85887544
ZFHX3	zinc finger homeobox 3, transcript variant B	Chr.16: 72816784 – 73092534
ZXDC	zinc finger C, transcript variant 2	Chr.3: 126156444 – 126194762
ZZZ3	zinc finger, ZZ-type containing 3	Chr.1: 78030190 – 78148343

Preoperative blood samples were collected (0–24 hours before treatment) and 1 month post-therapy. Blood was analyzed according to standard institutional review board protocols (Yale University: 6/17/2013 [HIPAA compliant] and Imperial College London: REC reference number 07/MRE09/54) in accordance with the World Medical Association Declaration of Helsinki regarding ethical conduct of research involving human subjects.[12] Follow-up (including imaging) was undertaken on all patients at 3 and 6 months. Imaging studies used to evaluate disease status included CT scan, magnetic resonance imaging (MRI), and 1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid-PET (United Kingdom alone). The clinical criteria used to determine recurrence or progression were Response Evaluation Criteria In Solid Tumors (ie, RECIST) 1.1 wherever applicable. In instances in which DOTA was not available (United States), criteria related to clinical assessment and anatomic imaging were used. Blood samples were collected per standard molecular diagnostics protocols for polymerase chain reaction (PCR)-based studies. A second aliquot (2 mL) was spun (300 g 10 min), and the plasma collected for enzyme-linked immunosorbent assay (ELISA).[11,12]

Multianalyte algorithm analysis (MAAA) PCR-based test (NETest). A 2-step manual technique protocol (RNA isolation with cDNA production and qPCR for 51 target genes; Table I) was used as described.[11,12] The overall efficiency of the PCR probes was 1.94 ± 0.11.[15] The interassay variability for clinical samples was 0.5–1.2%, and the intra-assay reproducibility was 0.4–1.0%.[15] A NET Disease Activity Risk Score (0–100%) was derived from the PCR data. This was derived from previously described training set (n = 130 [67 controls, 63 NETs]),[12] as well as samples from an independent set (n = 159: clinically stable disease n = 111; progressive disease n = 48) and includes biologically relevant gene cluster information that accurately predicts neuroendocrine tumor activity.[14] The cut-off for controls is 10%.[14] The cut-off for stable disease is 47%. Levels >47% are regarded as transcript evidence of "progressive" disease. A separate historical surgical group (n = 12; appendix: n = 5, pancreas: n = 4, ileum: n = 3; all T1N0M0, Ki67<1%) underwent curative (R0) operative resection and are disease free at 5 years. In this group 14% was identified as the cut-off value. The NETest is therefore currently scaled as minimal activity risk <14%, low activity risk 14–47%, and high activity risk >47%.

CgA ELISA. CgA was measured in duplicate by use of the DAKO ELISA kit (K0025, DAKO North America, Inc, Carpinteria, CA).[11,12] The coefficient of variation for the test is <10%.

Statistical analyses. Sensitivity comparisons using, respectively, χ-square and nonparametric measurements (Wilcoxon matched paired signed rank test and Mann-Whitney U test [unpaired] where necessary) were made between the MAAA-PCR test and the single-analyte plasma ELISA using Prism 6.0 for Windows (GraphPad Software, La Jolla, CA; www.graphpad.com). Additional analyses (including metrics [sensitivity, specificity], 2-tailed χ^2 tests) were undertaken in group I (R0 resections with no postoperative evidence of disease), group II (any R resection in which residual disease was identified), and group III.

RESULTS

Patient details are included in Table II. Fifteen of the 25 subjects were included in group I (R0: no postoperative evidence of disease), 12 in group II (any resection with residual disease), and 8 in group III (ablation/embolization). Overall, 48% of surgery patients were imaged by CT/MRI (United States); 52% were evaluated by ^{68}Ga-PET (UK). All ablation patients were imaged by CT/MRI. Volumetric measures pre- and postsurgery are included in Table III.

MAAA-PCR NETest. For the entire operative cohort (n = 27), the presurgery NETest scores were increased (79.8 ± 5.1%). Twenty-three (85%) of

Table II. Clinical and treatment (operative or ablation) characteristics

Code	*Sex*	*Age, y*	*Primary site*	*CgA,* U/L*	*NETest score†*	*Surgery type*	*Grade*	*Group*
1	F	33	A	**19.2**	**93.4**	Primary and lymph node resection	G1	Group I - R0‡
2	M	42	A	**19.6**	**93.4**	Surgery for diverticulitis, incidental finding of appendiceal NET	G1	Group I - R0§ ^{68}Ga DOTA-PET (6 mo)
3	M	42	P	9.5	**46.7**	Primary resection	G1	Group I - R0
4	F	59	P	12.0	33.4	Primary and lymph node resection	G1	Group I - R0§ ^{68}Ga DOTA-PET (6 mo)
5	F	40	P	10.1	40.0	Primary	G1	Group I - R0†
6	M	62	P	9.8	**46.7**	Primary	G1	Group I - R0
7	M	58	P	13.2	**86.7**	Primary resection	G2	Group I - R0
8	F	63	SI	15.5	**100**	Primary and lymph node resection	G1	Group I - R0
9	F	80	SI	**21.3**	**100**	Primary and lymph node resection	G1	Group I - R0
10	M	68	SI	14.6	**93.4**	Primary and lymph node resection	G1	Group I - R0
11	F	46	SI	13.0	**100**	Primary and lymph node resection	G1	Group I - R0
12	F	68	SI	**21.6**	**80.0**	Primary and lymph node resection	G1	Group I - R0§ ^{68}Ga DOTA-PET (6 mo)
13	M	69	SI	16.0	**93.4**	Primary and lymph node resection	G1	Group I - R0
14	M	58	SI	12.5	**100**	Primary resection for incidental NET	G2	Group I - R0
15	F	55	ST	6.9	**93.4**	Partial gastrectomy and lymph node resection	G3	Group I - R0§ ^{68}Ga DOTA-PET (6 mo)
16	M	37	P	9.5	40.0	Primary resection	G1	Group II - residual disease (lymph node metastases)
17	M	37	P	14.2	**100**	Primary and lymph node resection	G1	Group II - residual disease - MEN1
18	F	74	SI	**25.0**	**86.7**	Primary and lymph node resection	G1	Group II - residual disease (liver metastases)
19	F	64	SI	12.7	**86.7**	Primary and lymph node resection	G1	Group II - residual disease (liver metastases)
20	F	47	SI	**598.3**	**93.4**	Primary and liver resection y	G2	Group II - Residual disease (liver metastases)
21	F	72	SI	**120.0**	26.7	Primary, lymph node and liver resection	G1	Group II - residual disease (liver metastases) [debulked]
22	F	74	SI	14.5	**100**	Primary and lymph node resection	G1	Group II - residual disease (nonresectable liver metastases)
23	M	59	SI	14.2	26.7	Primary and lymph node resection	G1	Group II - Residual disease (liver metastases)
24	F	46	SI	7.9	**93.4**	Primary	G1	Group II - residual disease (lymph nodes)
25	F	69	SI	**21.0**	**100**	Primary, lymph node and liver resection	G2	Group II - residual disease (unresectable liver metastases) [debulked]
26	M	46	SI	**41.6**	**100**	Primary resection and ~90% debulking.	G2	Group II - residual mesenteric disease [debulked]

(*continued*)

Table II. (*continued*)

Code	*Sex*	*Age, y*	*Primary site*	*CgA,* U/L*	*NETest score†*	*Surgery type*	*Grade*	*Group*
27	M	48	R	9.5	**100**	Resection with liver debulking	G2	Group II - residual disease (liver metastases) [debulked]
28	M	52	P	**34.7**	**73.4**	Bland embolization	G1	Group III - embolization
29	F	51	SI	11.3	**79.6**	Bland embolization	G1	Group III - embolization
30	M	48	R	13.6	**92.1**	Bland embolization	G2	Group III -embolization
31	F	63	GB	12.3	**87.0**	RFA	G1	Group III - RFA
32	F	64	SI	**35.0**	**80.0**	RFA	G1	Group III - RFA
33	F	61	SI	**185.6**	**64.8**	RFA	G1	Group III - RFA
34	F	48	SI	**30.2**	**79.6**	TACE	G1	Group III - Chemoembolization
35	F	49	SI	**35.8**	**52.8**	TACE	G1	Group III - chemoembolization

*Upper limit of normal = 19 U/L. Numbers in bold reflect increased levels.
†Upper limit of normal = 10% Upper limit of minimal disease = 14%. Numbers in bold reflect increased levels.
‡R0* = normal, postresection NETest levels.
§R0 = subsequently developed image-positive recurrence.
A, Appendix; *F*, female; *^{68}Ga DOTA-PET*, ^{68}Ga-somatostatin receptor-based PET; *GB*, gall bladder; *M*, male; *P*, pancreas; *R*, rectum; *RFA*, radiofrequency embolization; *SI*, small intestine (ileum and jejunum); *ST*, stomach; *TACE*, transarterial chemoembolization.

Table III. Volumetric measurements of tumor in the operative groups.

Operative group	*Number*	*Before resection,* cm^3*	*After resection,* cm*	*Percentage change*
I	15	11.4 ± 5.6	0 ± 0	100%
II	12	34.0 ± 24.6	18.8 ± 25.9	−57 ± 32%

*Mean ± SD

the 27 subjects exhibited a decreased score postsurgery (4 patients were unchanged). For group I, the postoperative NETest scores were 28.9 ± 5.5% (presurgery: 80 ± 6.3%) (Fig 1, *A*) and for group II: it was 47.2 ± 9.9% (presurgery 79.5 ± 8.5%) (Fig 1, *B*). Surgery significantly reduced scores in each of these groups (group I: $P < .0001$; group II: $P < .002$). Postoperative NETest levels were significantly lower in group I (R0 resection) than group II ($P < .05$). For group III, the preablation NETest scores were elevated (76.2 ± 4.4%) and reduced after treatment (40.2 ± 4.1, $P < .0001$) (Fig 1, *C*).

We next compared percentage changes in the NETest score. Assessment of overall decrease in postoperative percentage change was −43.1 ± 5.3% in the combined surgery group. The NETest PCR score was significantly decreased ($P < .05$) in Group I compared with Group II (Fig 2). The percentage decrease also was greater in group I than group II (−64 ± 6% vs −37 ± 11%, $P < .05$). For group III, the percentage change was −47 ± 5%.

No patient had minimal NETest activity (<14%) presurgery or preablation. Postsurgery, however, 2 (7.4%) had a minimal activity scores (<14%) and 5 (19%) had a score of 14%. The 2 with minimal activity (postsurgery) had both undergone R0 resections and had no image evidence (^{68}Ga-PET) of disease. One was an appendiceal NET (patient 1) and the second an insulinoma ("benign") (patient 5). Both had low Ki-67 proliferation (<2%) tumors and no lymph node involvement. Postablation no patient exhibited minimal activity score.

CgA measurements. Levels were normal in 18 (67%) of the 27 subjects before surgery and in 3 (40%) of 8 subjects before ablation. For the complete operative cohort, the presurgery CgA levels were a mean of 40.9 ± 21.8U/L. For group I, this was 14.3±1.2 U/L (Fig 3, *A*) and for group II, this was 74 ± 48.5U/L (Fig 3, *B*). For the ablation group (III), this was 44.8 ± 20.5 U/L (Fig 3, *C*). For group I, the postoperative CgA levels were 12.3 ± 1.7U/L and for group II 73.3 ± 54.6U/L. The postoperative levels of the 2 groups were not significantly different ($P = .28$). Surgery was not associated with significantly reduced levels in either of group I ($P = .09$) or group II ($P = .1$) compared with individual presurgery scores (Fig 3). For group III, the postablation levels were 37 ± 17U/L, which was not reduced greatly ($P = .17$).

Overall, 6 (22%) of the 27 subjects exhibited a decrease in CgA levels postsurgery. Of the 9 patients with increased preoperative CgA levels, 6

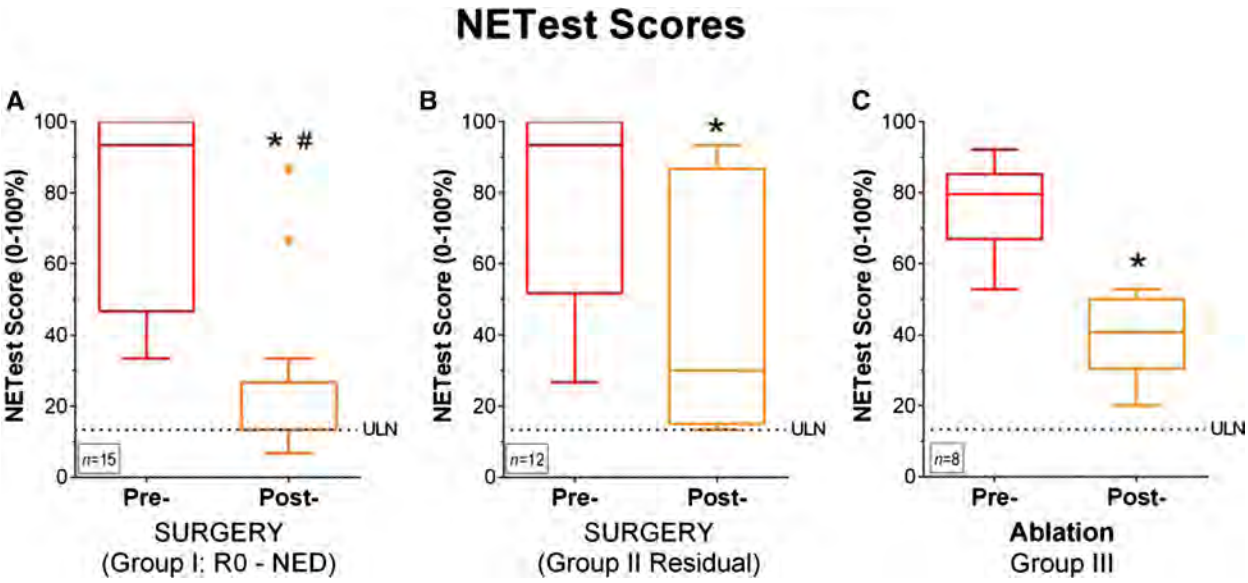

Fig 1. Alterations in the NETest after surgery or ablation. (*A*) Disease activity scores were decreased greatly in Group I (R0: no evidence of disease). The reduction was greater than in group II. (*B*) Levels of disease activity were decreased greatly in group II. (*C*) Ablation decreased NETest scores greatly. *$P < .002$ vs pretreatment (either surgery or radiofrequency embolization), #$P < .05$ vs group II (paired 2-tailed, nonparametric test). *NED*, No evidence of disease (by imaging, postoperatively); *pre*, pretherapy scores; *post*, posttherapy scores; *ULN*, upper limit of normal. Normal score is <10% for the PCR test and is 14% for minimal disease.

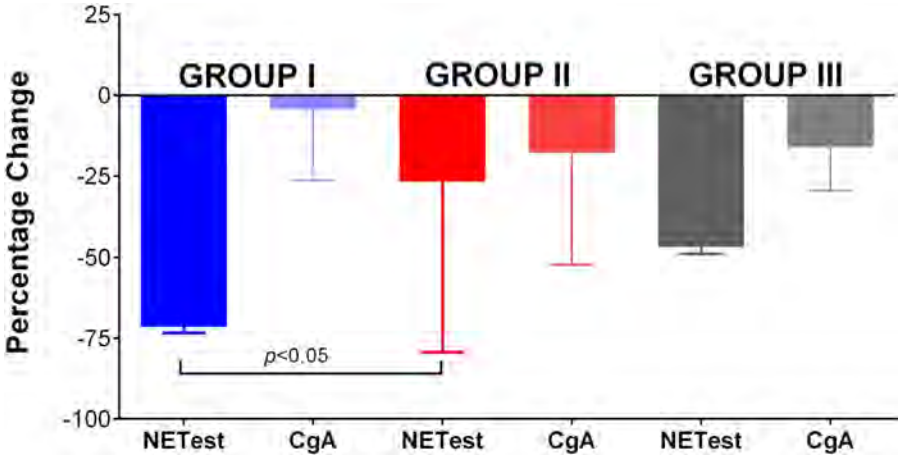

Fig 2. Alterations measured as a percentage change from baseline (pretreatment) to posttreatment (either surgery [groups I/II] or ablation [group III]) in the NETest score and chromogranin A. The change from baseline for the NETest was significantly greater in Group I (R0: no postoperative evidence of disease, $n = 15$) compared with Group II ($n = 12$). No significant changes from baseline were noted for CgA measurements for any of the treatment groups. $P < .05$ vs residual (unpaired 2-tailed, nonparametric test). Negative values (below 0) signify a posttherapy decrease in the biomarker.

(67%) exhibited a normalization of values after surgery. Four subjects (26%) in group I had increased preoperative CgA levels; 3 exhibited normalization after surgery (1 patient with increased CgA was patient 9 [Table II] who had a resection of a small intestinal NET and locoregional lymph nodes). Five patients (42%) in group II had increased preoperative CgA levels; 3 exhibited normalization after surgery (patients 20, 22, and 25; Table II). In group III, 1 (12%) of the 8 ablation subjects exhibited a significant decrease (to normal values) after treatment (patient 28 [Table II] bland embolization, pancreatic insulinoma).

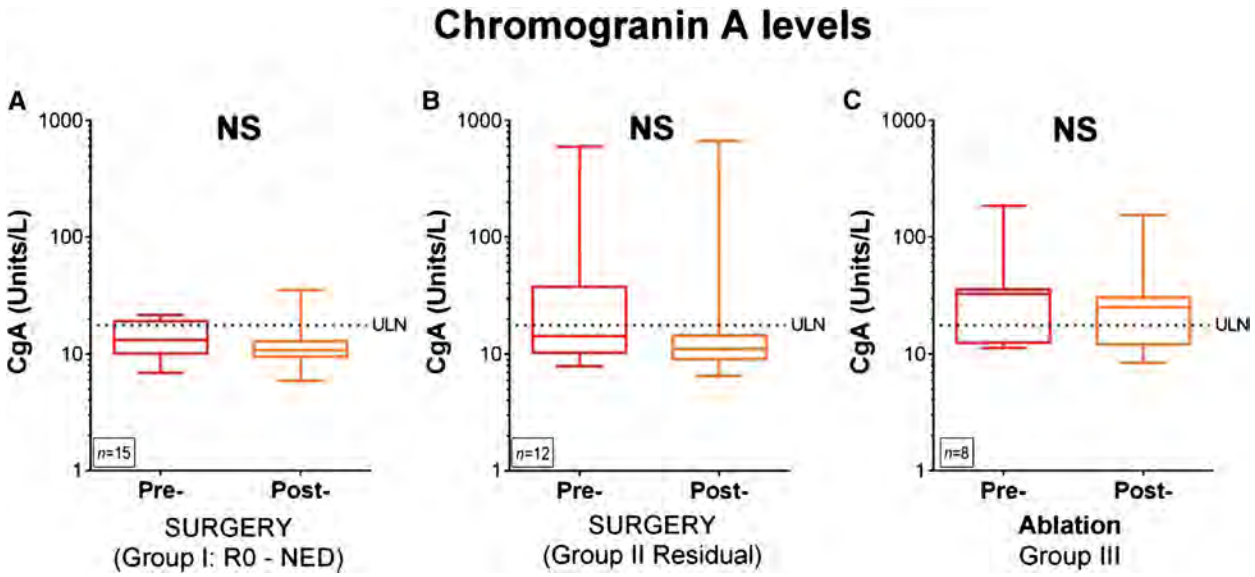

Fig 3. CgA level alterations after surgery or ablation. (*A–B*) Pre- and postoperative CgA levels were not decreased greatly in either group I or group II. (*C*) CgA levels were not decreased after treatment in the ablation group. *NED*, No evidence of disease (imaging: postoperative); *Pre*, presurgery scores; *post*, postsurgery scores; *NS*, not significant; *ULN*, upper limit of normal (19 U/L for CgA).

Assessment of overall percentage decrease in CgA postsurgery was −15.4 ± 5.7% in the complete cohort. A comparison of percentage changes in CgA in Group I versus Group II identified no significant differences (−5% vs −27%) (Fig 2). The change in the ablation group was −20%.

Follow-up assessment. Of the 15 patients in Group I, 4 (27%) developed disease recurrence loco-regionally at 6 months identified by imaging (^{68}Ga-somatostatin receptor-based PET) (patients 2, 4, 12, and 15; Table II, Fig 4). These included an appendiceal NET (G1), a high-grade (G3, Ki67 25%) type III gastric NET, a pancreatic NET (G1, lymph node metastases), and a small intestinal NET (G1, lymph node metastases). All recurrences were loco-regional. At 1 month after surgery, all 4 patients exhibited increased NETest scores (median, 30%; range 13-87%). Two of the 4 exhibited significantly increased CgA preoperatively. Both had significant reductions in CgA postoperatively to normal levels; however, all 4 who developed recurrent disease had normal CgA levels at the time of recurrence. The 2 with normal postoperative NETest scores (patients 1 and 5; Table I, Fig 4) remain disease-free at 18 and 47 months. In the ablation group, all 8 exhibited image-positive disease at 6 months.

Summary. All 35 patients (100%) exhibited an increased preoperative NETest score compared with 14 (40%) with increased CgA levels ($\chi^2 = 30$, $P < 2 \times 10^{-8}$). Residual or recurrent disease (groups I–III) was accurately identify by the NETest in 26/26 cases (100%) compared with 6 (23%) by elevated CgA ($\chi^2 = 32.5$, $P < 4 \times 10^{-9}$). The metrics for detecting disease were: sensitivity 100% versus 18%, specificities: 20% versus 50%, PPV: 73% versus 86% and NPV: 100% versus 4%.

The mean presurgery tumor volume was 22 ± 5 cm^3, which decreased to 8.8 ± 4.8 cm^3 after surgery. Tumor volumes were reduced in both groups (group I: 11.4 ± 1.8 to 0 cm^3, a 100% decrease; group II: 34.0 ± 8.7 to 18.8 ± 9.2 cm^3, −57.1 ± 11.4%). Decreases in tumor volume correlated with decreases in the NETest ($R^2 = 0.29$, $P = .023$) but not with CgA ($R^2 = 0.01$, $P = .9$). The NETest was significantly more effective than CgA for identifying the completeness of tumor resection. Overall decreases were noted in 23 of 27 compared with 6 of 27 by CgA ($\chi^2 = 19.1$, $P < .0001$ 2-tailed). This was also noted for ablation (8/8 vs 1/8; $P < .0001$). Increased NETest scores after surgery were identified in all group I patients who recurred, whereas CgA levels were normal in all. All group II and group III patients exhibited elevated NETest scores at 1 month.

DISCUSSION

It is accepted that operative resection is the only therapeutic modality most likely to provide cure for NET. Pharmacotherapy and embolization procedures are effective in diminishing tumor burden

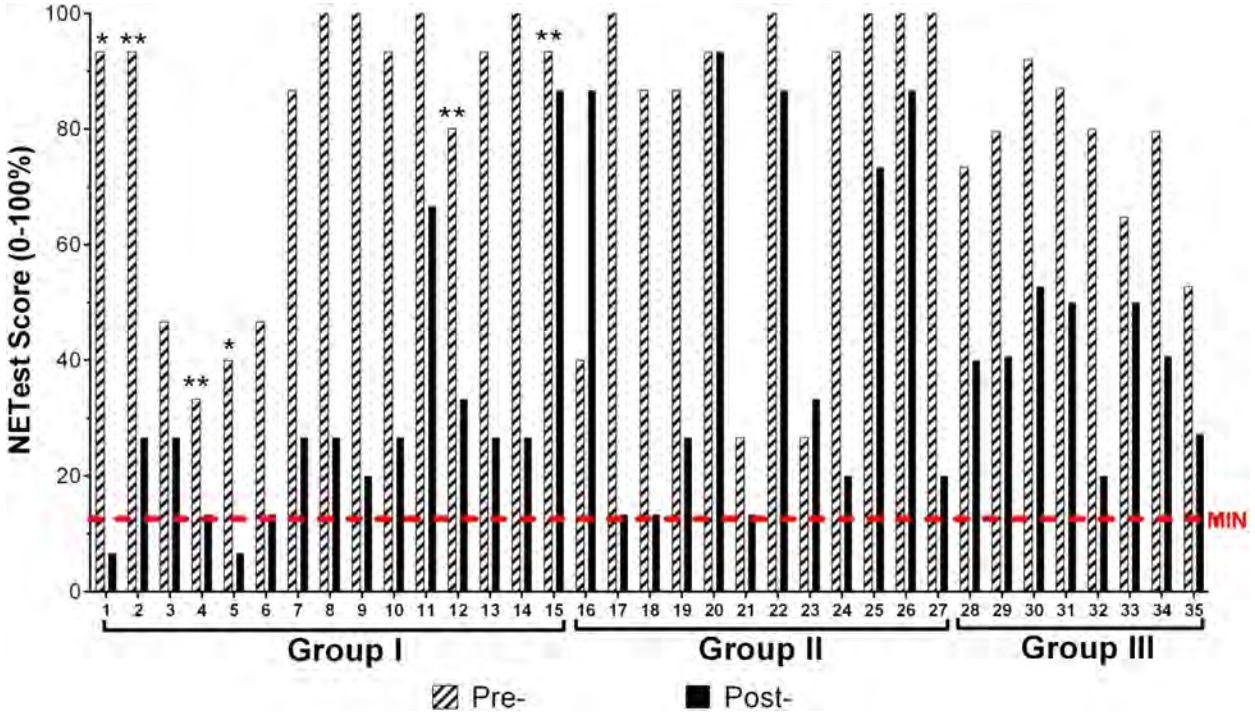

Fig 4. Individual NETest scores (pre- and postoperative levels) in group I and group II as well as in group III. In Group I, only 2 of 15 (1 and 5) exhibited minimal NETest activity levels postoperatively (appendiceal NET $n = 1$; insulinoma $n = 1$). In the known residual tumor group, all had abnormal scores postoperatively. Three patients (16, 20, and 23) did not exhibit a decreased score after operative resection. All patients exhibited a decrease after ablation; all had abnormal scores post-therapy. *MIN*, Minimal disease activity. This is <14% for the PCR test. *R0, normal postresection NETest levels. **R0, subsequently developed image-positive recurrence.

as are ablation protocols. The latter, however, in some circumstances may be curative. A key limitation, however, is the absence of techniques that define completeness of tumor removal or identify recurrence. Imaging has limitations but is particularly difficult at the site of surgical resection. Given the limited discriminant index of imaging technology, this is a problem if the residual disease sought is, as it often the case, of minimal size.[9] preoperative hepatic imaging of neuroendocrine metastases understages the disease in more than 50% of patients when compared with histopathologic examination.[16] Frozen section and subsequent formal assessment of margins are of considerable efficacy but these only evaluate the local area submitted for evaluation. Although a positive resection margin (R1) was not associated with poor survival in some series on liver resection for neuroendocrine metastases, others have reported that the completeness of surgery is significantly correlated with the disease free survival.[17] As a result of imaging limitations, it has become apparent that biomarker measurements may provide a viable strategy as a harbinger of residual disease. Considerable evidence has accumulated in other neoplastic conditions to support the concept of low disease burden as an index of treatment effectiveness.[18]

We developed a multianalyte PCR-based circulating gene test, NETest, that accurately and efficiently identifies NET. This multianalyte test is more sensitive and specific than either CgA[11,12] or other single-biomarker assays, eg, pancreastatin or neurokinin A (ROC analysis: NETest AUC for differentiating NETs from controls: 0.96 vs 0.56–0.67 for ELISAs, $P < .0001$).[11] In this investigation, we report the clinical utility of this MAAA in the evaluation of surgical resection and ablation therapies of NET disease.

In instances in which specific biomarkers for a NET are available, such as insulinoma or gastrinoma, biomarker identification of residual disease is a key determinant of the completeness of surgery and forms the basis for defining further therapy. These tumor types, however, represent only 1–2% of NET disease. For the majority of GEP-NETs, accurate and specific biomarker identification post-surgery is unavailable.[1] The example

of gastrinoma, which can exhibit a number of forms, including solitary, regional spread, distant metastatic spread, and multifocal primaries, provides an illustrative example of the efficacy of a sensitive and specific biomarker (gastrin) in determining the completeness of surgery and defining future management. In many instances, a sensitive and specific gastrin biomarker assay identifies the fact that ~10% are biochemically cured even after "complete" tumor resection.[19] In our study, one R0 (#5) gastrinoma was normalized by surgery (no evidence of recurrence at 6 months on imagery and by gastrin levels); in the second (#4) transcripts remained elevated and disease recurrence was identified by imagery and gastrin levels at 6 months. Although this is informative, the precise status of the majority of GEP-NETs (>98%) are not accurately identifiable with a monoanalyte marker such as gastrin or insulin. Thus, the utility of a diverse neuroendocrine cell system MAAA marker capable of providing post-surgery information for all NET subtypes should provide appropriate information and facilitate management.

Biomarker approaches to identify and predict disease recurrence after NET surgery have largely focused on monoanalyte measurements, eg, CgA or pancreastatin. In a retrospective Swedish study of 56 patients (1985–2004), increased CgA was noted in 28 of the 33 patients (85%) that exhibited disease recurrence; three of whom were noted to be radiology positive for a recurrence.[20] In a study in the United States, increased postoperative CgA was associated with a decreased survival in 49 metastatic midgut carcinoid patients who underwent primary tumor resection.[21] In a Danish study, patients with normal postoperative CgA levels had a 100% 5-year survival rate.[22] In contrast, a large, multinational retrospective study of 339 patients who underwent operative management for hepatic metastases between 1985 and 2009 failed to identify a role for CgA.[23] In a smaller study (n = 22), a ≥80% reduction in CgA levels after cytoreductive surgery for carcinoid tumors was noted to be predictive of subsequent symptom relief and disease control, despite incomplete cytoreduction.[24]

The limited utility of CgA is reflected in the fact that only 12 (34%) of the 35 subjects exhibited elevated pretreatment levels. Thus, posttreatment CgA values could provide no relevant clinical information. In a subgroup with increased preoperative values, operative reduction was associated with a decrease in CgA in 6 (67%). This finding is consistent with reports of CgA as a marker of tumor bulk. However, no differences were noted between patients with no evidence of disease postoperatively and those with residual disease, although the latter exhibited greater postoperative reductions. This may reflect the fact that more extensive cytoreduction was undertaken in this group. In particular, a decrease >80%, noted by Jensen et al[24] to represent a critical level commensurate with clinical advantage was not identified.

In contrast to monoanalyte measurements, the NETest PCR score was significantly more sensitive and changes accurately reflected differences between the two groups. Thus, levels were significantly lower in group I, who demonstrated no evidence of disease at postoperative imaging. In group II all patients resected with known residual disease exhibited abnormal scores. Of note, however, is that the majority (87%) of subjects in group I exhibited disease activity (≥14%), ie, were not disease free at a transcript level. We have interpreted this as evidence of residual NET disease and such individuals did not *ipse facto* have a biochemical R0 status. This is consistent with the general clinical appreciation of the limited ability to ensure at surgery that all neoplastic tissue has been resected.

In a separate review of our database we identified 12 patients (see the section "Patients and methods") who were operatively "cured" (R0) and had no clinical or radiologic evidence of disease recurrence after 5 years. This group had a mean NETest value of 6.6 ± 2.2% (upper 95% confidence interval 11.6%), with a maximum of 14%. For the ablation group, all patients exhibited increased scores after treatment. Similarly, molecular strategies have been reported to be more sensitive than histologic approaches for detecting residual disease. Thus, PCR for CgA identified more positive lymph nodes (73%) than either standard histology or immunohistochemistry (53–57%) after small intestinal NET surgery.[25] Fifty percent (14 of 28) lymph nodes previously considered negative were molecularly positive leading to patient upstaging.[25] Overall, these data are consistent with high recurrence rates noted despite R0 resections of neuroendocrine disease. In the current study, the clinical utility of positive blood transcript levels was determined when four patients (of 13 with increased scores) were identified to have disease recurrence within a 6 month follow-up period with functional imaging after R0 as well as all patients positive after ablation protocols.

The Ki-67 index and tumor grade are used as surrogates for biologic behavior of neuroendocrine neoplasia, with greater levels associated with aggressive behavior. In the current study, the

majority of subjects (27/35, 77%) were grade 1. Three of the four R0 resected patients who subsequently developed recurrent disease had G1 tumors; the fourth was a highly proliferating type III gastric NET (G3: Ki-67 25%). Seven of the 8 ablation subjects were also G1. The 2 patients with G2 lesions in group I, a multifocal insulinoma and a small intestinal NET, respectively, both remain disease-free at 18 and 47 months postoperatively. Overall, although the numbers are small, grade was not predictive of tumor recurrence.

In conclusion, our aim was to evaluate the effect of surgery and ablation on the circulating NET transcript signature. These data demonstrate that the signature was reduced by operative resection and by ablation and that this decrease was reflective of the extent of resection. Blood transcript levels 1 month after surgery identified individuals with increased scores who underwent a R0 tumor resection and subsequently developed clinical recurrence within a 6-month period. Our results suggest that a PCR-based blood test will be useful in the assessment of the adequacy of operative resection, but a further long-term prospective study is needed to establish the most accurate timing of blood collection (postsurgery) as well as the metrics of the NETest in the prediction of residual/recurrent disease. Under such circumstances, the role of adjuvant therapy may, in the future, become a relevant consideration for NET patients with R0 resected disease.

REFERENCES

1. Modlin IM, Oberg K, Chung DC, Jensen RT, de Herder WW, Thakker RV, et al. Gastroenteropancreatic neuroendocrine tumours. Lancet Oncol 2008;9:61-72.
2. Lawrence B, Gustafsson BI, Chan A, Svejda B, Kidd M, Modlin IM. The epidemiology of gastroenteropancreatic neuroendocrine tumors. Endocrinol Metab Clin North Am 2011;40:1-18; vii.
3. Frilling A, Modlin I, Kidd M, Russell C, Breitenstein S, Salem R, et al. Recommendations for management of patients with neuroendocrine liver metastases. Lancet Oncol 2014;15:e8-21.
4. Caplin ME, Pavel M, Cwikla JB, Phan AT, Raderer M, Sedlackova E, et al. Lanreotide in metastatic enteropancreatic neuroendocrine tumors. N Engl J Med 2014;371:224-33.
5. Yang J, Kan Y, Ge BH, Yuan L, Li C, Zhao W. Diagnostic role of Gallium-68 DOTATOC and Gallium-68 DOTATATE PET in patients with neuroendocrine tumors: a meta-analysis. Acta Radiol 2014;55:389-98.
6. Frilling A, Sotiropoulos GC, Radtke A, Malago M, Bockisch A, Kuehl H, et al. The impact of 68Ga-DOTATOC positron emission tomography/computed tomography on the multimodal management of patients with neuroendocrine tumors. Ann Surg 2010;252:850-6.
7. Hodul PJ, Strosberg JR, Kvols LK. Aggressive surgical resection in the management of pancreatic neuroendocrine tumors: when is it indicated? Cancer Control 2008;15:314-21.
8. Akerstrom G, Hellman P, Hessman O, Osmak L. Surgical treatment of endocrine pancreatic tumours. Neuroendocrinology 2004;80(Suppl 1):62-6.
9. Bodei L, Sundin A, Kidd M, Prasad V, Modlin I. The status of neuroendocrine tumor imaging: from darkness to light? Neuroendocrinology 2015;101:1-17.
10. Modlin I, Kidd M, Taylor A, Drozdov I, Bodei L. Neuroendocrine tumor biomarkers: current status and perspectives. Neuroendocrinology 2014;100:265-77.
11. Modlin I, Drozdov I, Alaimo D, Callahan S, Teixeira N, Bodei L, et al. A multianalyte PCR blood test outperforms single analyte ELISAs for neuroendocrine tumor detection. Endocr Relat Cancer 2014;21:615-28.
12. Modlin I, Drozdov I, Kidd M. The identification of gut neuroendocrine tumor disease by multiple synchronous transcript analysis in blood. PLoS One 2013;8:e63364.
13. Walenkamp A, Crespo G, Fierro Maya F, Fossmark R, Igaz P, Rinke A, et al. Hallmarks of gastrointestinal neuroendocrine tumours: implications for treatment. Endocr Relat Cancer 2014;21:R445-60.
14. Kidd M, Drozdov I, Modlin I. Blood and tissue net gene cluster analysis correlate, define hallmarks and predict disease status. Endocr Relat Cancer 2015;22:561-75.
15. Modlin I, Drozdov I, Kidd M. Gut neuroendocrine tumor blood qPCR fingerprint assay: characteristics and reproducibility. Clin Chem Lab Med 2014;52:419-29.
16. Elias D, Lefevre JH, Duvillard P, Goere D, Dromain C, Dumont F, et al. Hepatic metastases from neuroendocrine tumors with a "thin slice" pathological examination: they are many more than you think. Ann Surg 2010;251:307-10.
17. Elias D, Lasser P, Ducreux M, Duvillard P, Ouellet JF, Dromain C, et al. Liver resection (and associated extrahepatic resections) for metastatic well-differentiated endocrine tumors: a 15-year single center prospective study. Surgery 2003;133:375-82.
18. Corless CL, Ballman KV, Antonescu CR, Kolesnikova V, Maki RG, Pisters PW, et al. Pathologic and molecular features correlate with long-term outcome after adjuvant therapy of resected primary GI stromal tumor: the ACOSOG Z9001 trial. J Clin Oncol 2014;32:1563-70.
19. Norton JA, Alexander HR, Fraker DL, Venzon DJ, Gibril F, Jensen RT. Possible primary lymph node gastrinoma: occurrence, natural history, and predictive factors: a prospective study. Ann Surg 2003;237:650-7; discussion 7-9.
20. Welin S, Stridsberg M, Cunningham J, Granberg D, Skogseid B, Oberg K, et al. Elevated plasma chromogranin A is the first indication of recurrence in radically operated midgut carcinoid tumors. Neuroendocrinology 2009;89:302-7.
21. Curran T, Tulin-Silver S, Patel K, Ward S, Schneiderman M, Harpaz N, et al. Prognostic clinicopathologic factors in longitudinally followed patients with metastatic small bowel carcinoid tumors. Pancreas 2011;40:1253-7.
22. Nykjaer KM, Gronbaek H, Nielsen DT, Christiansen P, Astrup LB. Description of patients with midgut carcinoid tumours: clinical database from a Danish centre. In Vivo 2007;21:679-84.
23. Mayo SC, de Jong MC, Pulitano C, Clary BM, Reddy SK, Gamblin TC, et al. Surgical management of hepatic neuroendocrine tumor metastasis: results from an international multi-institutional analysis. Ann Surg Oncol 2010;17:3129-36.

24. Jensen EH, Kvols L, McLoughlin JM, Lewis JM, Alvarado MD, Yeatman T, et al. Biomarkers predict outcomes following cytoreductive surgery for hepatic metastases from functional carcinoid tumors. Ann Surg Oncol 2007;14:780-5.
25. Lawrence B, Kenney B, Svejda B, Schimmack S, Alaimo D, Barbieri A, et al. Comparison of PCR-based detection of chromogranin A mRNA with traditional histological lymph node staging of small intestinal neuroendocrine neoplasia. BMC Res Notes 2012;5:318.

DISCUSSION

Dr James R. Howe (Iowa City, IA): Your group has really led the way with coming up with these new markers to follow patients with neuroendocrine tumors. How much does this test cost? Did you compare it with other markers, say, like pancreastatin? If I have a patient with liver metastases who, say, is being treated with octreotide, could I use your test to determine that he's progressing and I should change my therapy?

Dr Mark Kidd: Thank you for your generous remarks in respect of our primacy in developing this field. They are much appreciated. The cost of the test has not yet been determined by the developers. However molecular tests of a similar nature such as Mammaprint® cost approximately $4,000, so I assume that this will be the likely range. I presume that multiple iterations of the test in patient follow up will have a different price schedule but I ask your forbearance in accepting my comments on the subject of pricing since this is not my field of expertise.

As you might appropriately enquire, we did compare this test to pancreastatin and other commonly used NET biomarkers. In this particular study, we compared it with CgA. The multianalyte (>95% accuracy) we developed significantly outperforms CgA (60% accuracy). In a separate study published in 2014, we ran a head-to-head comparison of 4 assays, NETest, CgA, pancreastatin, and neurokinin A. The NETest significantly ($P<0.0001$) outperformed all three of the single analytes. CgA was more sensitive and specific than pancreastatin and NKA. Based on this data, we elected not to investigate pancreastatin in this particular study since it was less effective than both CgA and NETest.

Your question regarding the role of the test in respect of somatostatin analogues therapy is prescient. Blood transcript analysis very effectively defines the efficacy of the somatostatin analogs, octreotide and lanreotide. The blood signature accurately identifies patients who are stable on an analog compared with those who have progressive disease, despite being treated with an analog. When the gene cluster analysis of the signature is assessed, the transcript profile defines the biological activity of the evolving tumor and enables quantification of the specific components including genes regulating proliferation, metabolism and growth factor signaling, amongst others. Since these clusters biologically define the level of neoplasia (malignancy index) they are of considerable relevance in determining prognosis. In terms of being specific, our current data indicate that elevations in signature activity levels to 80% activity or above actually precede image-positive recurrence by at least 3–4 months. Our impression is that the test is a sensitive predictor of progression and we think that when used in conjunction with imaging, will provide a very accurate predictive quotient.

In respect of your specific question regarding a patient with liver metastases being treated with an SSA analog, I can assure you that alteration of the molecular profile 4–6 months before a demonstrable image change will certainly allow you to identify progression and alter therapy accordingly. I anticipate that identifying changes in the blood at a molecular level well before imaging changes are evident will have obvious advantages for both patients and clinicians seeking to target or modify therapy in a timely fashion.