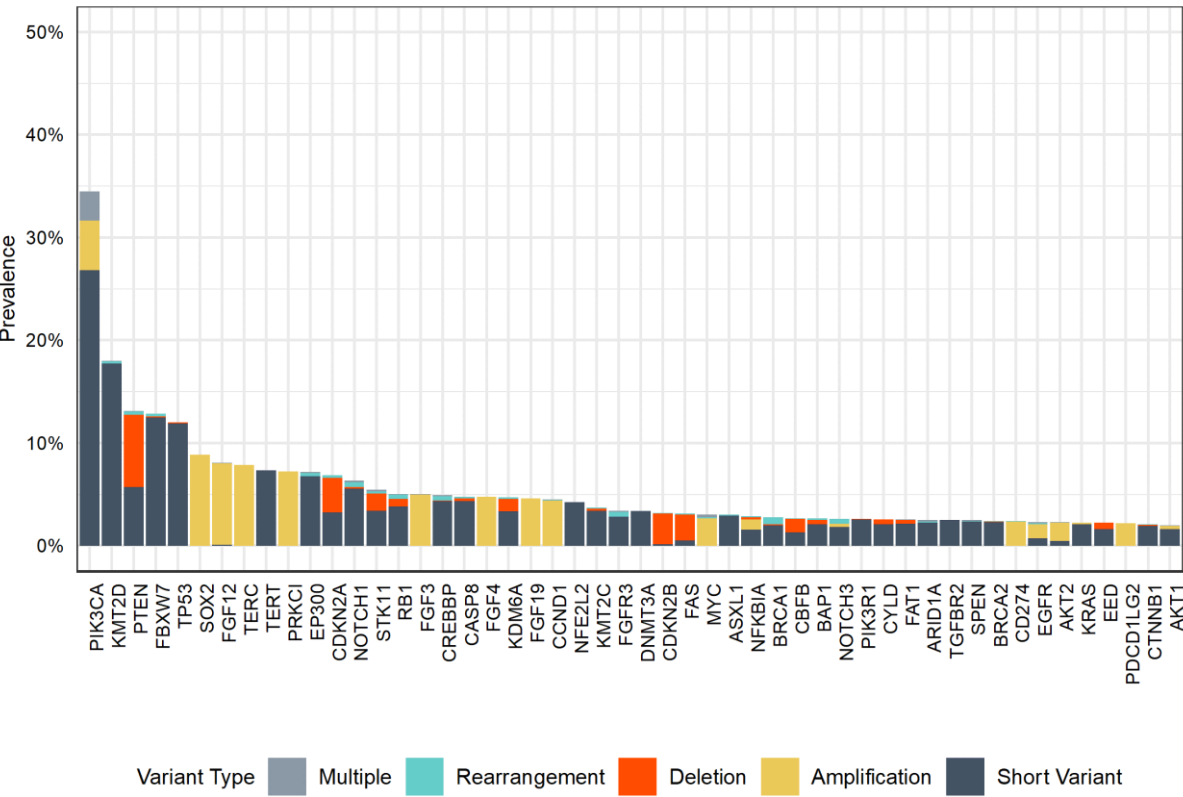


SUPPLEMENTARY FILE

Comprehensive Molecular Landscape of Anal Squamous Cell Carcinoma: Analysis of Tissue and Liquid Biopsies from 1,844 Patients

SUPPLEMENTARY FIGURES

Supplementary Figure 1

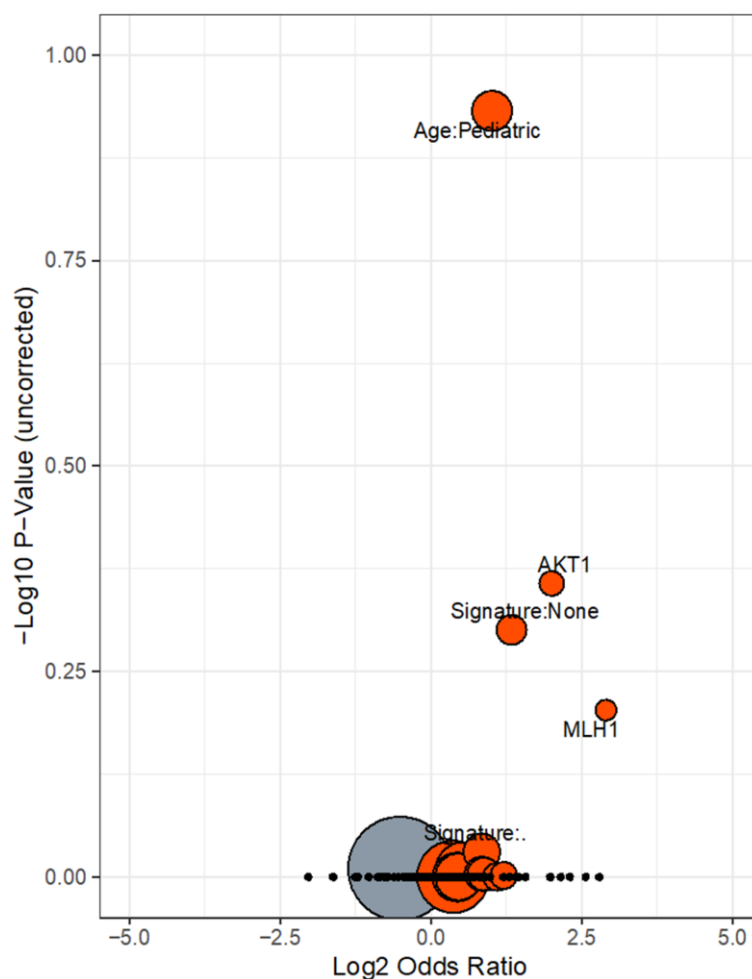


Supplementary Figure 1. 50 most altered genes in the tissue biopsy cohort

Bar plot displaying the prevalence of alterations in the 50 most frequently altered genes within the tissue biopsy cohort

Supplementary Figure 2

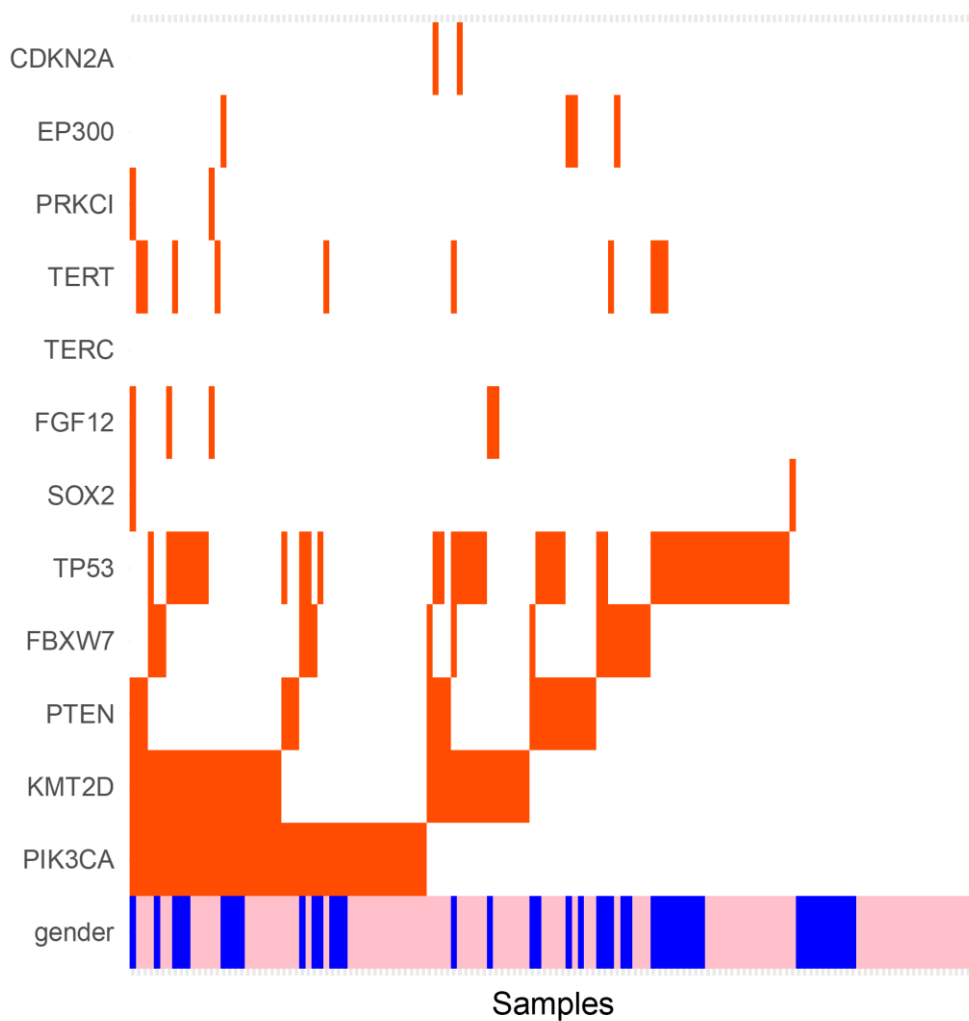
Negatively enriched in metastatic disease ← → Positively enriched in metastatic disease



Supplementary Figure 2. Enriched characteristics in metastatic vs. primary tissue

Volcano plot highlighting patterns of enrichment and depletion of recurrent clinical and molecular parameters in metastatic sites compared to primary tumors

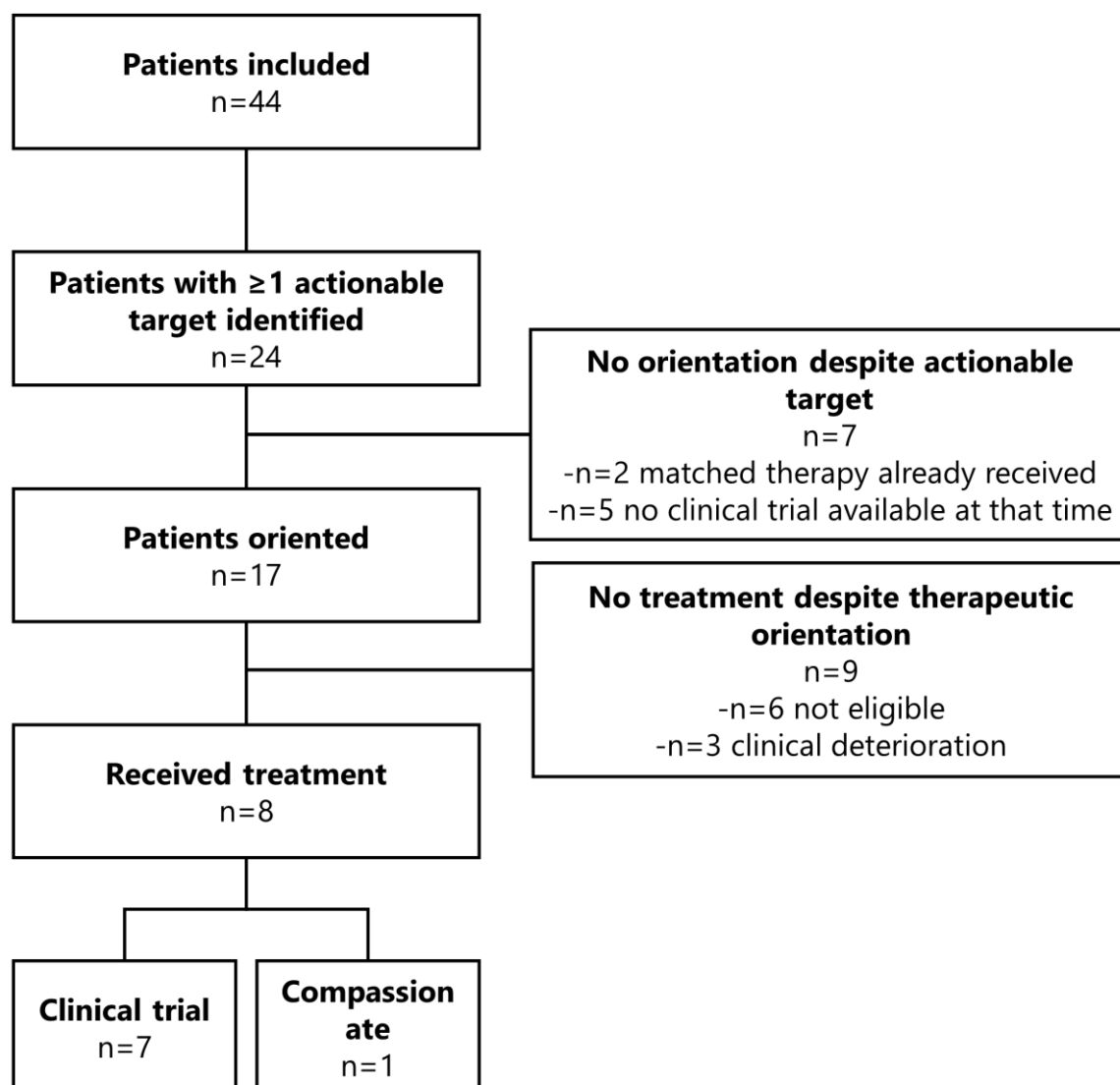
Supplementary Figure 3



Supplementary Figure 3. Distribution of genomic alterations in the liquid biopsy cohort

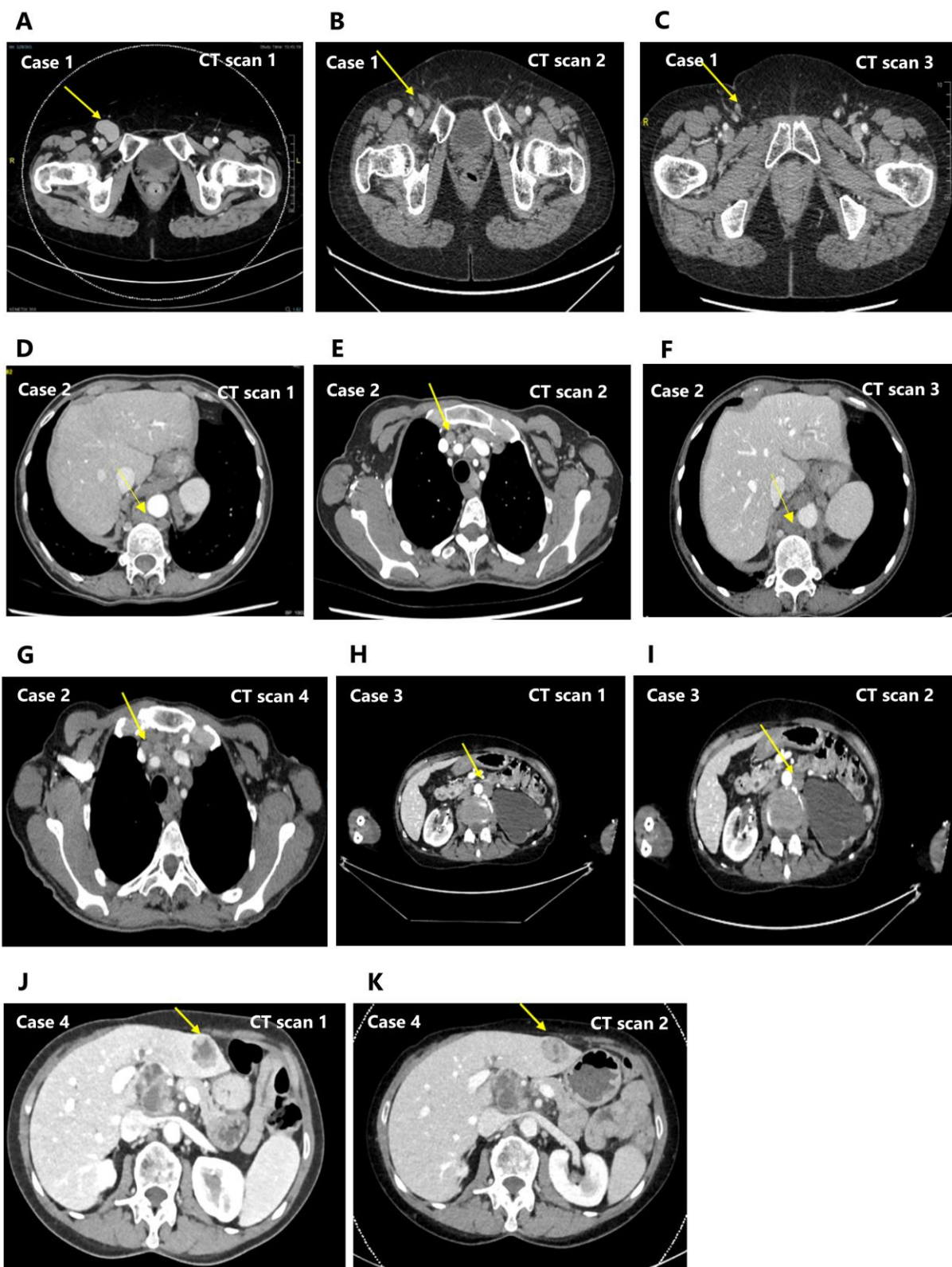
Oncoplot showing the distribution of the 12 most frequently altered genes in aSCC within the liquid biopsy cohort

Supplementary Figure 4



Supplementary Figure 4. Flow chart of the clinically annotated cohort

Supplementary Figure 5



Supplementary Figure 5. CT scan from the four clinical cases presented

a, b, and c. CT scans from Case 1, showing the baseline, nadir RECIST (-72%), and nadir iRECIST (-90%) CT scans during treatment, respectively.

d, e, f and g. CT scans from Case 2, showing the baseline CT scan with mediastinal lymph node involvement, followed by the best response (-21%), which reveals more hypodense lesions suggestive of a therapeutic response.

h and i. Showing the baseline CT scan and the first follow-up CT scan respectively for Case 3, which indicates stable disease as the best response.

j and k. Showing the baseline liver disease and the first CT scan evaluation for Case 4, which indicates stable disease.

SUPPLEMENTARY TABLES

Genes targeted by Foundation One CDx and Foundation One Liquid CDx assays (n=324)								
ABL1	CARD11	DDR2	FGFR1	JAK3	MUTYH	POLE	SMARCA4	BTG2
ACVR1B	CASP8	DIS3	FGFR2	JUN	MYB	PPARG	SMARCB1	CALR
AKT1	CBFB	DNMT3A	FGFR3	KDM5A	MYC	PPP2R1A	SMO	CD22
AKT2	CBL	DOT1L	FGFR4	KDM5C	MYCL	PRDM1	SNCAIP	CD70
AKT3	CCND1	EGFR	FH	KDM6A	MYCN	PRKAR1A	SOC51	CD74
ALK	CCND2	EMSY	FLCN	KDR	MYD88	PRKCI	SOX2	CSF3R
ALOX12B	CCND3	EP300	FLT1	KEAP1	NBN	PRKN	SOX9	CXCR4
AMER1	CCNE1	EPHA3	FLT3	KEL	NF1	PTCH1	SPEN	EED
APC	CD274	EPHB1	FOXL2	KIT	NF2	PTEN	SPOP	EZR
AR	CD79A	EPHB4	FUBP1	KLHL6	NFE2L2	PTPN11	SRC	HDAC1
ARAF	CD79B	ERBB2	GABRA6	KMT2A	NFKBIA	QKI	STAG2	ID3
ARFRP1	CDC73	ERBB3	GATA3	KMT2D	NKX2-1	RAC1	STAT3	MAF
ARID1A	CDH1	ERBB4	GATA4	KRAS	NOTCH1	RAD51	STK11	MAPK1
ASXL1	CDK12	ERCC4	GATA6	LTK	NOTCH2	RAD51B	SUFU	MSH3
ATM	CDK4	ERG	GID4	LYN	NOTCH3	RAD51C	SYK	MTAP
ATR	CDK6	ERRFI1	GNA11	MAP2K1	NPM1	RAD51D	TBX3	NSD2
ATRX	CDK8	ESR1	GNA13	MAP2K2	NRAS	RAD52	TEK	NSD3
AURKA	CDKN1A	ETV4	GNAQ	MAP2K4	NTRK1	RAD54L	TERC	NT5C2
AURKB	CDKN1B	ETV5	GNAS	MAP3K1	NTRK2	RAF1	TERT	NUTM1
AXIN1	CDKN2A	ETV6	GRM3	MAP3K13	NTRK3	RARA	TET2	P2RY8
AXL	CDKN2B	EWSR1	GSK3B	MCL1	PALB2	RB1	TGFBR2	PDCD1
BAP1	CDKN2C	EZH2	H3F3A	MDM2	PARP1	RBM10	TIPARP	PIM1
BARD1	CEBPA	FAM46C	HGF	MDM4	PARP2	REL	TMPRSS2	PPP2R2A
BCL2	CHEK1	FANCA	HNF1A	MED12	PARP3	RET	TNFAIP3	PTPRO
BCL2L1	CHEK2	FANCC	HRAS	MEF2B	PAX5	RICTOR	TNFRSF14	RAD21
BCL2L2	CIC	FANCG	HSD3B1	MEN1	PBRM1	RNF43	TP53	SDC4
BCL6	CREBBP	FANCL	IDH1	MERTK	PDCD1LG2	ROS1	TSC1	SGK1
BCOR	CRKL	FAS	IDH2	MET	PDGFRA	RPTOR	TSC2	SLC34A2
BCORL1	CSF1R	FBXW7	IGF1R	MITF	PDGFRB	RSPO2	TYRO3	
BCR	CTCF	FGF10	IKBKE	MKNK1	PDK1	SDHA	U2AF1	
BRAF	CTNNA1	FGF12	IKZF1	MLH1	PIK3C2B	SDHB	VEGFA	
BRCA1	CTNNB1	FGF14	INPP4B	MPL	PIK3C2G	SDHC	VHL	
BRCA2	CUL3	FGF19	IRF2	MRE11	PIK3CA	SDHD	WT1	
BRD4	CUL4A	FGF23	IRF4	MSH2	PIK3CB	SETD2	XPO1	
BRIP1	CYP17A1	FGF3	IRS2	MSH6	PIK3R1	SF3B1	XRCC2	
BTG1	DAXX	FGF4	JAK1	MST1R	PMS2	SMAD2	ZNF217	
BTK	DDR1	FGF6	JAK2	MTOR	POLD1	SMAD4	ZNF703	

Supplementary Table 1: Genes analyzed as part of this study

		Tissue (n=1733)	Liquid (n=140)	Clinical (n=44)
Age	Median [IQR]	61 [54-69]	63 [55-69]	58 [54-63]
Gender	Males	532 (30.7%)	45 (32.1%)	15 (34.1%)
	Females	1201 (69.3%)	95 (67.9%)	29 (65.9%)

Supplementary Table 2: Age and Gender of the Overall Cohort (n=1844 patients)

Genotype	Count	Percent
HPV-16	1315	75.9
HPV-18	62	3.6
HPV-6	33	1.9
HPV-45	22	1.3
HPV-33	21	1.2
HPV-35	13	0.8
HPV-31	9	0.5
HPV-11	6	0.3
HPV-67	6	0.3
HPV-58	5	0.3
HPV-59	2	0.1
HPV-73	2	0.1
HPV-34	1	0.1
HPV-39	1	0.1
HPV-44	1	0.1
HPV-68	1	0.1
negative	233	13.4

Supplementary Table 3: HPV-genotypes identified in the tissue biopsy cohort (n=1733 patients)

Count of males			Count of females		
532 (30.7%)			1201 (69.3%)		
HPV positive	HPV negative		HPV positive	HPV negative	
400 (75.2%)	132 (24.8%)		1100 (91.6%)	101 (8.4%)	
Males	Count	Percent	Females	Count	Percent
HPV-16	329	61.8	HPV-16	986	82.1
HPV-18	18	3.4	HPV-18	44	3.7
HPV-33	5	0.9	HPV-33	16	1.3
HPV-45	12	2.3	HPV-45	10	0.8
HPV-6	21	3.9	HPV-6	12	1
Other HPV	15	2.8	Other HPV	32	2.7
negative	132	24.8	negative	101	8.4

Supplementary Table 4: HPV-genotypes by gender in the tissue biopsy cohort (n=1733 patients)

Gene	SNV count	Amplification count	Homozygous deletion count	Rearrangement count	Multiple alteration count	Total Alt percent
<i>PIK3CA</i>	465	83	0	0	50	34.5
<i>KMT2D</i>	307	0	0	4	1	18.0
<i>PTEN</i>	99	0	122	5	1	13.1
<i>FBXW7</i>	216	0	2	5	0	12.9
<i>TP53</i>	206	0	2	0	0	12.0
<i>SOX2</i>	0	154	0	0	0	8.9
<i>FGF12</i>	2	137	0	0	1	8.1
<i>TERC</i>	0	136	0	0	0	7.8
<i>TERT</i>	127	0	0	0	0	7.3
<i>PRKCI</i>	0	125	0	0	0	7.2
<i>EP300</i>	117	0	0	6	1	7.2
<i>CDKN2A</i>	56	0	58	5	0	6.9
<i>NOTCH1</i>	96	0	3	8	3	6.3
<i>STK11</i>	59	0	29	4	2	5.4
<i>RB1</i>	66	0	13	7	1	5.0

Supplementary Table 5: Prevalence of the 15 most altered genes in the tissue biopsy cohort (n=1733 patients)

Aneuploidy	Type	Percent	Aneuploidy	Type	Percent
chr1p	loss	3.8	chr1p	gain	16.8
chr1q	loss	1.4	chr1q	gain	32.4
chr2p	loss	3.1	chr2p	gain	8.2
chr2q	loss	6.7	chr2q	gain	4.5
chr3p	loss	39.4	chr3p	gain	3.0
chr3q	loss	1.3	chr3q	gain	67.2
chr4p	loss	26.8	chr4p	gain	4.4
chr4q	loss	13.5	chr4q	gain	6.9
chr5p	loss	3.1	chr5p	gain	29.4
chr5q	loss	11.3	chr5q	gain	9.0
chr6p	loss	10.1	chr6p	gain	11.2
chr6q	loss	13.5	chr6q	gain	6.8
chr7p	loss	5.5	chr7p	gain	13.4
chr7q	loss	9.6	chr7q	gain	10.3
chr8p	loss	26.1	chr8p	gain	13.5
chr8q	loss	1.9	chr8q	gain	41.3
chr9p	loss	11.3	chr9p	gain	15.3
chr9q	loss	5.5	chr9q	gain	15.1
chr10p	loss	17.6	chr10p	gain	3.4
chr10q	loss	18.8	chr10q	gain	1.4
chr11p	loss	17.7	chr11p	gain	6.7
chr11q	loss	49.6	chr11q	gain	4.1
chr12p	loss	7.9	chr12p	gain	8.5
chr12q	loss	5.0	chr12q	gain	5.8
chr13q	loss	20.9	chr13q	gain	9.5
chr14q	loss	11.4	chr14q	gain	10.6
chr15q	loss	11.4	chr15q	gain	7.0
chr16p	loss	8.5	chr16p	gain	13.7
chr16q	loss	28.2	chr16q	gain	6.5
chr17p	loss	21.3	chr17p	gain	3.0
chr17q	loss	4.9	chr17q	gain	12.4
chr18p	loss	6.6	chr18p	gain	17.1
chr18q	loss	10.0	chr18q	gain	16.0
chr19p	loss	14.7	chr19p	gain	5.3
chr19q	loss	8.9	chr19q	gain	7.9
chr20p	loss	5.4	chr20p	gain	24.0
chr20q	loss	5.8	chr20q	gain	23.4
chr21q	loss	8.5	chr21q	gain	8.9
chr22q	loss	13.7	chr22q	gain	7.1

Supplementary Table 6: Prevalence of chromosomal copy-number loss and gain in the tissue biopsy cohort (n=1733 patients)

Count of samples without alteration			Count of samples with alterations		
113 (6.5%)			1620 (93.5%)		
HPV positive	HPV negative		HPV positive	HPV negative	
109 (96.5%)	4 (3.5%)		1391 (85.9%)	229 (14.1%)	
No alteration	Count	Percent	Alterations	Count	Percent
HPV-16	101	89.4	HPV-16	1214	74.9
HPV-18	3	2.7	HPV-18	59	3.6
HPV-33	1	0.9	HPV-33	20	1.2
HPV-45	3	2.7	HPV-45	19	1.2
HPV-6	1	0.9	HPV-6	32	2
Other HPV	0	0	Other HPV	47	2.9
negative	4	3.5	negative	229	14.1

Supplementary Table 7: Prevalence of molecular alteration by HPV status in the tissue biopsy cohort (n=1733 patients)

Genotype	<i>PIK3CA</i>	<i>KMT2D</i>	<i>PTEN</i>	<i>FBXW7</i>	<i>TP53</i>	<i>TERT</i>	<i>CDKN2A</i>	TMB-high
HPV-16	39.0	20.8	14.5	13.8	4.3	1.9	1.3	17.4
HPV-45	13.6	9.1	4.5	9.1	0.0	0.0	0.0	9.1
HPV-6	18.2	15.2	6.1	9.1	39.4	45.5	54.5	24.2
HPV-18	24.2	4.8	16.1	14.5	11.3	3.2	0.0	21.0
negative	19.3	8.6	6.9	5.6	53.2	34.8	33.9	12.9
HPV-33	23.8	9.5	19.0	23.8	0.0	0.0	0.0	19.0

Supplementary Table 8: Proportion of molecular alteration by HPV status in the tissue biopsy cohort (n=1733 patients)

Alteration	count	Prevalence	OncoKB	ESCAT
PIK3CA_E545_or_E542	407	23.5	3B	3A
TMB-high	296	17.1	1	1C
TP53_non_Y220	250	14.4	4	4
PTEN	237	13.7	4	3A
PIK3CA_other_mut	165	9.5	4	3A
CDKN2A	121	7	4	4
CDKN2B	55	3.2	4	4
BRCA1	53	3.1	3B	3A
FGFR3_mutation	52	3	4	1C
ARID1A	47	2.7	4	4
BRCA2	45	2.6	3B	3A
CD274_amplification	41	2.4	4	3B
KRAS_non_G12C	35	2	4	4
AKT1	35	2	3B	3B
NF1	32	1.8	4	4
CDK12	26	1.5	4	4
EGFR_amplification	25	1.4	4	3B
ERBB2_amplification	17	1	3A	3A
PALB2	17	1	3B	3A
MSI-H	15	0.9	1	1C
BRAF_non_V600E	15	0.9	4	3B
EGFR_mutation	15	0.9	4	3B
FGFR1_amplification	13	0.8	4	1C
MTOR_mutation	12	0.7	4	4
NRAS	12	0.7	3B	4
FGFR3_fusion	10	0.6	3B	1C
CCNE1	9	0.5	4	4
ERBB2_mutation	9	0.5	3A	3A
TSC2	7	0.4	3B	4
FGFR2_mutation	7	0.4	4	1C
MTOR_E2014K/2419K/2209V/2427Q/2223K	5	0.3	4	4
MDM2	4	0.2	4	3B
NTRK1	3	0.2	1	1C
KRAS_G12C	3	0.2	3A	3A
IDH1	3	0.2	3B	3A
KIT_mutation	3	0.2	3B	3A
TP53_Y220	3	0.2	3A	3B
FLT3	2	0.1	4	4
NTRK2	2	0.1	1	1C
NTRK3	2	0.1	1	1C
FGFR1_mutation	2	0.1	4	1C
FGFR2_amplification	2	0.1	4	1C
MAP2K1	1	0.1	3B	4
BRAF_V600E	1	0.1	1	1C
RET_fusion	1	0.1	1	1C
FGFR3_amplification	1	0.1	4	1C
ALK_fusion	1	0.1	3B	3A
SMARCB1_deletion	1	0.1	3B	3B

Supplementary Table 9: Prevalence of actionable alterations in the tissue biopsy cohort (n=1733 patients)

Gene	SNV count	Amplification count	Homozygous deletion count	Rearrangement count	Multiple alteration count	Total Alt percent
<i>PIK3CA</i>	33	1	0	0	15	35.0
<i>KMT2D</i>	34	0	0	0	8	30.0
<i>PTEN</i>	13	0	3	2	3	15.0
<i>FBXW7</i>	16	0	0	0	2	12.9
<i>TP53</i>	37	0	0	0	13	35.7
<i>SOX2</i>	0	2	0	0	0	1.4
<i>FGF12</i>	0	5	0	0	0	3.6
<i>TERC</i>	0	0	0	0	0	0.0
<i>TERT</i>	10	0	0	0	0	7.1
<i>PRKCI</i>	0	2	0	0	0	1.4
<i>EP300</i>	4	0	0	0	0	2.9
<i>CDKN2A</i>	2	0	0	0	0	1.4
<i>NOTCH1</i>	8	0	0	0	0	5.7
<i>STK11</i>	3	0	0	0	2	3.6
<i>RB1</i>	11	0	0	2	0	9.3

Supplementary Table 10: Prevalence of the 15 most altered genes in the liquid biopsy cohort (n=140 patients)

Alteration	count	Prevalence	OncoKB	ESCAT
TP53_non_Y220	62	44.3	4	4
PIK3CA_E545_or_E542	46	32.9	3B	3A
TMB-high	43	30.7	1	1C
PTEN	25	17.9	4	3A
PIK3CA_other_mut	20	14.3	4	3A
NF1	6	4.3	4	4
ARID1A	6	4.3	4	4
BRAF_non_V600E	5	3.6	4	3B
TP53_Y220	5	3.6	3A	3B
KRAS_non_G12C	4	2.9	4	4
NRAS	4	2.9	3B	4
EGFR_mutation	3	2.1	4	3B
CD274_amplification	3	2.1	4	3B
NTRK3	2	1.4	1	1C
CDKN2A	2	1.4	4	4
CDK12	2	1.4	4	4
FGFR3_mutation	2	1.4	4	1C
ERBB2_mutation	2	1.4	3A	3A
MAP2K1	2	1.4	3B	4
BRCA2	2	1.4	3B	3A
PALB2	2	1.4	3B	3A
AKT1	2	1.4	3B	3B
BRAF_V600E	1	0.7	1	1C
NTRK2	1	0.7	1	1C
MTOR_mutation	1	0.7	4	4
FGFR1_mutation	1	0.7	4	1C
EGFR_amplification	1	0.7	4	3B
ERBB2_amplification	1	0.7	3A	3A
TSC2	1	0.7	3B	4
BRCA1	1	0.7	3B	3A
IDH1	1	0.7	3B	3A
KIT_mutation	1	0.7	3B	3A
FANCA_mutation	1	0.7	3B	3A
RAD51_mutation	1	0.7	3B	3A
BRIP1_mutation	1	0.7	3B	3A

Supplementary Table 11: Prevalence of actionable alterations in the liquid biopsy cohort (n=140 patients)

ID	Actionable target	Actionable target 1	ESCAT target 1	Actionable target 2	ESCAT target 2	Actionable target 3	ESCAT target 3	Actionable target 4	ESCAT target 4	Actionable target 5	ESCAT target 5	Actionable target 6	ESCAT target 6	MTB orientation	No orientation despite actionable target	Treatment recommended by MTB	Patient oriented - Modality of recommendation	Treatment received	No treatment despite therapeutic orientation	Best response	PFS
27	NO	-	-	-	-	-	-	-	-	-	-	-	-	NO	-	-	-	-	-	-	-
159	YES	ERBB2 amplification (21 copies)	IIIB	PIK3CA E545K (0.1%)	IIIA	-	-	-	-	-	-	-	-	YES	-	HER2 inhibitor	Compassionate drug	Trastuzumab	-	PD	NA
339	NO	-	-	-	-	-	-	-	-	-	-	-	-	NO	-	-	-	-	-	-	-
501	YES	TMB high (15 mut/Mb)	IC	BRCA2 P1171fs*8 (0.14%)	IIIA	PIK3CA E545K (21.0%)	IIIA	-	-	-	-	-	-	NO	Matched therapy already received	-	-	-	-	-	-
608	YES	TMB high (18 mut/Mb)	IC	PTEN loss	IIIA	-	-	-	-	-	-	-	-	NO	Matched therapy already received	-	-	-	-	-	-
910	NO	-	-	-	-	-	-	-	-	-	-	-	-	NO	-	-	-	-	-	-	-
1000	NO	-	-	-	-	-	-	-	-	-	-	-	-	NO	-	-	-	-	-	-	-
1030	YES	PTEN R130Q	IIIA	PTEN loss	IIIA	-	-	-	-	-	-	-	-	NO	No clinical trial available at that time	-	-	-	-	-	-
1188	NO	-	-	-	-	-	-	-	-	-	-	-	-	NO	-	-	-	-	-	-	-
1227	YES	TMB high (18 mut/Mb)	IC	KIT K558N (1.7%)	IIIA	CDK12 E187_E189>KR* (0.35%)	IV	PIK3CA E542K (3.0%), E726K (1.5%)	IIIA	-	-	-	-	YES	-	Immunotherapy	Clinical trial	Durvalumab + Tremelimumab	-	PR	4 years
1470	YES	BRAF G469R (9.7 %)	IIIB	-	-	-	-	-	-	-	-	-	-	YES	-	MEK inhibiteur	Clinical trial	MEK inhibitor	-	PD	NA
1482	YES	RAD51C A354fs*34 (48.7 %)	IIIA	EGFR V765M (13.6 %)	IIIB	-	-	-	-	-	-	-	-	YES	-	PARP inhibitor	Clinical trial	Niraparib + Dostarlimab	-	SD	6 months
1588	YES	PIK3CA E542K (0.20 %)	IIIA	-	-	-	-	-	-	-	-	-	-	NO	No clinical trial available at that time	-	-	-	-	-	-
1823	NO	-	-	-	-	-	-	-	-	-	-	-	-	NO	-	-	-	-	-	-	-
2358	YES	PIK3CA H1047L (4.9 %)	IIIA	-	-	-	-	-	-	-	-	-	-	NO	No clinical trial available at that time	-	-	-	-	-	-
2367	NO	-	-	-	-	-	-	-	-	-	-	-	-	NO	-	-	-	-	-	-	-
2521	NO	-	-	-	-	-	-	-	-	-	-	-	-	NO	-	-	-	-	-	-	-
2664	YES	TMB high (21 mut/Mb)	IC	PIK3CA E545K (26.7 %)	IIIA	-	-	-	-	-	-	-	-	YES	-	Immunotherapy	Clinical trial	Durvalumab + Tremelimumab	-	PD	NA
2790	YES	TMB high (18 mut/Mb)	IC	PIK3CA E542K (20.2 %)	IIIA	-	-	-	-	-	-	-	-	YES	-	Immunotherapy	Clinical trial	NO	Not eligible for a trial	-	-
3106	NO	-	-	-	-	-	-	-	-	-	-	-	-	NO	-	-	-	-	-	-	-
3288	YES	TMB high (11 mut/Mb)	IC	PTEN loss	IIIA	-	-	-	-	-	-	-	-	YES	-	Immunotherapy	Clinical trial	NO	Clinical deterioration	-	-
3307	NO	-	-	-	-	-	-	-	-	-	-	-	-	NO	-	-	-	-	-	-	-
3634	YES	TMB high (11 mut/Mb)	IC	PIK3CA E542K (5.9 %)	IIIA	AKT1 E17K (2.7 %)	IIIB	-	-	-	-	-	-	YES	-	Immunotherapy	Clinical trial	NO	Clinical deterioration	-	-
3785	YES	MAP2K4 splice site 1074_1086+43del56 (2.2 %)	4	PIK3CA E545K (2.7 %)	IIIA	-	-	-	-	-	-	-	-	NO	No clinical trial available at that time	-	-	-	-	-	-
3937	NO	-	-	-	-	-	-	-	-	-	-	-	-	NO	-	-	-	-	-	-	-
4037	YES	TMB high (20 mut/Mb)	IC	PIK3CA G914R (6.1 %), E542K (13.6 %)	IIIA	MTAP Q103* (0.54 %)	-	-	-	-	-	-	-	YES	-	Immunotherapy	Clinical trial	NO	Not eligible for a trial	-	-
4262	NO	-	-	-	-	-	-	-	-	-	-	-	-	NO	-	-	-	-	-	-	-
4552	NO	-	-	-	-	-	-	-	-	-	-	-	-	NO	-	-	-	-	-	-	-
4981	YES	TMB high (14 mut/Mb)	IC	ERBB2 V842I (14.9 %)	IIIA	BRAF R671Q (0.24 %)	IIIB	FANCA S4* (49 %)	IIIB	PIK3CA E726K (10 %), E453K (10.6 %), E542K (26.6 %)	IIIA	FGFR3 S249C (0.23 %)	IC	YES	-	PIK3CA	Clinical trial	NO	Not eligible for a trial	-	-
5519	NO	-	-	-	-	-	-	-	-	-	-	-	-	NO	-	-	-	-	-	-	-
5680	YES	PIK3CA E545K (5.7 %), E453K (0.15%)	IIIA	-	-	-	-	-	-	-	-	-	-	YES	-	PIK3CA	Clinical trial	PIK3CA inhibitor	-	SD	5 months
5801	NO	-	-	-	-	-	-	-	-	-	-	-	-	NO	-	-	-	-	-	-	-
6267	NO	-	-	-	-	-	-	-	-	-	-	-	-	NO	-	-	-	-	-	-	-
6546	YES	BRIP1 M1V (50 %)	IIIA	PIK3CA C420R (1.5 %)	IIIA	-	-	-	-	-	-	-	-	NO	No clinical trial available at that time	-	-	-	-	-	-
6860	NO	-	-	-	-	-	-	-	-	-	-	-	-	NO	-	-	-	-	-	-	-
6863	YES	FGFR2 K659N (0.43 %)	IC	-	-	-	-	-	-	-	-	-	-	YES	-	FGFR2 inhibitor	Clinical trial	FGFR2 inhibitor	-	SD	4 months
7303	YES	FGFR3 rearrangement intron 17 (0.62 %)	IC	MAPK1 E322K (0.68 %)	IV	-	-	-	-	-	-	-	-	YES	-	FGFR3 inhibitor	Clinical trial	NO	Clinical deterioration	-	-
7678	NO	-	-	-	-	-	-	-	-	-	-	-	-	NO	-	-	-	-	-	-	-
8095	NO	-	-	-	-	-	-	-	-	-	-	-	-	NO	-	-	-	-	-	-	-
8161	NO	-	-	-	-	-	-	-	-	-	-	-	-	NO	-	-	-	-	-	-	-
8452	YES	FGFR3 S249C (3.9 %)	IC	PIK3CA E542K (22.4 %)	IIIA	TMB high (28 mut/Mb)	IC	PD-L1 amplification	IIIB	-	-	-	-	YES	-	PIK3CA	Clinical trial	NO	Not eligible for a trial	-	-
8805	YES	PIK3CA E542K (0.44 %)	IIIA	-	-	-	-	-	-	-	-	-	-	YES	-	PIK3CA	Clinical trial	NO	Not eligible for a trial	-	-
9185	YES	PIK3CA H1047R (0.89 %)	IIIA	-	-	-	-	-	-	-	-	-	-	YES	-	PIK3CA	Clinical trial	NO	Not eligible for a trial	-	-
9302	YES	PIK3CA E453K (17.1 %)	IIIA	-	-	-	-	-	-	-	-	-	-	YES	-	PIK3CA	Clinical trial	PIK3CA inhibitor	-	SD	6 months

Supplementary Table 12: Gustave Roussy's liquid biopsy experience (n=44 patients)

SUPPLEMENTARY DATA

Case 1: TMB high & Immunotherapy

A 61-year-old female was initially diagnosed with aSCC in 2009. The patient underwent curietherapy as the primary treatment.

In 2011, she developed a local recurrence, which was successfully treated by excision of the lesion with sphincter preservation.

In December 2017, the patient presented with retroperitoneal metastatic lymph node recurrence, beginning at the left renal hilum and extending laterally along the left aortic and left ilio pelvic regions. Additionally, there was involvement of the left uterine sidewall, extending to the small sciatic notch and deep portions of the left gluteus muscle. Pelvic MRI revealed a tumor in the upper part of the anal canal, primarily involving the internal anal sphincter and extending to the lower rectum, measuring 14 mm in height. A second nodular lesion was identified on the posterior vaginal wall, measuring 20 mm in diameter. The MRI also showed extensive tumor infiltration of the left parametric region, following the course of the left uterosacral ligament and extending into the pelvic wall and surrounding muscle groups. Moreover, retroperitoneal lymphadenopathy was present, including lateral aortic, external iliac, and inguinal nodes.

Between February and September 2018, the patient was treated with chemotherapy (LV5FU2-Mitomycin), receiving a total of 7 cycles. A PET scan performed on September 25, 2018, demonstrated a complete metabolic response to the chemotherapy. Despite this positive response, a surgical approach involving abdominoperineal amputation with lymph node dissection was abandoned due to the patient's refusal and after receiving a second opinion from the multidisciplinary board.

In May 2020, a follow-up PET scan revealed disease progression with new right inguinal lymphadenopathy and persistent hypermetabolism in the anal canal region. As a result, chemotherapy with LV5FU2-Mitomycin was resumed, and the patient completed 8 cycles between June and December 2020.

In January 2021, a subsequent PET scan showed further progression, with the development of a presacral lymph node and worsening hypermetabolism in the right inguinal lymph node. The metabolic activity of the anal lesion remained stable. The patient experienced significant difficulty tolerating chemotherapy, and there was reluctance to switch to other regimens such as modified DCF or weekly Taxol. A PET scan was scheduled for April 16, 2021.

In May 2021, a liquid biopsy was performed using the F1LCDx assay, providing a comprehensive molecular profile of the tumor. The results revealed a high Tumor Mutational Burden (TMB) of 15 mutations per megabase (mut/Mb), as well as the following key mutations:

KIT K558N (1.7%)

PIK3CA E726K (1.5%) and E542K (3.0%)

CDK12 E187_E189>KR* (0.4%)

DNMT3A Q237fs*79 (2.7%)

KMT2D Q1927* (3.1%)

TP53 M237I (0.3%)

Based on these findings, the molecular tumor board (MTB) made the following recommendations, prioritized as follows:

1. High TMB (15 mut/Mb) – This may warrant consideration for enrollment in a clinical trial evaluating immunotherapy.

2. *KIT* mutation (K558N) – Suggests eligibility for a clinical trial assessing cabozantinib or imatinib.
3. *CDK12* mutation (E187_E189>KR*) – Supports potential inclusion in a clinical trial investigating PARP inhibitors (PARPi).
4. *PIK3CA* double mutations (E726K and E542K) – Opens the possibility for inclusion in a clinical trial evaluating PIK3CA inhibitors.

Following the MTB's recommendations, the patient was enrolled in a clinical trial and initiated treatment with a combination of anti-PD1 and anti-CTLA4 antibodies, in addition to radiotherapy targeting the right inguinal lymph node. The first cycle of immunotherapy (C1J1) was administered on May 27, 2021, and the patient completed a total of 27 cycles by April 25, 2023, achieving a radiological partial response with a reduction of 90%.

At the follow-up consultation on June 24, 2024, the PET scan revealed no suspicious lesions, and the patient will continue routine monitoring as part of her ongoing care.

This case highlights the potential of immunotherapy in advanced cases with high TMB. The patient's ongoing response to the combination of immunotherapy and radiotherapy offers hope for improved outcomes in similar patients with refractory disease.

Case 2: *RAD51C* A354fs*34 & PARP inhibitors

A 59-year-old female was initially diagnosed in February 2019 with aSCC. She was treated with concomitant chemoradiotherapy followed by a period of surveillance.

In May 2019, the patient experienced hepatic recurrence of the carcinoma. She underwent hepatic metastasectomy followed by chemotherapy with CarboTaxol. A total of 6 chemotherapy cycles were completed, and she was placed under routine surveillance afterward.

On a PET scan performed on January 18, 2021, there was an increase in the metabolic activity of a hepatic hilum lymph node, with the SUV max increasing from 5.7 to 6.2. Additionally, new hypermetabolic lymph nodes were detected in the celiac region and in the superior mediastinum and posterior submediastinal space.

An MRI of the liver on January 21, 2021, revealed no signs of recurrence in the previously resected segment IV metastasis cavity. However, compared to January 2020, there was an increase in lymph node size at the hepatic hilum, both anterior and posterior to the portal trunk (measuring 8 mm in short axis).

In June 2021, the patient showed further progression of the disease, with new lymphadenopathy in the left lateral cervical and subclavicular regions, as well as worsening lymphadenopathy at the hepatic hilum.

In July 2021, molecular profiling via liquid biopsy using the F1LCDx assay revealed a low TMB (9 mut/Mb) and the following mutations:

RAD51C A354fs*34 (48.7%),

EGFR V765M (13.6%),

KMT2D R5048H (15.4%),

NFE2L2 R34P (13.3%),

PBRM1 splice site 1542-127_1562del148 (9.5%).

Based on these alterations, the MTB recommended enrolling the patient in a clinical trial evaluating PARPi. Given the high variant allelic frequency (VAF) of the *RAD51C* mutation, which suggested a potential germline origin, the MTB also advised genetic counseling. In line with these recommendations, the patient was included in the trial, which combined an anti-PD1 antibody with a PARP inhibitor. The first cycle (C1J1) was administered on July 15, 2021.

A CT scan performed on December 21, 2021, showed a stable disease with a 21% reduction in the size of the metastatic hepatic and lymph node lesions. The patient continued on the same treatment regimen as per protocol.

However, a CT scan on February 10, 2022, demonstrated disease progression, with an increase in the size of previously detected lymph nodes and the emergence of at least three new hepatic lesions. The patient also reported notable epigastric pain and abdominal discomfort due to the enlarged peri-gastric lymph node.

On February 23, 2022, the decision was made to withdraw the patient from the clinical trial due to the progression of the disease.

This case highlights the aggressive nature of squamous cell carcinoma of the anal canal when metastatic spread occurs, particularly to the liver and lymph nodes. Despite initial success with chemoradiotherapy and subsequent metastasectomy, the recurrence of disease led to a more challenging clinical scenario. The use of targeted therapies, including anti-PD1 therapy and PARP inhibitors, showed promise in providing a partial response, though the disease ultimately progressed.

The molecular profiling of the tumor played a key role in determining eligibility for the anti-PD1 + PARP inhibitor combination, underscoring the importance of personalized treatment approaches in managing refractory and metastatic SCC. The patient's progression despite these therapies suggests a need for alternative therapeutic strategies and further exploration of novel treatments for advanced anal canal SCC.

Case 3: *FGFR2* K659N & *FGFRi*

A 62-year-old female presented in November 2019 with acute anal pain and rectal bleeding. A thorough workup revealed a lesion in the lower rectum, which was biopsied and confirmed to be squamous cell carcinoma of the anal canal. Staging investigations showed extensive lymph node involvement, classified as cT4N2M0.

The patient was treated with concomitant chemoradiotherapy using 5FU and Mitomycin, which was completed in February 2020. Following treatment, she achieved a complete response, with no signs of residual disease.

In January 2021, the patient experienced a recurrence of disease with lymph node metastases both above and below the diaphragm. She was treated with modified DCF (Docetaxel, Cisplatin, and 5FU) from January 27, 2021, to January 1, 2022, resulting in a complete metabolic response, as evidenced by a follow-up PET scan.

In December 2021, the disease progressed again, with new lymph node involvement. The patient was started on Carboplatin and Paclitaxel from December 2021 to April 2022. However, this treatment was poorly tolerated due to hematologic toxicity, which required blood transfusions, and severe diarrhea.

A treatment break was implemented in May 2022, during which the patient was monitored closely.

By the end of 2022 and into early 2023, the disease began to progress once again. The patient resumed treatment with Carboplatin and Paclitaxel from February 2023 to May 2023, with the last cycle administered on May 19, 2023.

A recent scan evaluation revealed progression of the disease, including pulmonary metastases and worsening lymphadenopathy in the Baréty region, as well as bilateral

mediastinal and perihilar lymph node infiltration. These findings were attributed to the patient's increasing symptoms. To manage these symptoms, the patient underwent palliative radiotherapy from May 25, 2023, to June 1, 2023.

In June 2023, a molecular profiling performed on a liquid biopsy using the F1LCDx assay, revealed the presence of low TMB (5 mut/Mb), along with the following mutations:

FGFR2 K659N (0.4%)

NF1 Q83* (3.8%)

PTEN S59* (0.2%)

TP53 Y220C (0.8%)

TP53 K139N (0.3%)

DNMT3A S663* (0.9%)

DNMT3A splice site 1667+1G>A (25.6%)

FGF6 V127M (48.5%)

KMT2D Q2540* (3.3%)

NBN K219fs*16 (51.3%)

Based on these findings, the molecular tumor board (MTB) recommended enrolling the patient in a clinical trial evaluating FGFR inhibitors. No specific recommendations were made for the *TP53* Y220C mutation, as no clinical trials targeting this alteration were available at the time. Given the high variant allele frequency (VAF) of the *DNMT3A* splice site 1667+1G>A mutation, the MTB also recommended hematologic consultation to assess the possibility of clonal hematopoiesis.

Following these recommendations, the patient was enrolled in a clinical trial evaluating FGFR inhibitors, and treatment was initiated on August 4, 2023.

The first follow-up evaluation in September 2023 showed stable disease, indicating some degree of disease control under the new therapy.

However, a CT scan performed on November 24, 2023, showed progression of the target lesions, and, due to the toxicity associated with the FGFR inhibitor, treatment was discontinued on November 30, 2023.

This case demonstrates the evolving treatment landscape for advanced squamous cell carcinoma of the anal canal, including the potential role of FGFR inhibitors in managing metastatic disease. Although the patient showed initial stability, the progression of disease and treatment-related toxicity reinforce the need for ongoing research to improve outcomes in patients with refractory and metastatic SCC. Further clinical trials and personalized treatment strategies are essential to enhance survival and quality of life in these patients.

Case 4: *PIK3CA* E453K & *PIK3CA* inhibitor

A 63-year-old female presented with anal pain and rectal bleeding in October 2017, which led to the diagnosis of aSCC, initially staged as T3 N+ M1, indicating metastatic disease. The patient's metastatic disease involved distant lymph nodes and organs at diagnosis.

The patient was treated with first-line chemotherapy comprising Cisplatin and 5-FU. She received a total of five cycles from October 2017 to February 2018. The treatment was complicated by grade 2 neuropathy and severe diarrhea. Despite these adverse effects, the patient experienced a partial response to treatment, after which she was placed under surveillance.

Second-Line Treatment: In September 2018, disease progression was noted, prompting the initiation of second-line chemotherapy with capecitabine, which began in October 2018. Despite this treatment, the disease continued to progress, and by April 2020, new lymph node metastases were identified.

Third-Line Treatment: As a result of the progression, the patient was started on FOLFIRI (folinic acid, fluorouracil, and irinotecan) from May 2020 to July 2020, receiving four cycles. Subsequently, she underwent chemoradiotherapy with LV5FU2 from July 2020 to September 2020, which provided further disease control.

Surgical Intervention and Continued Progression: In July 2022, the patient developed a mass invading the rectovaginal septum, signaling disease progression. A decision was made to resume FOLFIRI chemotherapy, which continued until October 2022, when the patient underwent abdominoperineal resection surgery on October 6, 2022. During surgery, a 7 cm residual mass was found, invading both the rectal and vaginal walls, with nerve sheath infiltration. The pathology confirmed ypT3N0R1.

Despite the surgery, the disease continued to progress, with ganglionic involvement identified at the hepatic hilum, retroperitoneal, and hepatic regions by January 2024. The patient was restarted on FOLFIRI chemotherapy, receiving six cycles until April 2024, although this was complicated by grade II diarrhea and vomiting.

On May 23, 2024, the patient underwent molecular profiling through a liquid biopsy using the F1LCDx assay. This analysis revealed a low TMB (5 mut/Mb) as well as a *PIK3CA* E453K mutation (17.1%), a *TP53* E294* (0.1%) mutation and a *BCL2L1* amplification. Given these findings, the MTB recommended the inclusion of this patient in a clinical trial targeting the *PIK3CA* mutation.

The patient was enrolled in a clinical trial and received her first dose of a *PIK3CA* inhibitor on October 1, 2024 (C1J1). A follow-up CT scan performed on November 22, 2024, showed stable disease compared to the baseline scan.

This case highlights the importance of molecular profiling in identifying potential targeted therapies for advanced and recurrent squamous cell carcinoma of the anal canal. The use of a *PIK3CA* inhibitor has provided the patient with disease stability after multiple lines of chemotherapy, underscoring the potential for personalized treatments in improving outcomes for patients with metastatic anal canal SCC. Continued monitoring and further clinical trials are essential to identify the most effective treatment strategies for this challenging disease.