Supplemental Materials - Methods

Supplemental Methods

Processing of esophageal string sections and biopsies.

Esophageal string test samples and biopsies were processed and extracted, respectively, for biomarker quantification by ELISAs as previously described.[1] Briefly, proximal and distal esophageal string samples were frozen in 360 µL of elution buffer (0.5M $NaPO_4$, 0.5% BSA, 10 mM EDTA, 10 mM EGTA, 1% protamine sulfate, 1% Triton X-100, pH 6.0 + 40 µL complete Mini Protease Inhibitor Cocktail Tablets, Roche, Indianapolis, IN) and stored at -80°C until ready for processing and analysis. String samples in elution buffer were thawed on ice, vortexed at maximum speed for 1 min and transferred to an 80-145 µm filter pore size Biomasher column (Bioexpress, Kaysville, UT) for homogenization. Following centrifugation of the Biomasher column, the flow through eluate was collected, aliquoted and used for protein biomarker quantification by ELISA. Esophageal biopsies were extracted by incubating each sample for 1h in 300 µL of cold lysis buffer (50 mM TRIS, 650 mM NaCl, 5% Triton X-100, 50 mM NaF, 50 mM sodium phosphate, 50 mM sodium pyrophosphate, pH 6.5 + complete Mini Protease Inhibitor Cocktail Tablets, Roche, Indianapolis, IN). Biopsies were homogenized and centrifuged in Biomasher columns (Bioexpress, Kaysville, UT). The extracted protein concentration in biopsy supernatants was determined using the Pierce BCA Protein Assay Kit (ThermoFisher Scientific, Waltham, MA) according to the manufacturer's instructions. Biomarker levels measured by ELISA in biopsy extracts were normalized based on their extracted protein concentration and are reported as ng biomarker/mg protein.

Quantitative biomarker measurements: eosinophil-derived granule (EDGPs) and cytosolic proteins, and eosinophil-associated chemokines.

The biomarkers measured in EST samples and biopsy extracts included the eosinophil-derived granule cationic proteins eosinophil-derived neurotoxin (EDN), eosinophil peroxidase (EPX) and major basic protein-1 (MBP-1), and the eosinophil cytosolic protein Charcot-Leyden Crystal

protein/Galectin-10 (CLC/GAL-10). MBP1 and CLC/GAL-10 were measured using double-antibody sandwich ELISAs developed and standardized in the Ackerman laboratory (UIC, Chicago, IL). A mouse monoclonal antibody was used to capture MBP-1 as previously described.[1] String and biopsy samples were reduced and alkylated prior to analysis and assayed at a final 1:22 dilution. A mouse monoclonal antibody (Clone B-F42, Cell Sciences, Canton, MA) was used to capture CLC/GAL-10 as previously described.[1, 2] The detection antibodies for MBP-1 and CLC/GAL-10 were biotinylated using a Biotin-XX Microscale Protein Labeling Kit (B30010; Molecular Probes, Invitrogen, Eugene, Oregon) according to the manufacturer's instructions, and detected using streptavidin-conjugated horseradish peroxidase (ExtraAvidin Peroxidase, E2886; Sigma-Aldrich, St. Louis, MO, USA). Standard curves were constructed using serum, containing previously determined concentrations of MBP-1 and CLC/Gal-10, obtained from a patient with the hypereosinophilic syndrome (HES).[2-5] The MBP1 and CLC/GAL-10 ELISAs had detection ranges of 5.9 – 750 ng/mL and 0.125 – 16 ng/mL, respectively, and coefficient of variation (CV) of 5.95% (MBP1) and 15.38% (CLC/GAL-10). EPX levels were determined using a double-antibody sandwich ELISA developed and standardized as previously described.[6] The EPX ELISA detected EPX in the range of 8–1024 ng/ml, with an assay CV of 13%. Quantitation of EDN, Eotaxin-2 and Eotaxin-3 was performed using commercially available ELISA kits according to the manufacturer's instructions. EDN was quantified using an EDN ELISA Kit (MBL International, Woburn, MA, USA) in EST samples diluted 1:22 and Eotaxin-2 using a Human CCL24/Eotaxin-2/MPIF-2 Quantikine ELISA Kit (R&D Systems, Minneapolis, MN) and Eotaxin-3 with Human CCL26/Eotaxin-3 DuoSet ELISA (R&D Systems, Minneapolis, MN) in EST samples diluted 1:5.

References

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Supplemental Materials - Tables

Table S1.A. Spearman[a] correlations for EREFS with histologic peak eosinophil counts

	EREFS		
Subject Group	**Proximal**	**Distal**	**Total[b]**
Children	0.31	0.63	0.56
Adults	0.71	0.71	0.74
All Subjects	0.48	0.58	0.57

[a] Spearman (r_s) correlations for histologic peak eosinophils/HPF vs. EREFS, all $p < 0.0.5$

[b] Total EREFS = Proximal + distal EREFS.

Table S1.B. Spearman correlations for eosinophil-associated proteins in EST samples and biopsy extracts with histologic eosinophil counts and EREFS

	Biomarkers											
	CLC (Gal-10)		***MBP1***		***EDN***		***EPX***		***Eotaxin-2***		***Eotaxin-3***	
	Bx	***EST***	***Bx***	***EST***	***Bx***	***EST***	***Bx***	***EST***	***Bx***	***EST***	***Bx***	***EST***
Eos/hpf[a]	**0.7**	**0.4**	**0.64**	**0.4**	**0.67**	**0.46**	**0.61**	**0.51**	**0.53**	**0.4**	**0.61**	**0.68**
EREFS[b]												
Distal	0.50	0.35	0.63	0.41	0.52	0.38	0.52	0.42	0.64	0.62	0.49	0.60
Proximal	0.48	0.31	0.56	0.32	0.49	0.45	0.52	0.46	0.66	0.66	0.53	0.56
Total[c]	**0.50**	**0.35**	**0.63**	**0.40**	**0.53**	**0.43**	**0.54**	**0.44**	**0.68**	**0.68**	**0.52**	**0.60**

[a] p-values for significance of correlations between Eos/hpf and EAPs are shown in Figure 1.

[b] $p < 0.0001$ for all pair-wise correlations of EREFS with EAPs, except for CLC(Gal-10) and proximal EREFS, $p = 0.002$.

[c] Correlations of EAPs to sum of Distal + Proximal EREFS as an indicator of total esophageal disease activity.

Table S2.A. Non-parametric ANOVA for differences in EST string and biopsy biomarker levels between patient groups[a,b]

Biomarker[c]: Source	EoE Active, Median (Range)	EoE Inactive, Median (Range)	Normal, Median (Range)	Overall KW test	EoE Active vs EoE Inactive	EoE Active vs Normal	EoE Inactive vs Normal
MBP1: Biopsy	1091.13 (0.00 to 5169.37)	11.64 (0.00 to 1561.36)	0.00 (0.00 to 324.67)	**<.001**	**<.001**	**<.001**	**0.010**
String	613.74 (0.00 to 26820.07)	379.85 (0.00 to 1485.82)	344.88 (0.00 to 4430.01)	**<.001**	**0.002**	**0.008**	0.999
EDN: Biopsy	2171.48 (0.00 to 10846.12)	132.31 (0.00 to 1232.98)	87.48 (0.00 to 1146.50)	**<.001**	**<.001**	**<.001**	0.138
String	820.32 (0.00 to 3250.69)	453.59 (63.56 to 2829.69)	240.89 (45.56 to 2000.12)	**<.001**	**0.021**	**<.001**	0.085
CLC/GAL10: Biopsy	1089.67 (14.99 to 11179.41)	214.43 (6.63 to 1116.37)	32.94 (7.78 to 1004.01)	**<.001**	**<.001**	**<.001**	**<.001**
String	392.41 (4.62 to 1944.13)	79.74 (0.88 to 2611.78)	69.26 (11.18 to 1548.43)	**<.001**	**0.006**	**<.001**	1.000
EPX: Biopsy	50.77 (0.00 to 5285.71)	1.06 (0.00 to 488.46)	0.00 (0.00 to 57.36)	**<.001**	**<.001**	**<.001**	0.055
String	242.00 (0.00 to 13626.00)	76.00 (7.00 to 876.00)	38.50 (0.00 to 575.22)	**<.001**	**<.001**	**<.001**	0.191
Eotaxin-2: Biopsy	590.71 (0.00 to 4345.39)	6.97 (0.00 to 7191.63)	0.00 (0.00 to 553.01)	**<.001**	**0.007**	**<.001**	**<.001**
String	427.86 (0.00 to 6358.78)	138.33 (0.00 to 1112.09)	111.03 (0.00 to 781.93)	**<.001**	**0.025**	**<.001**	0.453
Eotaxin-3: Biopsy	929.02 (0.00 to 17355.18)	0.00 (0.00 to 2905.66)	0.00 (0.00 to 3.51)	**<.001**	**<.001**	**<.001**	0.142
String	978.76 (0.00 to 16809.27)	108.86 (0.00 to 1145.00)	0.00 (0.00 to 507.50)	**<.001**	**<.001**	**<.001**	**0.048**

[a] Dwass, Steel, Critchlow-Fligner multiple comparison procedure was used to adjust *p*-values

[b] *p*-values shown in bold are significant for the indicated patient group comparisons as presented graphically in Figure 3.

[c] For MBP1, EDN, CLC/Gal10, EPX – levels are ng/ml; for Eotaxin-2 and Eotaxin-3 – levels are pg/ml

Table S2.B. Non-parametric ANOVA for differences in EST string and biopsy biomarker levels between patient groups[d]

Biomarker: Source	EoE Active, Median (Range)	EoE Inactive, Median (Range)	Normal, Median (Range)	Overall KW test	EoE Active vs Inactive	EoE Active vs Normal	EoE Inactive vs Normal
MBP1: Biopsy	1091.13 (0.00 to 5169.37)	11.64 (0.00 to 1561.36)	0.00 (0.00 to 324.67)	**<.001**	**<.001**	**<.001**	**0.005**
String	613.74 (0.00 to 26820.07)	379.85 (0.00 to 1485.82)	344.88 (0.00 to 4430.01)	**<.001**	**<.001**	**0.004**	0.967
EDN: Biopsy	2171.48 (0.00 to 10846.12)	132.31 (0.00 to 1232.98)	87.48 (0.00 to 1146.50)	**<.001**	**<.001**	**<.001**	0.063
String	820.32 (0.00 to 3250.69)	453.59 (63.56 to 2829.69)	240.89 (45.56 to 2000.12)	**<.001**	**0.009**	**<.001**	**0.038**
CLC/GAL10: Biopsy	1089.67 (14.99 to 11179.41)	214.43 (6.63 to 1116.37)	32.94 (7.78 to 1004.01)	**<.001**	**<.001**	**<.001**	**<.001**
String	392.41 (4.62 to 1944.13)	79.74 (0.88 to 2611.78)	69.26 (11.18 to 1548.43)	**<.001**	**0.003**	**<.001**	0.986
EPX: Biopsy	50.77 (0.00 to 5285.71)	1.06 (0.00 to 488.46)	0.00 (0.00 to 57.36)	**<.001**	**<.001**	**<.001**	**0.025**
String	242.00 (0.00 to 13626.00)	76.00 (7.00 to 876.00)	38.50 (0.00 to 575.22)	**<.001**	**<.001**	**<.001**	0.088
Eotaxin-2: Biopsy	590.71 (0.00 to 4345.39)	6.97 (0.00 to 7191.63)	0.00 (0.00 to 553.01)	**<.001**	**0.003**	**<.001**	**<.001**
String	427.86 (0.00 to 6358.78)	138.33 (0.00 to 1112.09)	111.03 (0.00 to 781.93)	**<.001**	**0.011**	**<.001**	0.237
Eotaxin-3: Biopsy	929.02 (0.00 to 17355.18)	0.00 (0.00 to 2905.66)	0.00 (0.00 to 3.51)	**<.001**	**<.001**	**<.001**	0.066
String	978.76 (0.00 to 16809.27)	108.86 (0.00 to 1145.00)	0.00 (0.00 to 507.50)	**<.001**	**<.001**	**<.001**	**0.022**

[d] Non-parametric ANOVA, no adjustments made for multiple comparisons

Table S3. Integrated Discrimination Improvement (IDI) by adding an additional biomarker (Model 2) to eotaxin-3 alone (Model 1) for differentiating active from inactive (treated) EoE

	Biomarkers				
	MBP-1	**CLC (Gal-10)**	**EPX**	**EDN**	**Eot-2**
IDI (SEM)	0.078 (0.024)	0.042 (0.019)	0.063 (0.021)	0.009 (0.006)	0.003 (0.007)
***p*[a]**	**0.0009**	**0.023**	**0.0024**	0.1214	0.5976
IDI 95% CI	(0.0319, 0.1249)	(0.0058, 0.0784)	(0.0224, 0.1039)	(-0.0025, 0.0213)	(-0.0094, 0.0163)
Mean Probability for Active EoE: Model 2[b]	0.766	0.739	0.747	0.727	0.726
Mean Probability for Active EoE: Model 1[c]	0.720	0.730	0.730	0.730	0.725
Mean Probability for Inactive EoE: Model 2[b]	0.439	0.447	0.434	0.468	0.478
Mean Probability for Inactive EoE: Model 1[c]	0.471	0.480	0.480	0.480	0.480
Prob. Δ for Active EoE	0.046	0.009	0.017	-0.003	0.001
Prob. Δ for Inactive EoE	-0.032	-0.033	-0.046	-0.012	-0.002
Relative IDI	0.315	0.168	0.253	0.038	0.014

[a] *p*-value for testing the null hypothesis of IDI = 0

[b] Model 2 – IDI index for adding the indicated additional biomarker to eotaxin-3 for distinguishing active from inactive (treated) EoE.

[c] Model 1 – IDI index for eotaxin-3 alone for distinguishing active from inactive (treated) EoE.

Table S4. Addition of Individual Biomarkers to Eotaxin-3: Change in AUC of ROC.

Biomarker	*Δ AUC[a] of ROC[b]*	*Standard Error*	*95% Wald Confidence Limits*		*Chi-Square*	*Pr > ChiSq*
MBP-1	0.0237	0.0236	-0.0225	0.0699	1.0121	0.3144
CLC/Gal-10	0.0348	0.0263	-0.0168	0.0863	1.7459	0.1864
EPX	0.0124	0.0232	-0.0330	0.0578	0.2857	0.5930
EDN	0.00190	0.0103	-0.0183	0.0221	0.0340	0.8537
Eot-2	-0.00492	0.0134	-0.0312	0.0213	0.1349	0.7134

[a] AUC, area under the ROC curve

[b] ROC, Receiver Operator Characteristic curve

Supplemental Materials – Figure Legends

Figure Legends

Figure S1. Receiver operating characteristic (ROC) curves show the sensitivity and specificity for distinguishing active EoE (≥15 Eos/hpf) amongst all patients with EoE (active, inactive) or patient controls with normal esophagi. ROC analyses used the concentration of the indicated ***individual*** eosinophil-associated protein biomarkers captured by the 1-hr EST (**A, left panels**) or measured in mucosal biopsy extracts (**B, right panels**). Panels are ordered (top to bottom) for highest to lowest AUCs from the EST for Eot3, eotaxin-3; EPX, eosinophil peroxidase; EDN, eosinophil-derived neurotoxin; CLC (Gal-10), Charcot-Leyden crystal protein/Galectin-10; MBP-1, major basic protein-1; and Eot2, eotaxin 2. The area under the curve (AUC, [lower, upper limit]) are indicated above each panel; AUC values >0.80 are considered highly predictive.

Figure S2. Receiver operating characteristic (ROC) curves for esophageal biopsy extracts show significant sensitivity and specificity for distinguishing active EoE (≥15 Eos/hpf) amongst all patients with EoE (active, inactive) or patient controls with normal esophagi. ROC analyses used the level of Eotaxin-3 ***combined with*** one additional eosinophil-associated protein biomarker as measured in esophageal mucosal biopsy extracts. Panels are ordered (left to right) for highest to lowest AUCs for Eot3, eotaxin-3 **plus** CLC (Gal-10), Charcot-Leyden crystal protein/Galectin-10; EDN, eosinophil-derived neurotoxin; MBP-1, major basic protein-1; EPX, eosinophil peroxidase; and Eot2, eotaxin 2. Points on the ROC curves are labeled by their predictive probabilities. The area under the curve (AUC) is indicated above each panel; AUC values >0.80 are considered highly predictive.

Figure S3. Receiver operating characteristic (ROC) curves for esophageal biopsy extracts distinguish subjects with active EoE (≥15 Eos/hpf) from those with inactive EoE (<15 Eos/hpf). ROC analyses used the level of Eotaxin-3 **combined with** one additional eosinophil-associated protein biomarker measured in mucosal biopsy extracts. Panels are ordered (left to right) for highest to lowest AUCs for Eot3, eotaxin-3 **plus** EDN, eosinophil-derived neurotoxin; CLC (Gal-10), Charcot-Leyden crystal protein/galectin-10; MBP-1, major basic protein-1; Eot2, eotaxin 2; and EPX,

eosinophil peroxidase. Points on the ROC curves are labeled by their predictive probabilities. The area under the curve (AUC) is indicated above each panel; AUC values >0.80 are considered highly predictive.

Figure S4. Comparisons of models with one versus two biomarker predictors. Improvement from the reference model with Eotaxin-3 (Eot3) only for discriminating subjects with active EoE (≥15 Eos/hpf) from those with inactive EoE (<15 Eos/hpf), by adding another EAP to the model, is shown. Plots on the left show the superimposed ROC curves of the reference (Eot3 only) versus new models (+ indicated EAP). If adding an EAP improves the model, the reference ROC curve moves toward the [0, 1] point (left upper corner). Table S3 shows the results of testing the change in ROC AUC. Each plot on the right is the risk assessment plot, showing the sensitivity (true positive) and 1-specificity (1 minus specificity) (false positive) for differentiating active EoE against the model estimated probability of being active EoE. If adding an EAP improves the model, the reference sensitivity curve moves toward the [1, 1] points (right upper corner) while the 1-specificity curve moves toward the [0, 0] point (left lower corner). The sum of the area between the curves is the Integrated Discrimination Index (IDI) (1) (see Table S2). TP, True Positive = sensitivity and FP, False Positive = 1-specificity. *(1) Pickering JW, Endre ZH. New metrics for assessing diagnostic potential of candidate biomarkers. Clin J Am Soc Nephrol 2012;7:1355-64).*

Figure S5. Nomogram for differentiating between patients with active vs. inactive EoE using concentrations of luminal eotaxin-3 and MBP-1 detected by the 1-hr esophageal string test. This nomogram, generated using eotaxin-3 and MBP-1 levels captured by the 1-hr EST amongst all patients with a confirmed diagnosis of EoE, provides the probability of their being in remission (<15 Eos/HPF) or continuing to have active esophageal inflammation (≥15 Eos/HPF). The nomogram is used by summing the points identified on the top scale for each of the two independent covariate biomarkers, eotaxin-3 (Eot3) and MBP-1. This point total is then identified on the total points scale at the bottom of the figure to determine the probability of the patient having active EoE as defined by a histologic eosinophil count of ≥15 Eos/HPF. The 'optimum' cutoff based on Youden J is a probability of ≥0.50, which is associated with 80% sensitivity and

75% specificity (see corresponding ROC curve for Eot3 + MBP1 in Figure 5). For example, an EOT3 level of 1000pg/ml ≙ 5 points; an MBP1 level of 5000ng/ml ≙ 11 points. Summing these 5 + 11 = 16 total points. This total points score is then identified on the total points scale in the bottom of the figure to determine the probability, in this example a probability of >0.95, for the patient having active EoE as defined by a histologic eosinophil count of ≥15 Eos/hpf.

Figure S1

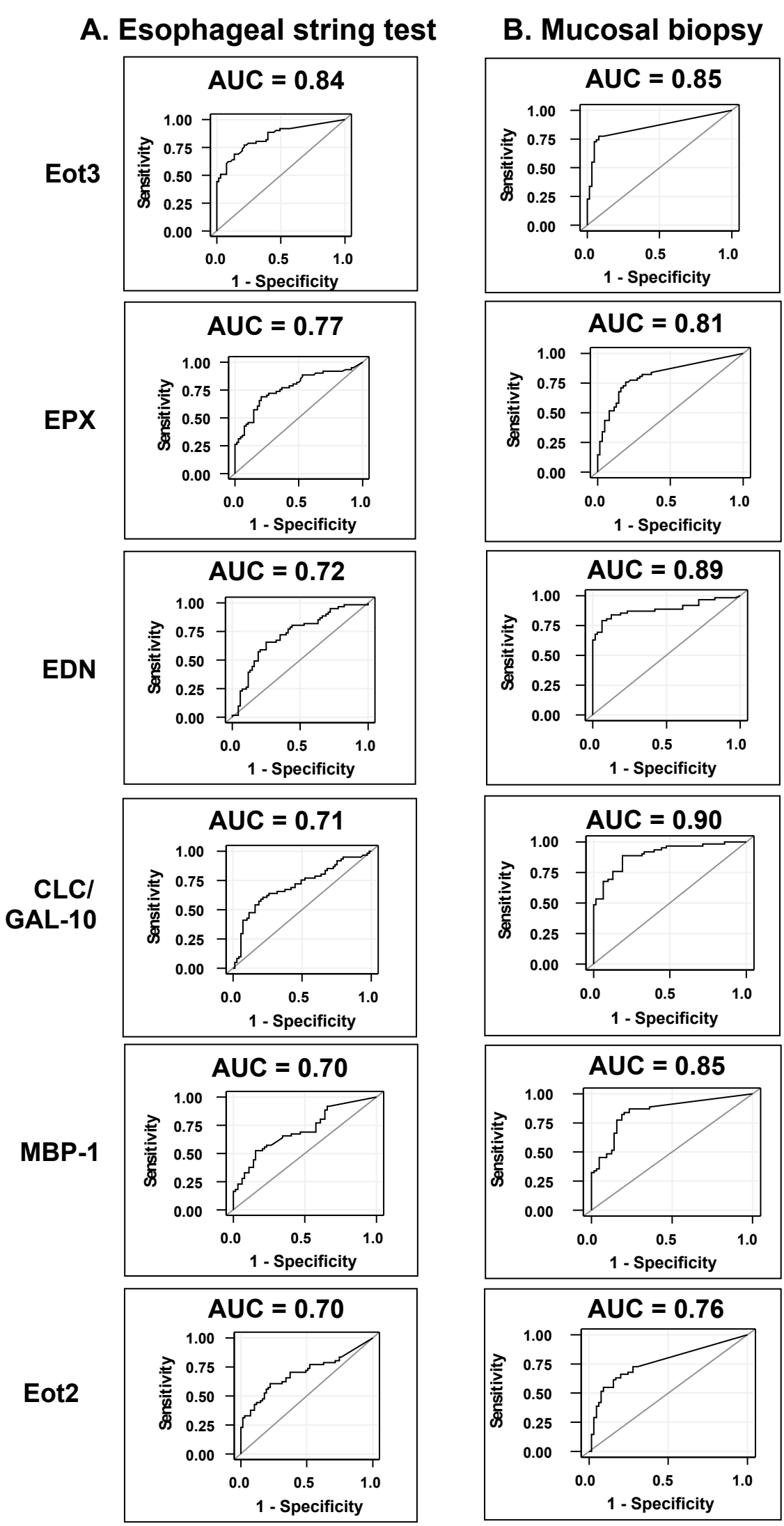
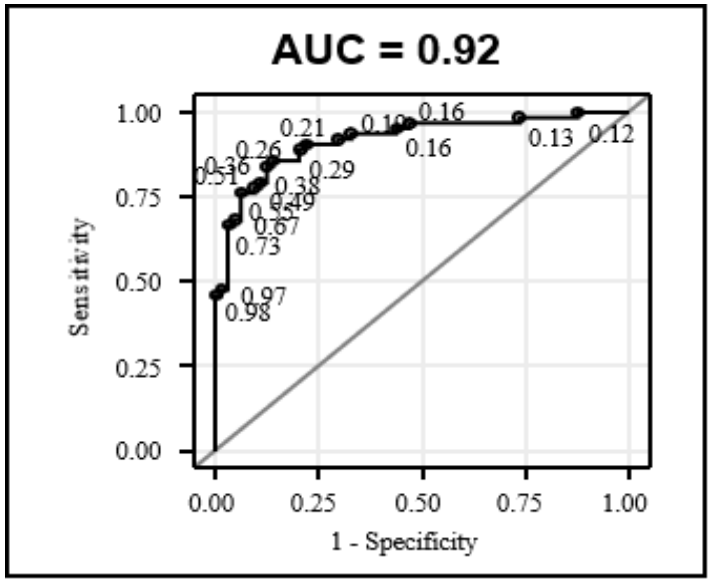
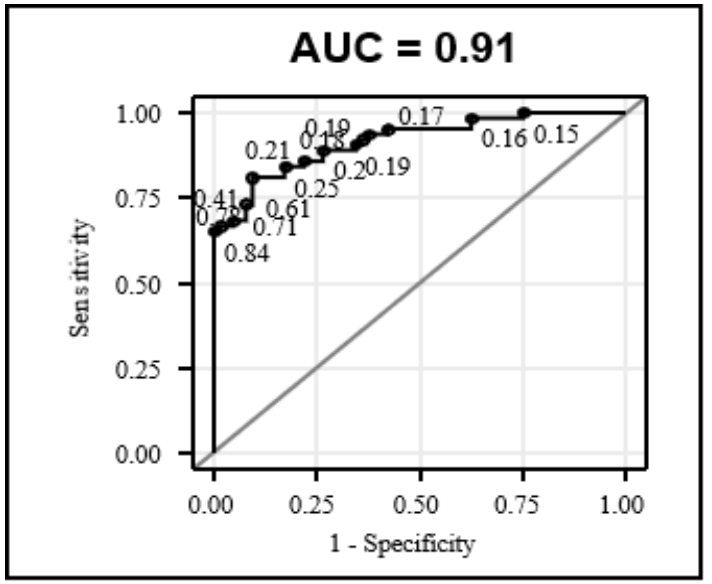

A. Esophageal string test
B. Mucosal biopsy
Eot3
AUC = 0.84
AUC = 0.85
EPX
AUC = 0.77
AUC = 0.81
EDN
AUC = 0.72
AUC = 0.89
CLC/
GAL-10
AUC = 0.71
AUC = 0.90
MBP-1
AUC = 0.70
AUC = 0.85
Eot2
AUC = 0.70
AUC = 0.76
Sensitivity
1 - Specificity
0.00
0.25
0.50
0.75
1.00
0.0
0.5
1.0

Figure S2

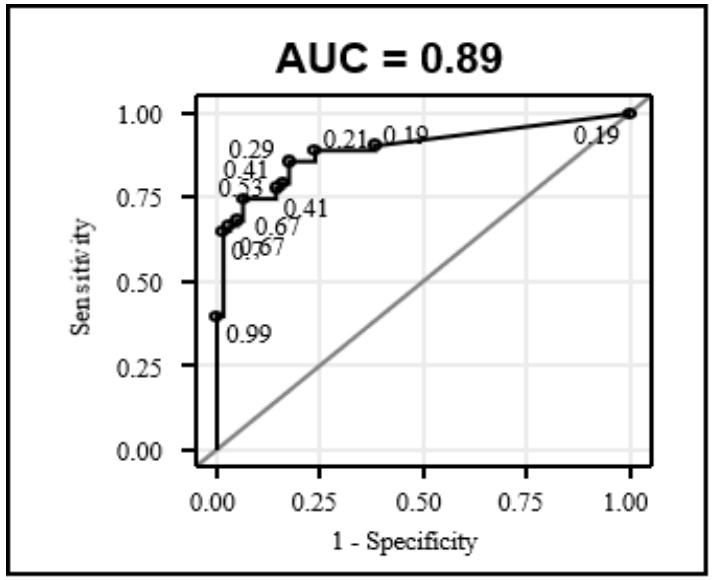
Eot3 + CLC/GAL-10



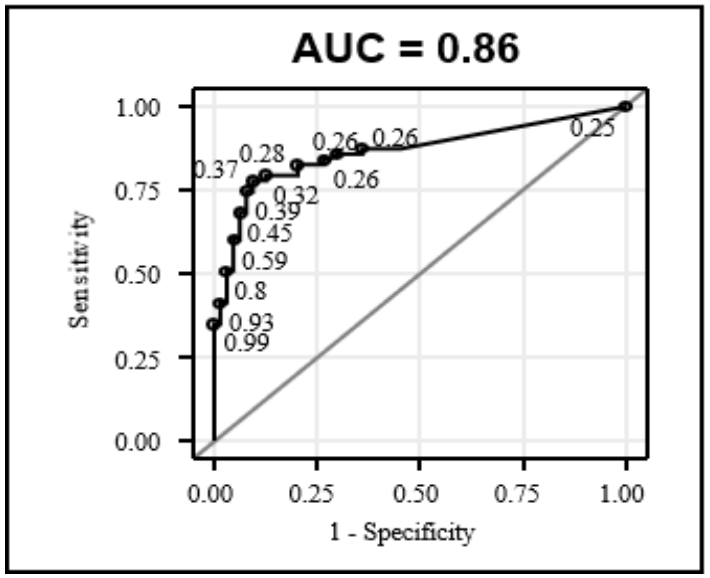
Eot3 + EDN



Eot3 + MBP1



Eot3 + EPX



Eot3 + Eot2

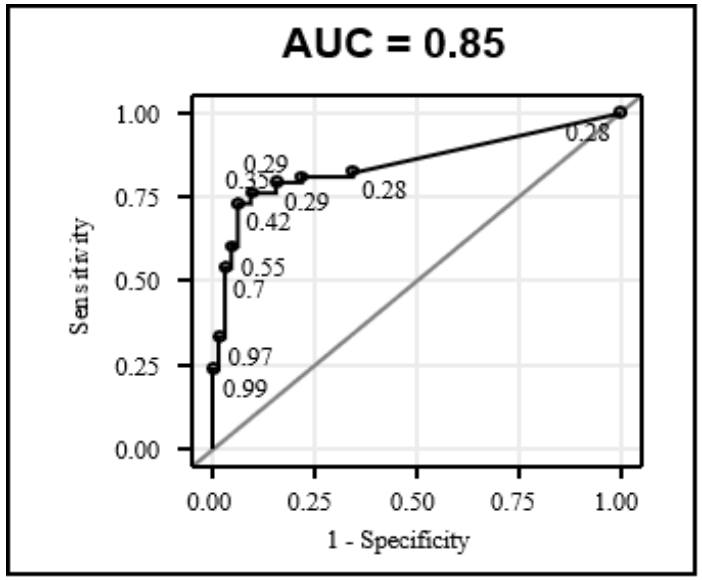
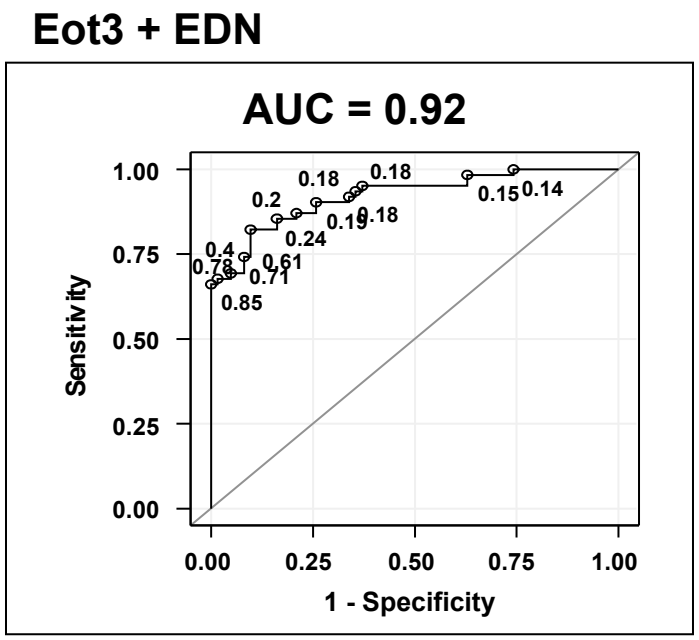
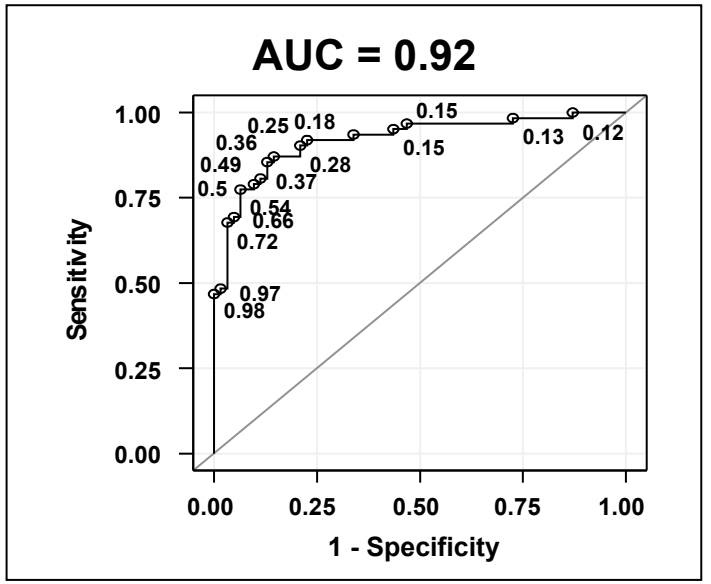
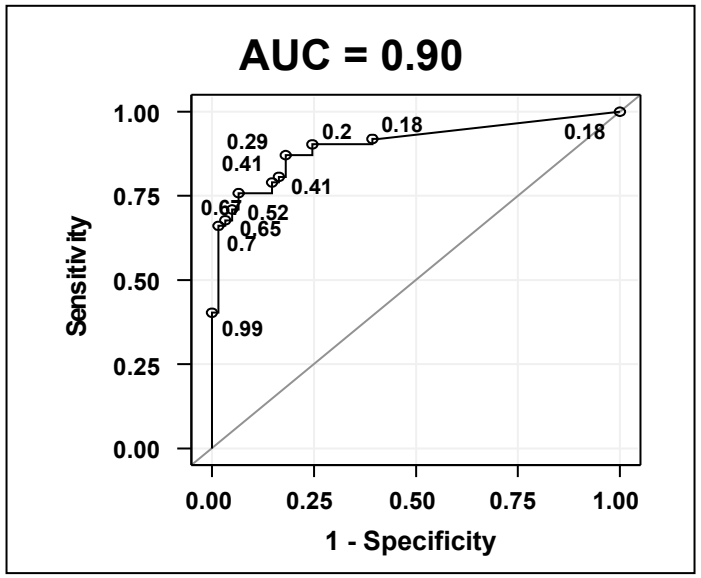
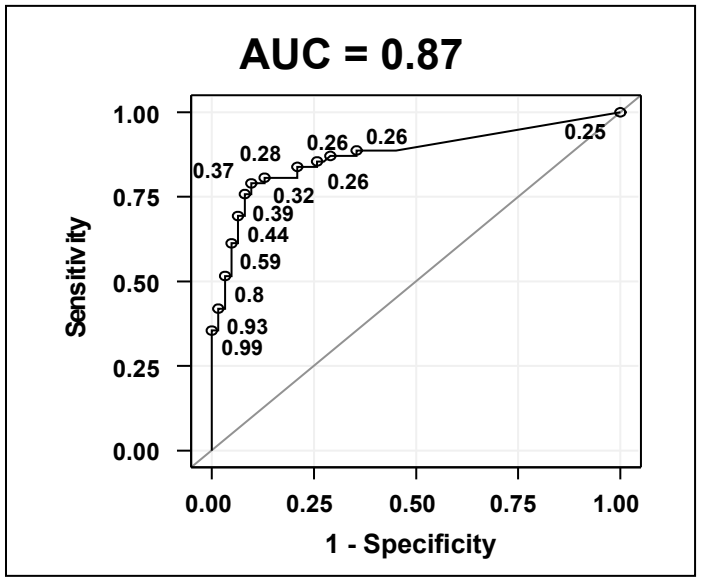


Figure S3


Eot3 + EDN
AUC = 0.92
Sensitivity
1 - Specificity
0.00
0.25
0.50
0.75
1.00
0.18
0.18
0.2
0.15
0.14
0.19
0.18
0.24
0.4
0.61
0.78
0.71
0.85


Eot3 + CLC/GAL-10
AUC = 0.92
Sensitivity
1 - Specificity
0.00
0.25
0.50
0.75
1.00
0.25
0.18
0.15
0.36
0.15
0.13
0.12
0.49
0.28
0.5
0.37
0.54
0.66
0.72
0.97
0.98


Eot3 + MBP1
AUC = 0.90
Sensitivity
1 - Specificity
0.00
0.25
0.50
0.75
1.00
0.29
0.2
0.18
0.18
0.41
0.41
0.52
0.65
0.7
0.99


Eot3 + EPX
AUC = 0.87
Sensitivity
1 - Specificity
0.00
0.25
0.50
0.75
1.00
0.26
0.26
0.25
0.37
0.28
0.26
0.32
0.39
0.44
0.59
0.8
0.93
0.99

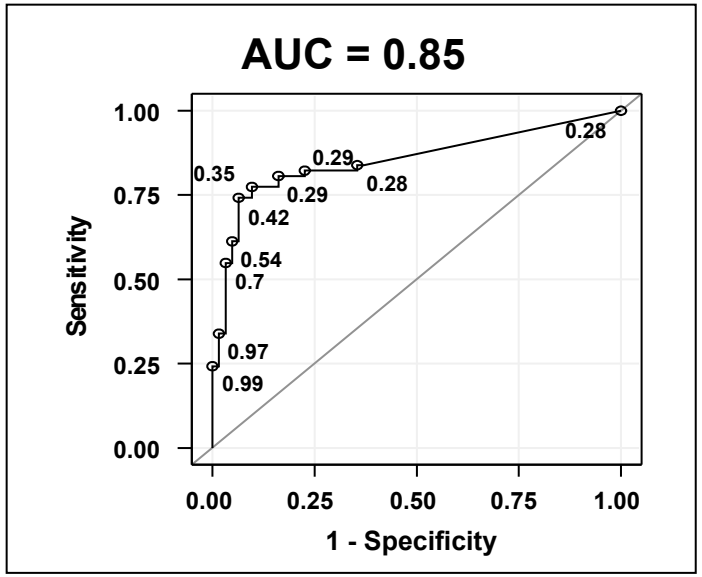

Eot3 + Eot2
AUC = 0.85
Sensitivity
1 - Specificity
0.00
0.25
0.50
0.75
1.00
0.29
0.28
0.35
0.29
0.28
0.42
0.54
0.7
0.97
0.99

Figure S4

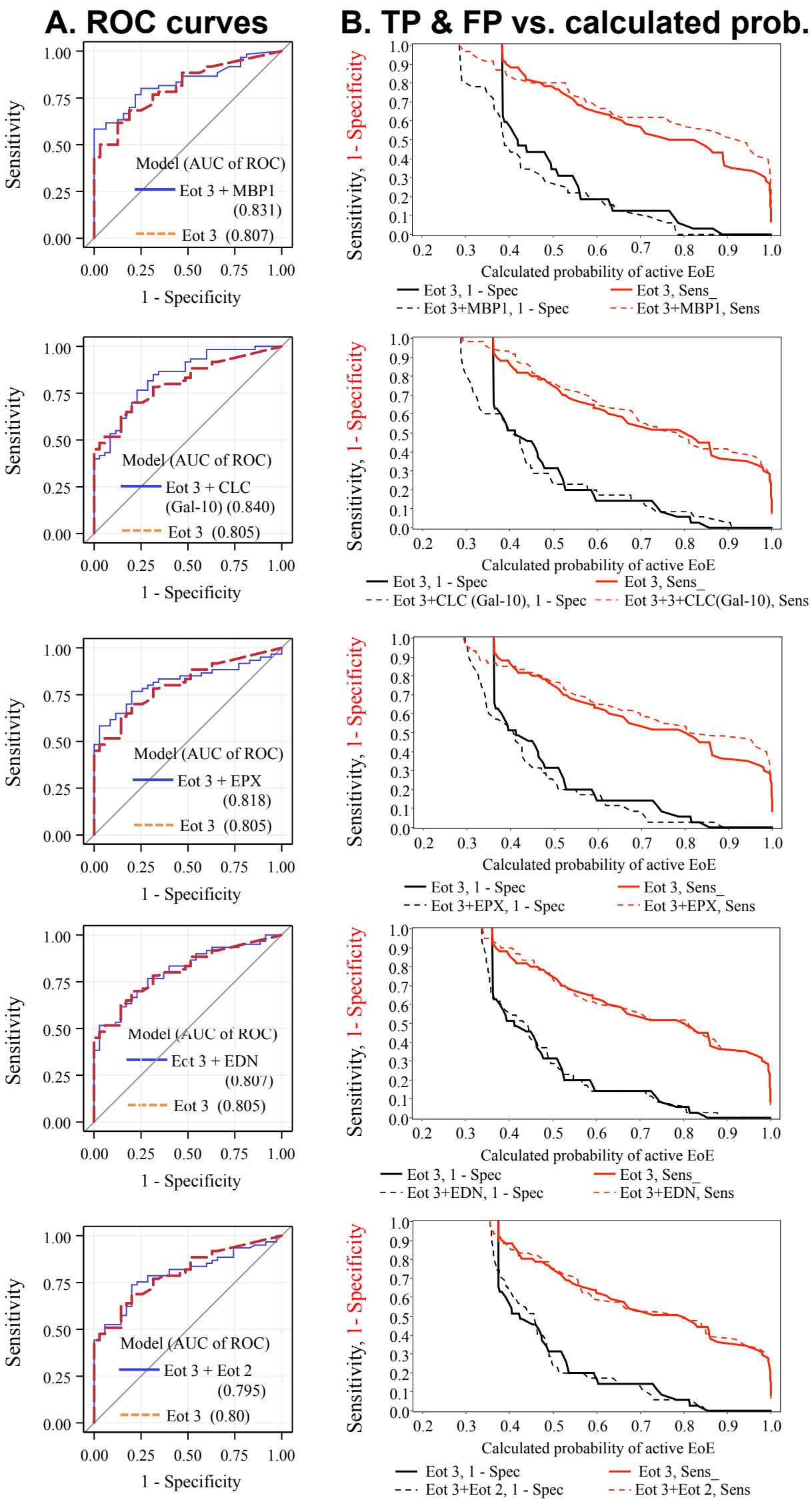
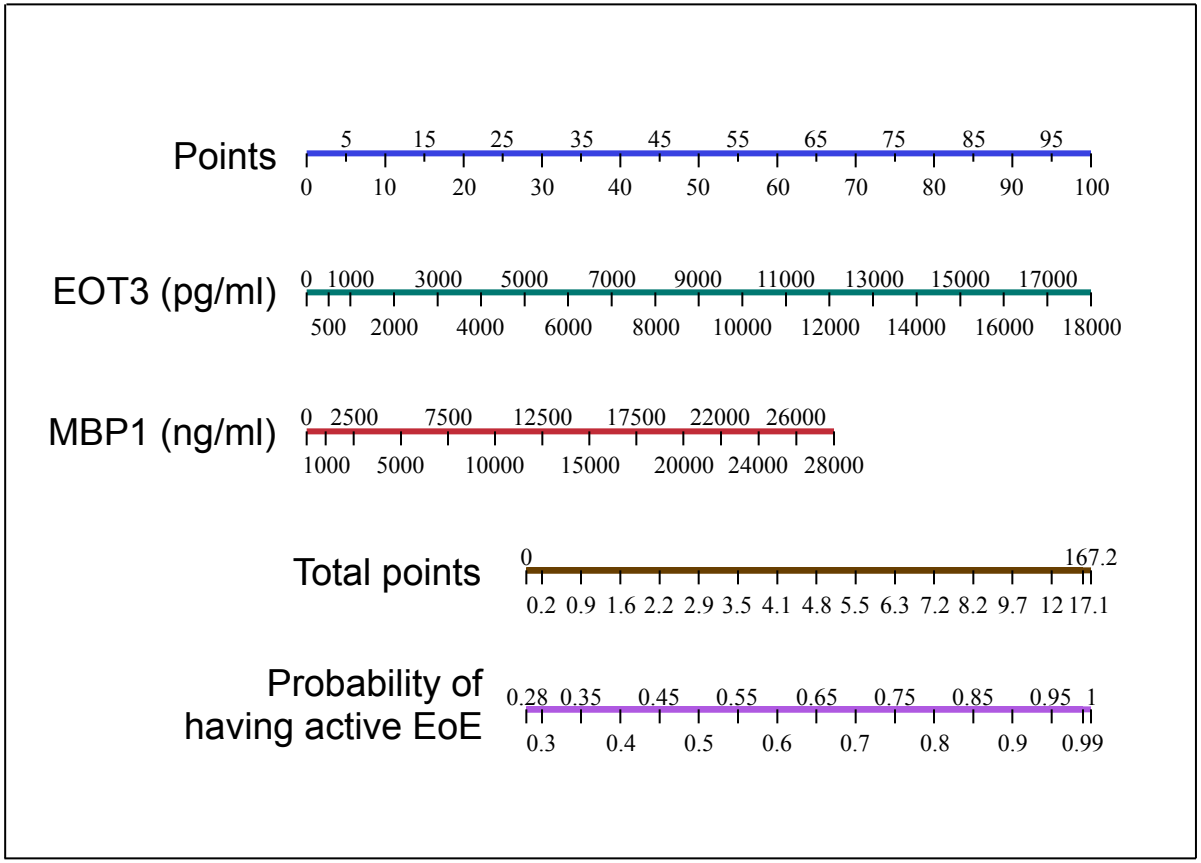

A. ROC curves
B. TP & FP vs. calculated prob.
Sensitivity
1 - Specificity
Model (AUC of ROC)
Eot 3 + MBP1 (0.831)
Eot 3 (0.807)
Sensitivity, 1- Specificity
Calculated probability of active EoE
Eot 3, 1 - Spec
Eot 3, Sens_
Eot 3+MBP1, 1 - Spec
Eot 3+MBP1, Sens
Model (AUC of ROC)
Eot 3 + CLC (Gal-10) (0.840)
Eot 3 (0.805)
Eot 3+CLC (Gal-10), 1 - Spec
Eot 3+3+CLC(Gal-10), Sens
Model (AUC of ROC)
Eot 3 + EPX (0.818)
Eot 3 (0.805)
Eot 3+EPX, 1 - Spec
Eot 3+EPX, Sens
Model (AUC of ROC)
Eot 3 + EDN (0.807)
Eot 3 (0.805)
Eot 3+EDN, 1 - Spec
Eot 3+EDN, Sens
Model (AUC of ROC)
Eot 3 + Eot 2 (0.795)
Eot 3 (0.80)
Eot 3+Eot 2, 1 - Spec
Eot 3+Eot 2, Sens

Figure S5


Points
5 15 25 35 45 55 65 75 85 95
0 10 20 30 40 50 60 70 80 90 100
EOT3 (pg/ml)
0 1000 3000 5000 7000 9000 11000 13000 15000 17000
500 2000 4000 6000 8000 10000 12000 14000 16000 18000
MBP1 (ng/ml)
0 2500 7500 12500 17500 22000 26000
1000 5000 10000 15000 20000 24000 28000
Total points
0 167.2
0.2 0.9 1.6 2.2 2.9 3.5 4.1 4.8 5.5 6.3 7.2 8.2 9.7 12 17.1
Probability of having active EoE
0.28 0.35 0.45 0.55 0.65 0.75 0.85 0.95 1
0.3 0.4 0.5 0.6 0.7 0.8 0.9 0.99