

Supplementary material

Supplementary Table 1: List of biomarkers evaluated in Panel A and prioritized for Panel B

	Biomarker	Unit	Assay	Platform	AUC	Specificity at 90 % sensitivity	Prioritization for Panel B
1	PIVKA-II	mAU/mL	Immunoassay	Fujirebio Lumipulse	0.961	0.831	Yes
2	PIVKA-II	ng/mL	Chip electrophoresis	Fujifilm Micro Total Analysis System Wako™	0.955	0.864	Yes
3	AFP-L3	%	Lectin chip electrophoresis	Fujifilm Micro Total Analysis System Wako™	0.917	0.69	Yes
4	ANG2	ng/mL	Immunoassay	cobas e 601	0.904	0.763	Yes
5	GGT	U/l	Clin Chem	cobas c 311	0.902	0.746	Yes
6	GPC3-N	pg/mL	Immunoassay	cobas e 601	0.897	0.695	Yes
7	TIMP1	ng/mL	Immunoassay	MTP ELISA	0.894	0.729	Yes
8	PIVKA-II	mAU/mL	Immunoassay	IMPACT	0.883	0.76	Yes
9	AFP	ng/mL	Chip electrophoresis	Fujifilm Micro Total Analysis System Wako™	0.874	0.621	Yes
10	AFP	IU/mL	Immunoassay	cobas e 601	0.870	0.576	Yes
11	HGF	pg/mL	Immunoassay	MTP ELISA	0.858	0.627	Yes
12	IL6	pg/mL	Immunoassay	cobas e 601	0.854	0.61	Yes
13	ALCAM/CD166	ng/mL	Immunoassay	MTP ELISA	0.845	0.42	No
14	AST	U/l	Clin Chem	cobas c 311	0.843	0.695	Yes
48	IGFBP3	ng/mL	Immunoassay	cobas e 601	0.84	0.523	Yes
49	IGFBP7	ng/mL	Immunoassay	cobas e 601	0.83	0.40	Yes
15	GP73 ver. 1	ng/mL	Immunoassay	MTP ELISA	0.812	0.356	No

16	fucAFP	ng/mL	Lectin-based Immunoassay	JOM MTP ELISA	0.811	0.633	No
50	IGF1	ng/mL	Immunoassay	cobas e 601	0.81	0.591	Yes
17	IL8	ng/mL	Immunoassay	IMPACT	0.802	0.576	Yes
18	AFP-L3	%	LCA lectin-based Immunoassay	IMPACT	0.802	0.38	No
19	Midkine	ng/mL	Immunoassay	MTP ELISA	0.798	0.62	Yes
20	OPG	ng/mL	Immunoassay	IMPACT	0.798	0.508	Yes
21	GP73 ver.2	ng/mL	Immunoassay	MTP ELISA	0.791	0.475	Yes
22	sGPC 3-FL	ng/mL	Immunoassay	cobas e 601	0.79	0.336	Yes
23	sAxl	ng/mL	Immunoassay	MTP ELISA	0.787	0.508	Yes
24	alpha-Fucosidase	U/l	Clin Chem	cobas c 311	0.785	0.322	Yes
25	Albumin	mg/mL	Clin Chem	cobas c 311	0.784	0.305	Yes
26	ALT	U/l	Clin Chem	cobas c 311	0.782	0.593	Yes
27	AFP-L3	%	PSA lectin-based Immunoassay	IMPACT	0.781	0.373	No
28	CES1 ver.1	ng/mL	Immunoassay	cobas e 601	0.78	0.54	No
29	CES1 ver.2	ng/mL	Immunoassay	cobas e 601	0.78	0.51	No
30	BILT	micromol/L	Clin Chem	cobas c 311	0.774	0.322	Yes
31	GLDH	U/l	Clin Chem	cobas c 311	0.771	0.475	Yes
32	Midkine	ng/mL	Immunoassay	Cellmid MTP ELISA	0.768	0.458	No
33	CES1 ver.3	ng/mL	Immunoassay	cobas e 601	0.76	0.48	No
34	CES1 ver.4	ng/mL	Immunoassay	cobas e 601	0.76	0.5	Yes
35	LRG1	microg/mL	Immunoassay	MTP ELISA	0.75	0.520	No
36	Ferritin	ng/mL	Immunoassay	cobas e 601	0.74	0.4	Yes
37	CES1	ng/mL	Immunoassay	MTP ELISA	0.736	0.102	No
38	MMP2	ng/mL	Immunoassay	MTP ELISA	0.735	0.271	Yes
39	COMP	ng/mL	Immunoassay	IMPACT	0.712	0.288	Yes
40	AFP-L3	ng/mL	PSA lectin-based	IMPACT	0.710	0.034	No

			Immunoassay				
41	Endoglin	ng/mL	Immunoassay	MTP ELISA	0.705	0.271	No
42	2-6-Sialyltransferase	ng/mL	Immunoassay	MTP ELISA	0.7	0.66	Yes
43	free IGFII	ng/mL	Immunoassay	MTP ELISA	0.7	0.412	No
44	CES1 ver.5	ng/mL	Immunoassay	cobas e 601	0.69	0.24	No
45	MMP3	ng/mL	Immunoassay	MTP ELISA	0.689	0.22	No
46	MMP3	ng/mL	Immunoassay	IMPACT	0.682	0.288	Yes
47	AFP-L3	ng/mL	LCA lectin-based Immunoassay	IMPACT	0.676	0.153	No
48	Midkine	ng/mL	Immunoassay	R&D Systems MTP ELISA	0.673	0.373	Yes
49	anti-p53	microg/mL	Immunoassay	cobas e 601	0.655	0.288	No
50	S100	ng/mL	Immunoassay	cobas e 601	0.64	0.28	No
51	MMP9	ng/mL	Immunoassay	MTP ELISA	0.634	0.237	No
52	HSP70	ng/mL	Immunoassay	MTP ELISA	0.625	0.39	No
53	Fucosylated Hp	ng/mL	Immunoassay	MTP ELISA	0.62	0.367	No
54	Calcitonin	pg/mL	Immunoassay	cobas e 601	0.59	0.16	No
55	BMP7	ng/mL	Immunoassay	IMPACT	0.577	0.068	Yes
56	Pro-GRP	pg/mL	Immunoassay	cobas e 601	0.56	0.08	No
57	DKK1	ng/mL	Immunoassay	IMPACT	0.540	0.068	Yes
58	TGF1 β	ng/mL	Immunoassay	MTP ELISA	0.532	0.085	No
59	Hp	mg/mL	Clin Chem	cobas c 311	0.51	0.136	No
60	TG-II	ng/mL	Immunoassay	cobas e 601	0.51	0.02	No
61	MMP2/MMP9	ng/mL	Immunoassay	MTP ELISA	0.502	0.085	No
62	SCC	ng/mL	Immunoassay	cobas e 601	0.493	0.017	No
63	CFHR1	microg/mL	Immunoassay	cobas e 601	0.473	0.017	No

AFP, alpha-fetoprotein; AFP-L3, *Lens culinaris* agglutinin-reactive fraction of AFP; ALCAM/CD166, activated leukocyte cell adhesion molecule; ALT, alanine aminotransferase; Ang2, angiopoietin-2; AST, aspartate aminotransferase; BILT, bilirubin; BMP-7, bone morphogenetic protein 7; CES1, carboxylesterase 1;

CFHR1, complement factor H-related protein 1; COMP, cartilage oligomeric matrix protein; DKK1, dickkopf-related protein 1; fucAFP, fucosylated alpha-fetoprotein; GGT2, gamma-lutamyltransferase-2; GP73, golgi membrane protein 1; HGF, hepatocyte growth factor; HR1, complement factor H related 1; HSP70 heat shock protein 70; Hp/fucHp, haptoglobin; IGF1, insulin-like growth factor-1; IGFBP3, Insulin-like growth factor-binding protein 3; IGFBP7, Insulin-like growth factor-binding protein 7; IL6, interleukin 6; IL8, interleukin 8; LRG1, leucine rich alpha-2-glycoprotein 1; midkine, neurite growth-promoting factor-2; MMP-2, matrix metalloprotease-2; MMP-3, matrix metalloprotease-3; MMP9, matrix metalloproteinase-9; OPG, osteoprotegerin; p53, tumour protein 53; PIVKA-II, protein induced by vitamin k absence-II; ProGRP, progastrin-releasing peptide; TG-II, thyroglobulin-II; TGF-1 β , transforming growth factor beta; TIMP1, tissue inhibitor of metalloproteinase-1.

Supplementary Table 2: List of biomarkers prioritized for Panel B without Panel A data

Biomarker	Assay	Platform	Rationale
CES1	Immunoassay	cobas e 601	Not available for panel A
OPN	Immunoassay	cobas e 601	Not available for panel A
MMP3	Immunoassay	cobas e 601	Not available for panel A
Cyfra 21-1	Immunoassay	cobas e 601	Oncology IVD assay
PIVKA-II	Immunoassay	cobas e 601	Oncology IVD assay
HE4	Immunoassay	cobas e 601	Oncology IVD assay
NSE	Immunoassay	cobas e 601	Oncology IVD assay
CA125	Immunoassay	cobas e 601	Oncology IVD assay
CA15-3	Immunoassay	cobas e 601	Oncology IVD assay
CA19-9	Immunoassay	cobas e 601	Oncology IVD assay
CA72-4	Immunoassay	cobas e 601	Oncology IVD assay
IL6	Immunoassay	IMPACT	Not available for panel A
IL10	Immunoassay	IMPACT	Not available for panel A

CA, cancer antigen; CES1, carboxylesterase 1; Cyfra 21-1, cytokeratin 19 fragments; HE4, epididymal protein 4; IL6, interleukin 6; IL10, interleukin 10; MMP3, matrix metalloproteinase-3; NSE, neuron-specific enolase 2; OPN, osteopontin; PIVKA-II, protein induced by vitamin k absence-II.

Supplementary Table 3: Biomarkers selected by lasso regression for diagnosis of all-stage and early-stage HCC

All-stage HCC (n=308) vs control (n=734) [†]						
Optimize	Panel size	Accuracy, % [*]	Sensitivity, % [*]	Specificity, % [*]	Selected marker panel (frequency≥3 shown)	Selected frequency in 100 runs
90 specificity	2	87.4	84.9	90.3	AFP + PIVKA-II	79
	3	86.8	82.2	90.3	AFP + PIVKA-II + GGT	7
	4	86.6	84.9	89.2	AFP + PIVKA-II + GGT + HE4	3
90 sensitivity	2	87.5	89.0	85.2	AFP + PIVKA-II	83
	3	86.7	89.0	85.8	AFP + PIVKA-II + GGT	3
	4	89.5	90.4	87.5	AFP + PIVKA-II + GGT + HE4	4
Early-stage HCC (n=125) vs control (n=734) [†]						
Optimize	Panel size	Accuracy, % [*]	Sensitivity, % [*]	Specificity, % [*]	Selected marker panel (frequency≥3 shown)	Selected frequency in 100 runs
90 specificity	1	70.1	53.3	89.2	AFP	9
	2	80.6	73.3	89.8	AFP + PIVKA-II	78
	3	84.7	80.0	89.5	-	
	4	78.0	66.7	90.3	-	
90 sensitivity	1	70.0	88.3	53.7	AFP	12
	2	80.9	90.0	73.9	AFP + PIVKA-II	76
	3	80.9	83.3	76.7	AFP + PIVKA-II + HE4	3
	4	78.2	83.3	73.3	AFP + PIVKA-II + HE4 + COMP	3

AFP, alpha-fetoprotein; COMP, cartilage oligomeric matrix protein; HCC, hepatocellular carcinoma; HE4, epididymal protein 4; GGT, gamma-lutamytransferase; PIVKA-II, protein induced by vitamin k absence-II.

[†] Samples with missing measurements were excluded from the multivariate analysis.

^{*}Calculated by median values of accuracy, sensitivity at 90% specificity and specificity at 90% sensitivity in the test dataset within 100 runs cross-validation.

- No marker panel shown because the selected frequency of the marker panel was below 3 times.

Supplementary Table 4: Biomarkers selected by exhaustive search for diagnosis of all-stage and early-stage HCC

All-stage HCC (n=308)						
Optimize	Panel size	Accuracy %*	Sensitivity %*	Specificity %*	Selected marker panel (frequency≥6 shown)	Selected frequency in 200 runs
90% specificity	2	86.2	83.6	89.8	AFP + PIVKA-II	133
					PIVKA-II+ COMP	13
					PIVKA-II + CEA	12
					PIVKA-II + ALB	6
90% specificity	3	87.0	84.9	89.8	AFP + PIVKA-II + HE4	16
					AFP + PIVKA-III + GGT2/CA19-9/sAXL	9
					AFP + PIVKA-II+ BILT/IL10/TIMP1	8
					AFP + PIVKA-II + CA72-4/GPC3/MMP2/Sialyltransferase	7
					AFP + PIVKA-II +MMP3/ALT/AST	6
90% specificity	4	87.2	84.9	89.8	AFP + PIVKA-II + OPG + IGFBP3/IL8/ALT	6
90% sensitivity	2	87.4	89.0	85.2	AFP + PIVKA-II	190
					PIVKA-II + GPC3-N	7
90% sensitivity	3	87.9	89.0	86.9	AFP + PIVKA-II + CYFRA	31
					AFP + PIVKA-II + OPG	25
					AFP + PIVKA-II + IGFBP3	14
					AFP + PIVKA-II + COMP	10
					AFP + PIVKA + GP73/HE4	9
					AFP + PIVKA-II + TIMP1	8
					AFP + PIVKA-II + CEA	7
					AFP + PIVKA-II+ CA72-4/ALB/OPN	6
90% sensitivity	4	88.0	89.0	86.9	AFP + PIVKA-II + OPG + IGFBP3/IL8/ALT	6

Early-stage HCC (n=125)						
Optimize	Panel size	Accuracy %*	Sensitivity %*	Specificity %*	Selected marker panel (frequency≥6 shown)	Selected frequency in 200 runs
90% specificity	2	77.9	66.7	89.2	AFP + PIVKA-II	118
					PIVKA-II + COMP	28
					PIVKA-II + ALB/CEA	7
90% specificity	3	80.0	70.0	89.5	AFP + PIVKA-II + MMP2	14
					AFP + PIVKA-II + COMP	13
					AFP + PIVKA-II + GP73	12
					AFP + PIVKA-II + BMP7/OPG	10
					AFP + PIVKA-II + MMP3/GGT/GLDH	9
					AFP + PIVKA-II + IL10	8
					AFP + PIVKA-II + BILT/CA125/CES1/GPC3-N/HGF/Sialyltransferase	6
90% specificity	4	79.5	70.0	89.2	AFP + PIVKA-II +OPG + ALT	9
					AFP + PIVKA-II +OPG + IL8	6
90% sensitivity	2	80.9	90.0	73.3	AFP + PIVKA-II	168
					PIVKA-II + GPC3	16
					AFP + HE4	9
90% sensitivity	3	80.8	88.3	74.4	AFP + PIVKA-II + COMP	34
					AFP + PIVKA-II + MMP3	28
					AFP + PIVKA-II + Midkine	19
					AFP + PIVKA-II + IGFBP3	16
					AFP + PIVKA-II + OPG	15
					AFP + PIVKA-II + HE4	14
					AFP + PIVKA-II + CYFRA	13
90% sensitivity	4	81.2	86.7	76.7	AFP + PIVKA-II + IGFBP3 + IGF1	11
					AFP + PIVKA-II + COMP + MMP3/AST	10

					AFP + PIVKA-II + HE4 + AST	7
					AFP + PIVKA-II + COMP + IL8	6

AFP, alpha-fetoprotein; ALB, albumin; ALT, alanine aminotransferase; AST, aspartate aminotransferase; BMP-7, bone morphogenetic protein 7; CA, cancer antigen; CEA, carcinoembryonic antigen; CYFRA, cytokeratin; GPC3, glypican-3; HE4, epididymal protein 4; IGFBP3, Insulin-like growth factor-binding protein 3; IL8, interleukin 8; MMP3, matrix metalloproteinase-3; OPG, osteoprotegerin; PIVKA-II, protein induced by vitamin k absence-II; HCC, hepatocellular carcinoma.

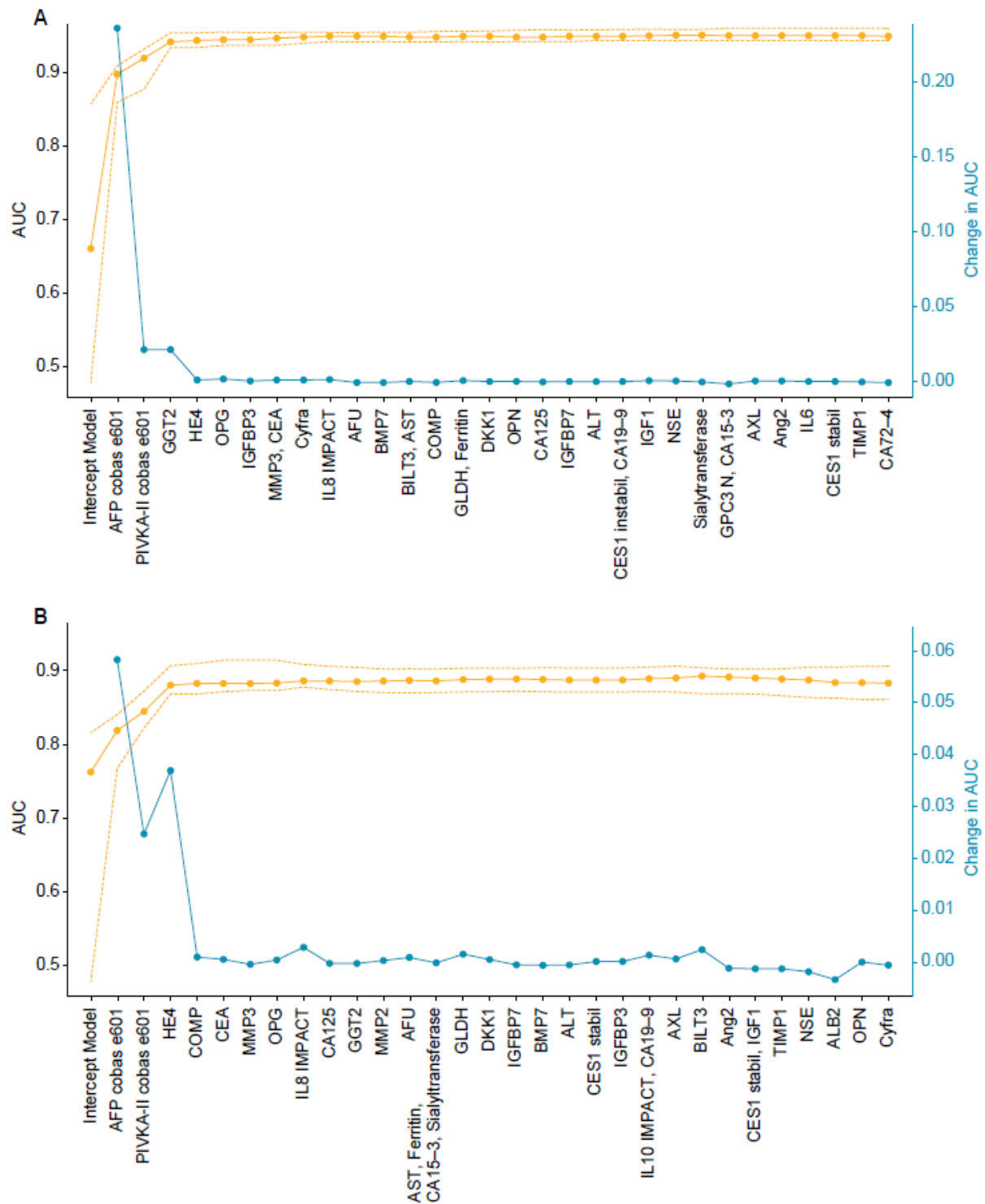
*Calculated by median values of accuracy, sensitivity at 90% specificity and specificity at 90% sensitivity in the test dataset within 200 runs cross-validation.

Supplementary Table 5: Sensitivity and specificity of regression models using three-biomarker combinations with and without age and gender

Biomarker combinations without age and gender										
	All-stage HCC (n=308) vs control (n=734)*					Early-stage HCC (n=125) vs control (n=734)*				
	AUC, %	Specificity, % when sensitivity is fixed at:		Sensitivity, % when specificity is fixed at:		AUC, %	Specificity, % when sensitivity is fixed at:		Sensitivity, % when specificity is fixed at:	
		90%	95%	90%	95%		90%	95%	90%	95%
AFP+PIVKA-II+MMP3	94.4	84.7	74.7	83.8	73.4	89.1	72.9	57.9	65.6	52.8
AFP+PIVKA-II+IGFBP3	94.3	85.8	75.7	85.4	74.0	88.5	74.3	53.8	68.0	52.8
AFP+PIVKA-II+COMP	94.4	85.6	74.8	84.1	73.7	89.0	74.1	50.8	67.2	52.0
AFP+PIVKA-II+AFP-L3	94.1	82.3	73.6	84.4	75.3	88.1	69.1	44.6	68.0	52.0
Biomarker combinations with age and gender										
AFP+PIVKA-II+MMP3	95.3	85.9	78.5	84.4	75.6	90.7	75.1	67.9	67.2	54.4
AFP+PIVKA-II+IGFBP3	95.3	85.8	80.8	87.0	76.0	90.6	77.4	64.7	71.2	52.0
AFP+PIVKA-II+COMP	95.4	85.5	80.2	85.7	74.4	90.9	80.2	68.0	72.8	52.8
AFP+PIVKA-II+AFP-L3	95.2	84.1	78.6	86.0	76.6	90.5	77.7	65.4	71.2	53.6

*Samples with missing measurements were excluded from the multivariate analysis.
 AFP, alpha-fetoprotein; AFP-L3, *Lens culinaris* agglutinin-reactive fraction of AFP; AUC, area under the curve; COMP, cartilage oligomeric matrix protein; IGFBP3, Insulin-like growth factor-binding protein 3; MMP3, matrix metalloproteinase-3; PIVKA-II, protein induced by vitamin k absence-II; HCC, hepatocellular carcinoma.

Supplementary Figure 1: Biomarker selected by lasso regression for diagnosis of all-stage HCC (A) and early-stage HCC (B)

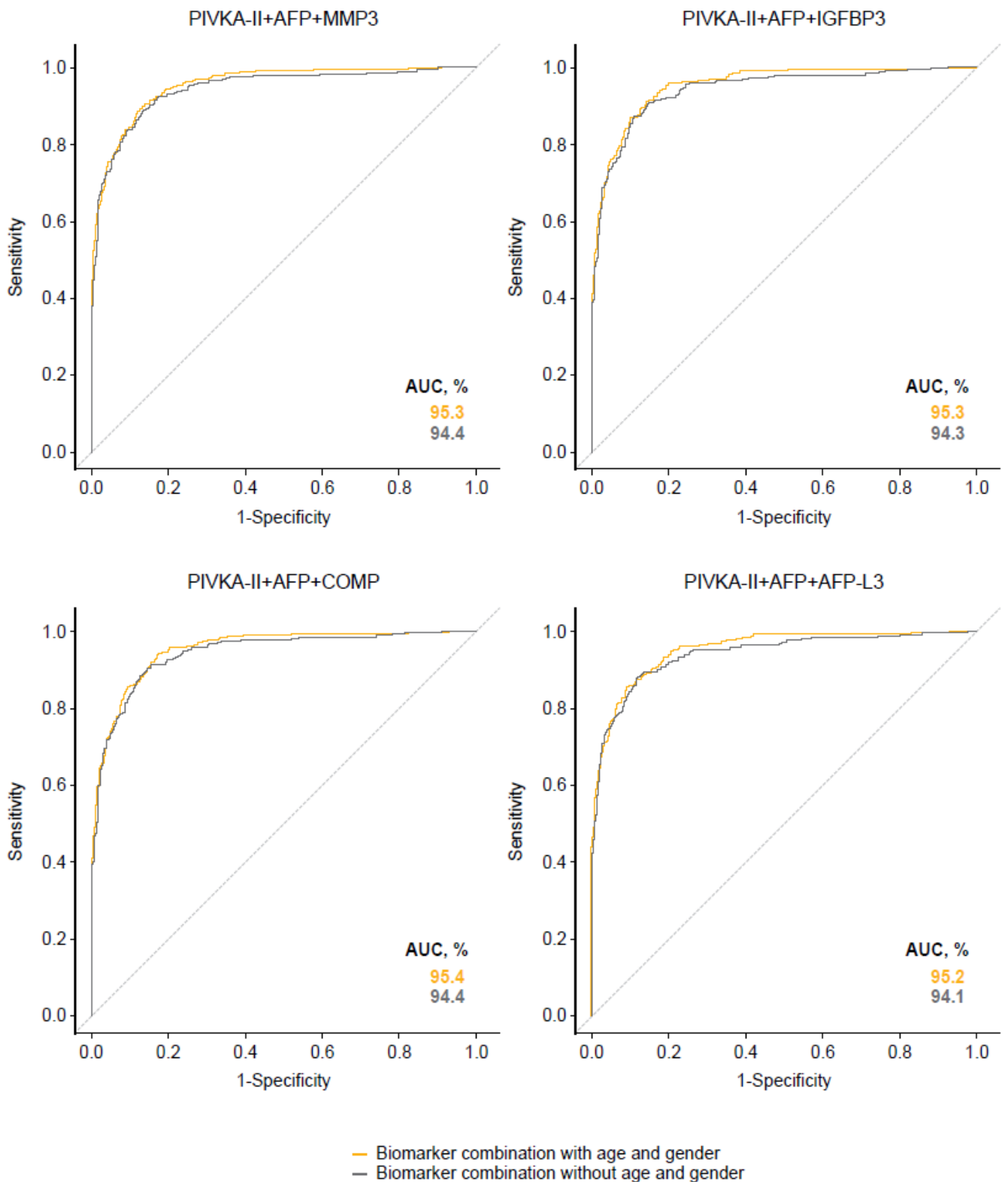


AFP, alpha-fetoprotein; AFU, alpha-1-fucosidase; ALB2, serum albumin; ALT, alanine aminotransferase; Ang2, angiopoietin-2; AST, aspartate aminotransferase; AUC, area under the curve; BILT3, bilirubin total generation 3; BMP-7, bone morphogenetic protein 7; CA15-3, cancer antigen 15-3; CA19-9, cancer antigen 19-9; CA125, cancer antigen 125; CEA, carcinoembryonic antigen; CES1, carboxylesterase 1; COMP, cartilage oligomeric matrix protein; Cyfra, cytokeratin; DKK1, dickkopf-related protein 1; GGT2, gamma-lutamyltransferase-2; GLDH, glutamate

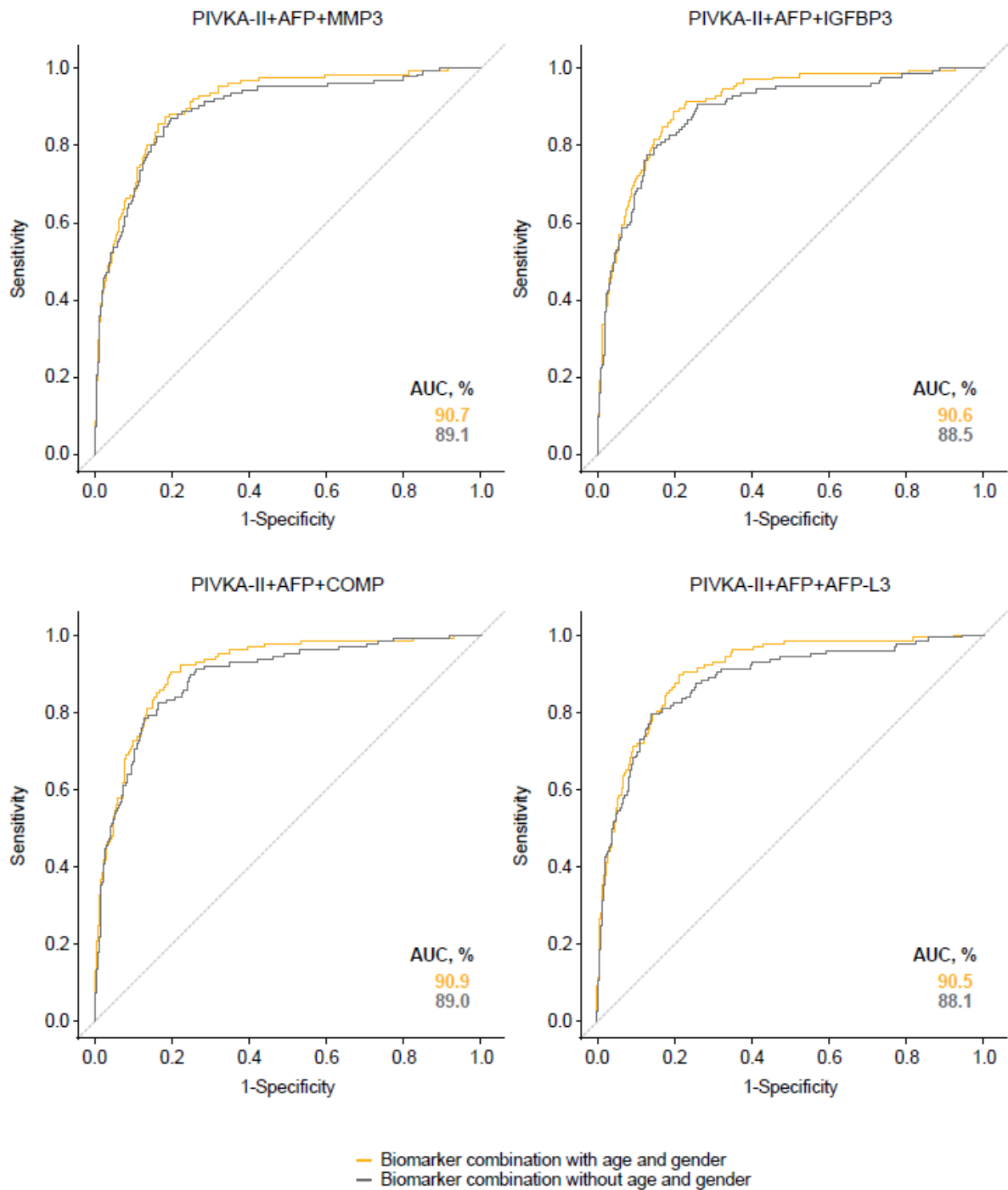
dehydrogenase; GP73, golgi membrane protein 1; GPC3 N, glypican 3; HCC, hepatocellular carcinoma; HE4, human epididymis protein 4; IGF1, insulin-like growth factor-1; IGFBP3, Insulin-like growth factor-binding protein 3; IGFBP7, Insulin-like growth factor-binding protein 7; IL6, interleukin 6; IL8, interleukin 8; IL10, interleukin 10; MMP-2, matrix metalloprotease-2; MMP-3, matrix metalloprotease-3; NSE, neuron-specific endolase; OPG, osteoprotegerin; OPN, osteopontin; PIVKA-II, protein induced by vitamin k absence-II; TIMP1, tissue inhibitor of metalloproteinase-1.

Supplementary Figure 2: Three-biomarker combinations with age and gender vs without age and gender for all-stage HCC (A) and early-stage HCC (B)

A. All-stage HCC vs control



B. Early-stage HCC vs control



AFP, alpha-fetoprotein; AFP-L3, *Lens culinaris* agglutinin-reactive fraction of AFP; AUC, area under the curve; COMP, cartilage oligomeric matrix protein; IGFBP3, Insulin-like growth factor-binding protein 3; MMP3, matrix metalloproteinase-3; PIVKA-II, protein induced by vitamin k absence-II; HCC, hepatocellular carcinoma.

Red curve biomarker panel with age and gender, Black curve biomarker panel without age and gender.