Supplementary Information

Supplementary Methods

Development of Glycoprotein Units (GPUs) standard curve for normalization of data from the SubB2M-$_{A12}$-SPR assays

To generate an internal calibration curve, the Neu5Gc-containing glycoproteins bovine Alpha-1-acid glycoprotein (bAGP) (Sigma-Aldrich, Cat No. G3643) and human cancer antigen 125 (CA125) purified from a human ovarian carcinoma cell line (MyBioSource, San Diego, USA, Cat No. MBS318371) were combined at starting concentrations of 15 µg/ml and 15 units/ml, respectively, in 0.5 % normal human serum (equivalent to 3000 µg/ml bAGP and 3000 units/ml CA125 in 100 % serum). This glycoprotein mixture was two-fold serially diluted down to 14.65 ng/ml and 0.0146515 units/ml, respectively, in 0.5 % normal human serum. This concentration range of glycoprotein standards was run before every set of serum samples analyzed. RUs for each concentration of the GPU standard mixture were determined by subtracting binding due to $SubB_{A12}$ (flow cell 4) from binding due to SubB2M on flow cell 2 and flow cell 3. The RUs obtained for the highest concentration standard was considered 100 GPUs. The resulting standard curve was used to convert SPR RUs, taken at the point of stability in the generated sensorgram, to GPUs. The presence of Neu5Gc on both standard glycoproteins was confirmed by mass spectrometry as described previously (17) and below.

Mass spectrometry glycomic analysis of standard glycoproteins

Glycoproteins (10 µg bAGP and 116.83 units CA125) were immobilised onto PVDF membrane (Millipore) and *N*-glycans released by overnight incubation with 0.5 µl PNGase F (New England BioLabs 500,000 units/ml) in 10 µl water at 37 °C. Released *N*-glycans were reduced to alditols with 0.5 M NaBH4 in 50 mM KOH for 3 h at 50 °C. The reduction was quenched with 1 µl glacial acetic acid desalted using AG50W-X8 cation exchange resin.

O-glycans were released from PNGase F treated proteins by reductive β-elimination. PVDF spots were incubated in 20 µL of 0.5 M $NaBH_4$ in 50 mM KOH at 50 °C for 16 hours and desalted as described for the *N*-glycans.

PGC-LC-ESI-MS *N*-glycans were analysed using a Hypercarb PGC column (3 µm, 100 mm × 180 µm, Thermo Scientific). *N*-glycans were separated over a 90 min and *O*-glycans over a 60 min gradient of 1–90 % of acetonitrile in 10 mM ammonium bicarbonate (vol/vol) at a flow rate of 1 µL/min using a Dionex ultimate HPLC (Thermo Scientific) interfaced with an amaZon Speed ESI-IT mass spectrometer (Bruker Bruker Daltonics, Germany).

The MS spectra were acquired in negative ion mode over a mass range of 450 to 2200 m/z. The following MS settings were used: drying gas temperature: 180 ºC, drying gas flow: 5 L/min, nebulizer gas: 9 psi, capillary 3400 V. Ions were detected in ion charge control (ICC) (target: 50,000 ions) with an accumulation time of 200 ms. Induced collision was performed at 35 % normalised collision energy and an isolation window of 4 m/z). Instrument control, data acquisition and processing were performed with Bruker DataAnalysis software version 4.2 (Bruker Daltonics, Germany).

Supplementary Figures

Figure S1. Glycan array analysis of SubB2M and $SubB_{A12}$ using a Z-Biotech Neu5Ac/Neu5Gc array. A) Glycan array result of SubB2M and $SubB_{A12}$ performed using the Z-Biotech Neu5Gc/Neu5Ac N-Glycan Array. Histogram represents the average relative fluorescent units of binding to each of the numbered structures shown in **B**. For structure ID see http://www.zbiotech.com/neu5gc-xenoantigen-microarray.html and http://nebula.wsimg.com/deda6829116ce09edb871bd7ce7cde6c?AccessKeyId=B5CD53DB37409833427C&disposition=0&alloworigin=1 for further information.

Figure S2. Characterization of human CA125 *O*-glycosylation and bovine Alpha-1-acid glycoprotein (bAGP) by PGC-LC-MS/MS. Annotated Base Peak Chromatogram of the total **A)** *O*-glycome released from CA125 and Extracted ion chromatogram of m/z 681.32^{-} (Neu5Gc) and 665.32^{-} (Neu5Ac) and **B)** *N*-glycome released from bAGP and Extracted ion chromatogram of m/z 1127.4^{2-} (Neu5Gc) and 1111.4^{2-} (Neu5Ac). Confirmation of **C)** Neu5Gc (m/z 681.32^{-}) and Neu5Ac (m/z 665.32^{-}) containing *O*-glycan structures by MS/MS fragmentation and **D**) Neu5Gc (m/z 1127.4^{2-}) and Neu5Ac (m/z 1111.4^{2-}) containing glycan structures by MS/MS fragmentation.

Figure S3. A representative Glycoprotein Units (GPUs) standard curve. Bovine AGP (MW = 41-43 kDa; ~50 %/50 % Neu5Ac/Neu5Gc; high total sialic acids) and human CA125 (MW = >200 kDa, 5-10 % Neu5Gc; low total sialic acid) were combined at starting concentrations of 15 µg/ml and 15 units/ml, respectively, in 0.5 % normal human serum. This glycoprotein mixture was

two-fold serially diluted down to 14.65 ng/ml and 0.0146515 units/ml, respectively, in 0.5 % normal human serum. The Response Units (RUs) for each concentration of the standard mixture were determined by subtracting binding due to $SubB_{A12}$ (flow cell 4) from binding due to SubB2M on flow cell 2 and flow cell 3. RUs obtained for the highest concentration standard was considered 100 GPUs. FC2 = flow cell 2; FC3 = flow cell 3.

Figure S4. ROC curves depicting the ability of serum Neu5Gc levels determined by the optimized SubB2M-$_{A12}$-SPR assay to distinguish Stage I – IV ovarian cancer patients from cancer-free (normal) individuals. Sensitivity% (true positive rate; ability to detect disease) is plotted against 100 %-specificity% (false positive rate or 100 %-true negative rate; ability to detect lack of disease). ROC analyses were performed with the data shown in Figure 2B using Graphpad Prism 8.0

Figure S5. ROC curves depicting the ability of serum Neu5Gc levels to distinguish Stage I – IV breast cancer patients from normal (cancer-free) individuals. Sensitivity% (true positive rate; ability to detect disease) is plotted against 100 %-specificity% (false positive rate or 100 %-true negative rate; ability to detect lack of disease). ROC analyses were performed with the data shown in Figure 3 using Graphpad Prism 8.0

Figure S6. Serum Neu5Gc levels determined by SubB2M-$_{A12}$-SPR assay for A) relapse cases and B) remission cases from the Circ.BR cohort. The mean GPUs from duplicate analyses for each serum sample are shown.

818 Error bars = ± 1 SD from the mean for each group. Two independent assays
819 were performed with both showing the same trends. Results from one assay
820 are shown. Clinical information for each patient is shown in the top right of
821 each plot with treatment history and metastases overlaid. ALND: Axillary
822 lymph node dissection, ILC: Invasive Lobular Carcinoma Mast: mastectomy,
823 SNB: sentinel node biopsy, WLE: wide local excision, XRT: radiation therapy.
824 Detailed information for each patient in the Circ.BR cohort are shown in
825 Supplementary Table 3.
826
827

828 **Supplementary Table 4. Optimal cut-off values, sensitivity and**
829 **specificity for distinguishing Stage I, II, III and IV ovarian cancer patients**
830 **from normal (cancer-free) individuals using serum Neu5Gc levels**
831 **determined by optimized SubB2M-SPR assay before and after $SubB_{A12}$**
832 **subtraction.** Sensitivity and specificity were determined from the Receiver
833 operating characteristic (ROC) curves (**Figure S4**). Optimal cut-off values
834 were selected to give the maximum sum of sensitivity and specificity.
835

	Before $SubB_{A12}$ subtraction	**After $SubB_{A12}$ subtraction**
Normal vs Stage I	>9.02 GPUs (91.67 % sensitivity, 100 % specificity)	>6.90 GPUs (100 % sensitivity, 100 % specificity)

Normal vs Stage II	>8.83 GPUs (90.91 % sensitivity, 94.45 % specificity)	>6.88 GPUs (100 % sensitivity, 100 % specificity)
Normal vs Stage III	>14.50 GPUs (100 % sensitivity, 100 % specificity)	>16.40 GPUs (100 % sensitivity, 100 % specificity)
Normal vs Stage IV	>10.49 GPUs (100 % sensitivity, 100 % specificity)	>11.87 GPUs (100 % sensitivity, 100 % specificity)

836

837

838 **Supplementary Table 5. Optimal cut-off and area under the curve (AUC)**
839 **values for distinguishing Stage I, II, III and IV breast cancer patients from**
840 **normal (cancer-free) individuals using serum Neu5Gc levels.** Sensitivity
841 and specificity were determined from the receiver operating characteristic
842 (ROC) curves (**Figure S5**). Optimal cut-off values were selected to give the
843 maximum sum of sensitivity and specificity.

844

	Optimal cut-off	**ROC AUC**
Normal vs Stage I	>10.55 GPU (sensitivity = 95.83 %, specificity = 100 %)	0.9583
Normal vs Stage II	>10.49 GPU (sensitivity = 100 %, specificity = 100 %)	1.000
Normal vs Stage III	>14.54 GPU (sensitivity = 100 %, specificity = 100 %)	1.000

Normal vs Stage IV	>19.87GPU (sensitivity = 100%, specificity = 100%)	1.000

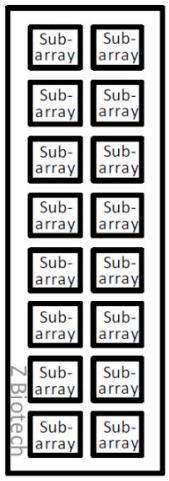
Supplementary Table S1. Supplementary glycan microarray document based on MIRAGE guidelines DOI: 10.1093/glycob/cww118.

Classification	Guidelines
1. Sample: Glycan Binding Sample	
Description of Sample	Sample names: *Escherichia coli* SubB2M and $SubB_{A12}$. Origin: B subunit pentameric toxins produced as a recombinant protein in *E. coli*. Method of preparation: The preparation of SubB2M and $SubB_{A12}$ are explained in the Materials and Methods section.
Sample modifications	SubB2M and $SubB_{A12}$ are hexahistidine-tagged proteins.
Assay protocol	Please see Materials and Methods and appended manufacture's manual.
2. Glycan Library	
Glycan description for defined glycans	Arrays used are the Z-Biotech Neu5Gc/Neu5Ac N-Glycan Array. Glycans in this study are listed in Supplementary Figure S1 and are outlined below.
Glycan description for undefined glycans	N/A.
Glycan modifications	N/A
3. Printing Surface; e.g., Microarray Slide	
Description of surface	NHS matrix slides
Manufacturer	Schott Nexterion
Custom preparation of surface	N/A.
Non-covalent Immobilisation	N/A.
4. Arrayer (Printer)	

Description of Arrayer	See Z-Biotech
Dispensing mechanism	See Z-Biotech
Glycan deposition	See Z-Biotech
Printing conditions	See Z-Biotech
5. Glycan Microarray with "Map"	
Array layout	See page 3.
Glycan identification and quality control	Arrays are quality controlled as described on page 6.
6. Detector and Data Processing	
Scanning hardware	Innopsys InnoScan 1100AL (Lasers: 488 nM, 532 nM with two filter sets for analysis at 532 and 595 nM), 635 nM) scanner.
Scanner settings	Scanning resolution: 10 μM Laser channel: 532 nM operating 532 nM excitation filter set. PMT: 20 % gain Scan powers: Low laser power.
Image analysis software	Innopsys MAPIX.
Data processing	Data was exported as a CSV file and exported to Microsoft Excel.
7. Glycan Microarray Data Presentation	
Data presentation	Data is presented as histograms in Figure S1.
8. Interpretation and Conclusion from Microarray Data	
Data interpretation	We only use glycan arrays as a yes/no binding tool. Due to this we look only at binding that is unambiguously above background vs lack of binding above background.
Conclusions	SubB2M is specific for Neu5Gc, $SubB_{A12}$ does not bind to any sialic acid containing glycans.

Print Layout

16-subarray Slide



Array Map:

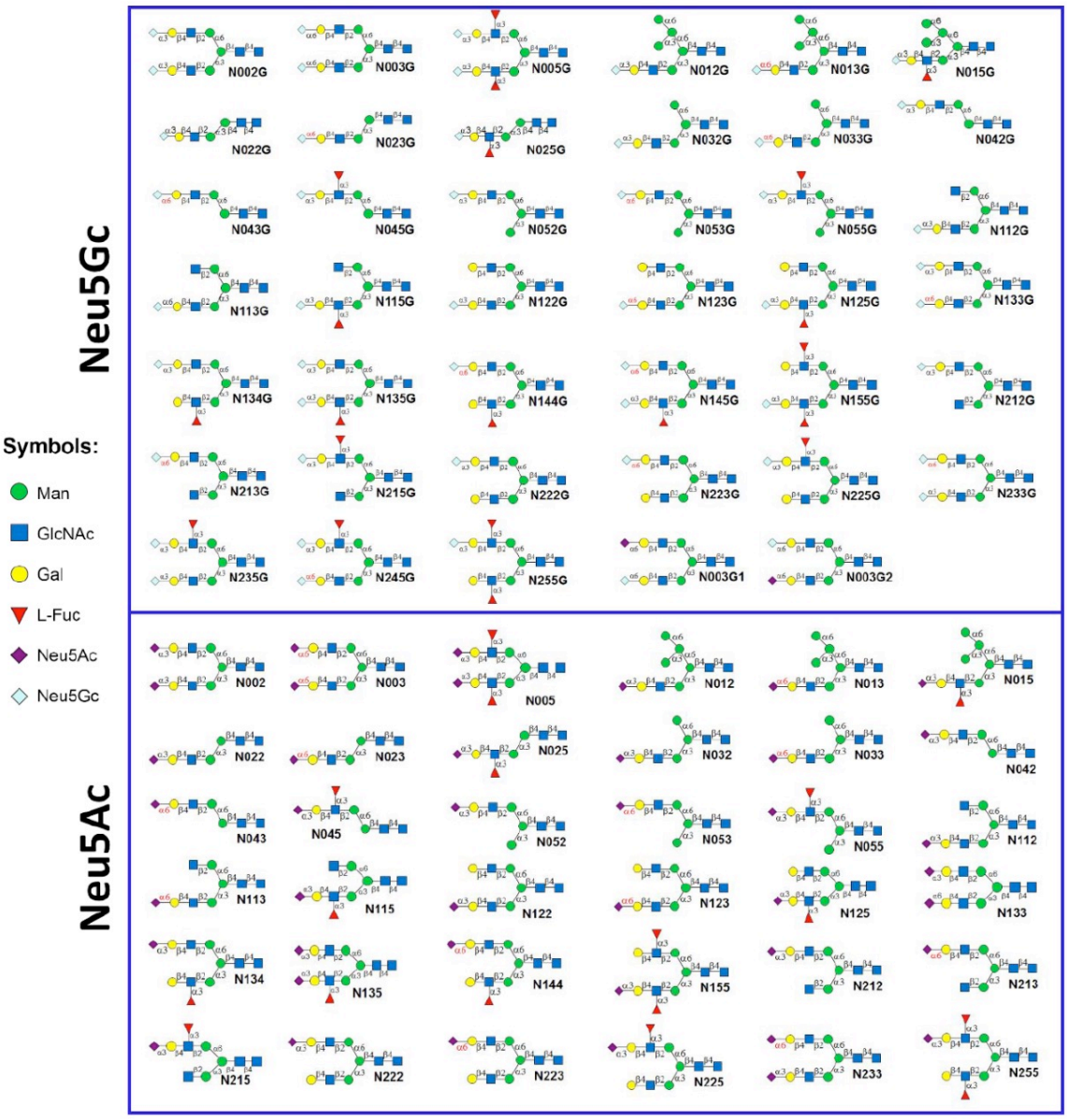
GC-1	GC-1	GC-1	GC-2	GC-2	GC-2	GC-3	GC-3	GC-3	GC-4	GC-4	GC-4	GC-5	GC-5	GC-5	NC1
GC-6	GC-6	GC-6	GC-7	GC-7	GC-7	GC-8	GC-8	GC-8	GC-9	GC-9	GC-9	GC-10	GC-10	GC-10	NC1
GC-11	GC-11	GC-11	GC-12	GC-12	GC-12	GC-13	GC-13	GC-13	GC-14	GC-14	GC-14	GC-15	GC-15	GC-15	NC1
GC-16	GC-16	GC-16	GC-17	GC-17	GC-17	GC-18	GC-18	GC-18	GC-19	GC-19	GC-19	GC-20	GC-20	GC-20	PC1
GC-21	GC-21	GC-21	GC-22	GC-22	GC-22	GC-23	GC-23	GC-23	GC-24	GC-24	GC-24	GC-25	GC-25	GC-25	PC1
GC-26	GC-26	GC-26	GC-27	GC-27	GC-27	GC-28	GC-28	GC-28	GC-29	GC-29	GC-29	GC-30	GC-30	GC-30	PC1
GC-31	GC-31	GC-31	GC-32	GC-32	GC-32	GC-33	GC-33	GC-33	GC-34	GC-34	GC-34	GC-35	GC-35	GC-35	PC2
GC-36	GC-36	GC-36	GC-37	GC-37	GC-37	GC-38	GC-38	GC-38	GC-39	GC-39	GC-39	GC-40	GC-40	GC-40	PC2
AC-1	AC-1	AC-1	AC-2	AC-2	AC-2	AC-3	AC-3	AC-3	AC-4	AC-4	AC-4	AC-5	AC-5	AC-5	PC2
AC-6	AC-6	AC-6	AC-7	AC-7	AC-7	AC-8	AC-8	AC-8	AC-9	AC-9	AC-9	AC-10	AC-10	AC-10	PC3
AC-11	AC-11	AC-11	AC-12	AC-12	AC-12	AC-13	AC-13	AC-13	AC-14	AC-14	AC-14	AC-15	AC-15	AC-15	PC3
AC-16	AC-16	AC-16	AC-17	AC-17	AC-17	AC-18	AC-18	AC-18	AC-19	AC-19	AC-19	AC-20	AC-20	AC-20	PC3
AC-21	AC-21	AC-21	AC-22	AC-22	AC-22	AC-23	AC-23	AC-23	AC-24	AC-24	AC-24	AC-25	AC-25	AC-25	PC4
AC-26	AC-26	AC-26	AC-27	AC-27	AC-27	AC-28	AC-28	AC-28	AC-29	AC-29	AC-29	AC-30	AC-30	AC-30	PC4
AC-31	AC-31	AC-31	AC-32	AC-32	AC-32	AC-33	AC-33	AC-33	AC-34	AC-34	AC-34	AC-35	AC-35	AC-35	PC4
AC-36	AC-36	AC-36	AC-37	AC-37	AC-37	AC-38	AC-38	AC-38	AC-39	AC-39	AC-39	GC-41	GC-41	GC-41	Marker

Glycan list:

N-Glycan Identification List:

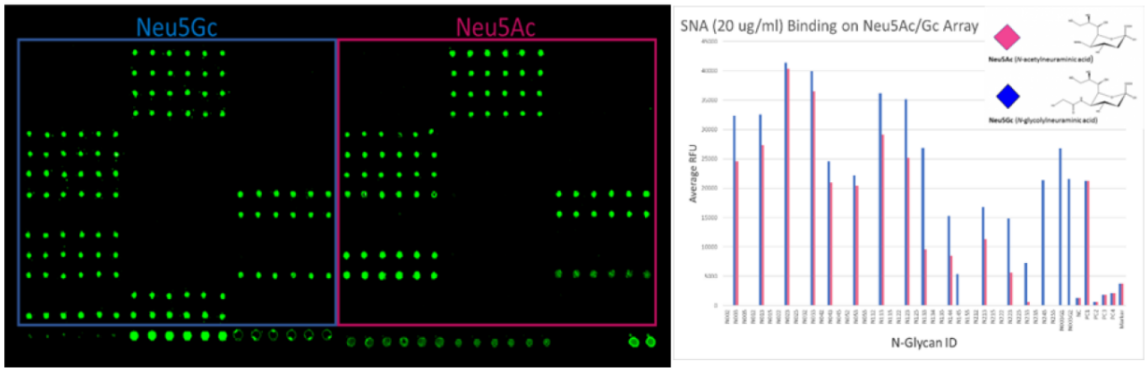
Gc Glycan ID	Neu5Gc Glycans	Ac Glycan ID	Neu5Ac Glycans
GC-1	N002G	AC-1	N002
GC-2	N003G	AC-2	N003
GC-3	N005G	AC-3	N005
GC-4	N012G	AC-4	N012
GC-5	N013G	AC-5	N013
GC-6	N015G	AC-6	N015
GC-7	N022G	AC-7	N022
GC-8	N023G	AC-8	N023
GC-9	N025G	AC-9	N025
GC-10	N032G	AC-10	N032
GC-11	N033G	AC-11	N033
GC-12	N042G	AC-12	N042
GC-13	N043G	AC-13	N043
GC-14	N045G	AC-14	N045
GC-15	N052G	AC-15	N052
GC-16	N053G	AC-16	N053
GC-17	N055G	AC-17	N055
GC-18	N112G	AC-18	N112
GC-19	N113G	AC-19	N113
GC-20	N115G	AC-20	N115
GC-21	N122G	AC-21	N122
GC-22	N123G	AC-22	N123
GC-23	N125G	AC-23	N125
GC-24	N133G	AC-24	N133
GC-25	N134G	AC-25	N134
GC-26	N135G	AC-26	N135
GC-27	N144G	AC-27	N144
GC-28	N145G		
GC-29	N155G	AC-29	N155
GC-30	N212G	AC-30	N212
GC-31	N213G	AC-31	N213
GC-32	N215G	AC-32	N215
GC-33	N222G	AC-33	N222
GC-34	N223G	AC-34	N223
GC-35	N225G	AC-35	N225
GC-36	N233G	AC-36	N233
GC-37	N235G		
GC-38	N245G		
GC-39	N255G	AC-39	N255
GC-40	N003G1		
GC-41	N003G2		

Neu5Gc and Neu5Ac N-Glycans

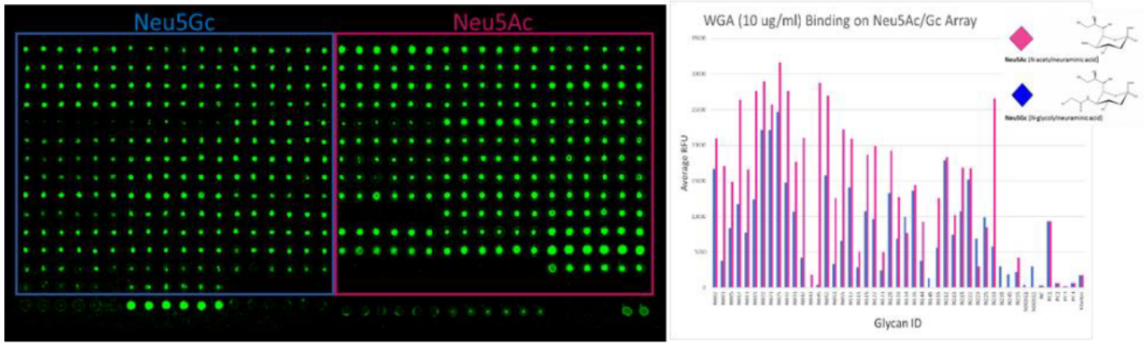


QC

Example 1: Neu5Gc/Neu5Ac array on 8 subarray format. A subarray assayed with a biotinylated SNA target (20 µg/ml), followed by streptavidin-Cy3 (1 µg/ml). The array was scanned with GenePix scanner at 500 PMT and 100% laser power at 532 nm wavelength. The positive control shows binding as expected. N-glycans containing α-2,6 Neu5Gc and α-2,6 Neu5Ac show binding as expected. Analysis of the fluorescence intensity reveals that Neu5Gc-sialylated glycans bind more strongly.



Example 2: Neu5Gc/Neu5Ac array on 8 subarray format. A subarray assayed with a biotinylated WGA target (10 µg/ml), followed by streptavidin-Cy3 (1 µg/ml). The array was scanned with GenePix scanner at 450 PMT and 100% laser power at 532 nm wavelength. The positive control shows binding as expected. Most N-glycans show binding as expected. Analysis of the fluorescence intensity reveals that Neu5Ac-sialylated glycans bind more strongly.



Supplementary Table S2. Details for each of the normal (cancer-free) individuals and breast cancer patients used in this study. Patient details were provided by the Victorian Cancer Biobank with informed written consent from each subject. Abbreviations: ALND: axillary lymph node dissection; DCIS: ductal carcinoma in-situ; G1: Grade 1; G2: Grade 2; G3: Grade 3; LCIS: lobular carcinoma in-situ; LVSI: lymph vascular space invasion; NST: no special type. Information on vital status and/or recurrence was not available from the VCB for all patients.

Specimen no.	Age	Breast cancer stage	Type of breast cancer	Vital status	Date of death	Survival time (years)*	Recurrence	Neu5Gc levels (GPUs)
07AH130	50	N/A	Cancer-free					6.083
08AH714	42	N/A	Cancer-free					6.326
09AH320	47	N/A	Cancer-free					4.972
09AH434	42	N/A	Cancer-free					4.278
09AH794	57	N/A	Cancer-free					10.077
09AH796	52	N/A	Cancer-free					8.653
09AH820	38	N/A	Cancer-free					5.076
12EH0028	53	N/A	Cancer-free					4.694
12EH0114	72	N/A	Cancer-free					5.563
15EH0238	51	N/A	Cancer-free					7.785
15EH0234	79	N/A	Cancer-free					7.125
15EH0228	62	N/A	Cancer-free					6.257
16EH0287	61	N/A	Cancer-free					2.472
16EH0217	76	N/A	Cancer-free					5.806
16EH0396	74	N/A	Cancer-free					3.965
16EH0397	50	N/A	Cancer-free					0.423
17EH0084	54	N/A	Cancer-free					4.556
17EH0260	46	N/A	Cancer-free					7.299
17EH0268	63	N/A	Cancer-free					5.528
17EH0314	46	N/A	Cancer-free					3.792
17EH0349	93	N/A	Cancer-free					3.792
17EH0211	52	N/A	Cancer-free					4.937
02PM1046	80	I	Invasive lobular carcinoma (G2)					20.702

04PM1332	55	I	Invasive ductal carcinoma (G2) with associated DCIS (high grade)	A	N/A			37.856
09PM0065	53	I	Invasive ductal carcinoma (G3) with associated DCIS (high grade)	A	N/A			24.904
15PM0686	38	I	Invasive ductal carcinoma (G2) with associated DCIS (high grade)	A	N/A			11.014
15PM0997	59	I	Invasive mixed micropapillary and ductal/NST carcinoma (G3) and DCIS (high grade)					24.349
11MH0317	43	I	Invasive ductal carcinoma NST (G3)	A	N/A			25.460
12MH0314	44	I	Invasive ductal carcinoma NST (G2) with associated DCIS (high grade)	A	N/A			25.251
11MH0052	68	I	Invasive ductal carcinoma (G2) with associated DCIS (low grade)					16.188
11MH0554	61	I	Invasive ductal carcinoma NST (G2) with associated DCIS (high grade)	A	N/A			22.508
13MH1003	66	I	Invasive ductal carcinoma (G1) with associated DCIS (low grade)					17.299
10RMH737	58	I	Invasive ductal carcinoma NST (G2) with associated DCIS (high grade)					14.799
11MH0567	47	I	Invasive ductal carcinoma with lobular features (G2) with associated DCIS (low grade)					12.369
09RMH083	60	I	Invasive ductal carcinoma NST (G2) with extensive DCIS (high grade)					35.634
10RMH281	54	I	Invasive ductal carcinoma NST (G3)	A	N/A			22.717
10RMH383	77	I	Invasive mucinous adenocarcinoma (G2)					30.391
10RMH514	81	I	Invasive lobular carcinoma NST (G2) with associated LCIS	A	N/A			35.947
12MH0215	35	I	Invasive ductal carcinoma NST (G2)					14.869
12MH0481	56	I	Invasive ductal carcinoma NST (G2) with associated DCIS (high grade)					23.133
12MH1013	65	I	Invasive ductal adenocarcinoma NST (G1) with DCIS (low grade)	A	N/A			-3.674
12MH1291	56	I	Invasive ductal adenocarcinoma NST (G2)	A	N/A			26.849

13MH1223	57	I	Invasive ductal carcinoma NST (G2) with DCIS (intermediate grade)					24.210
14MH0232	51	I	Invasive ductal carcinoma NST (G1)	A	N/A			22.091
14MH0240	43	I	Invasive ductal carcinoma NST (G1) with LCIS (moderate)	A	N/A			28.446
16MH1550	48	I	Lobular carcinoma NST (G3) with associated LCIS	A	N/A			20.772
02PM0520	58	II	Invasive ductal carcinoma NST (G3) with associated DCIS (high grade) & metastatic adenocarcinoma	A	N/A			23.967
04PM0880	45	II	Invasive ductal carcinoma (G3) with associated DCIS (minor high grade) & metastatic carcinoma	A	N/A			17.543
05PM1100	31	II	Mixed invasive ductal carcinoma (NTS and mucinous) (G2) with associated DCIS (high grade) & metastatic tumor of sentinel nodes	A	N/A			11.605
05PM1168	55	II	Invasive ductal carcinoma (G3) with associated DCIS (high grade) & metastatic carcinoma	D	30/05/2008	3		13.410
06PM0542	26	II	Invasive mucinous carcinoma (G1 & G2) with associated DCIS (high grade)					11.257
08PM1308	59	II	Invasive ductal carcinoma NST (G3) & metastatic carcinoma	A	N/A			17.786
08PM1949	49	II	Invasive lobular carcinoma (G2) (post chemotherapy) & metastatic carcinoma	A	N/A			15.772
15PM0781	44	II	Invasive ductal carcinoma NST (G3) & metastatic carcinoma	A	N/A			10.910
14PM0388	67	II	Invasive ductal carcinoma NST (G3) with associated DCIS (high grade)					12.542
15PM1021	53	II	Invasive ductal carcinoma NST (G2) with associated DCIS (intermediate to high grade)	A	N/A			10.945
15PM1119	51	II	Mixed invasive carcinoma (micropapiliary and NST) (G3) with associated DCIS (low and high grade) & metastatic carcinoma	A	N/A			18.619
13MH0429	51	II	Invasive ductal carcinoma (G2) with associated DCIS (intermediate grade) & metastatic carcinoma	D	26/04/2017	4.08		13.063
16MH1592	47	II	Invasive ductal carcinoma NST (G3) with minor DCIS	A	N/A			28.585

01PM0361	75	II	Invasive ductal carcinoma NST (G3) with DCIS (high grade)	D	03/01/2003	1.33		20.286
01PM0632	71	II	Invasive ductal carcinoma NST (G3)	D	06/06/2003	1.75	No	23.411
03PM0734	47	II	Invasive ductal carcinoma NST (G2) & metastatic carcinoma					22.091
05PM2377	49	II	Invasive ductal carcinoma NST (G1) with DCIS (low grade)	A	N/A		Yes	30.738
08PM1773	79	II	Invasive ductal carcinoma NST (G3) with DCIS (high grade)					20.598
08PM1785	54	II	Invasive ductal carcinoma NST (G3) with DCIS (high grade) & metastatic carcinoma	A	N/A			20.771
09PM0040	51	II	Invasive ductal carcinoma NST (G1) with DCIS (high grade)	A	N/A		Yes	16.154
10PM0659	31	II	Invasive ductal carcinoma (G2) with neuroendocrine differentiation					27.092
10PM2156	78	II	Invasive ductal carcinoma NST (G3) with DCIS (high grade)	D	18/03/2017	6.33	No	17.022
12PM0321	46	II	Invasive ductal carcinoma NST (G1) with DCIS (low to intermediate grade) & metastatic carcinoma	A	N/A			15.945
13PM0443	39	II	Invasive ductal (NST) (G3) and lobular carcinoma with DCIS (intermediate to high grade)	D	20/02/2015	1.83	Yes	16.223
01PM0503	49	III	Invasive ductal carcinoma (G3) with associated DCIS (high grade) of right breast	D	09/03/2002	0.42		65.254
03PM0187	57	III	Invasive ductal carcinoma NTS (G3) with associated DCIS (high grade) of left breast with invasion of skeletal muscle and lymphatic channels					30.530
03PM1038	32	III	Invasive ductal carcinoma (G3) with associated DCIS (high grade) of right breast & metastatic carcinoma	D	18/12/2004	1.42		31.016
04PM0931	51	III	Invasive ductal carcinoma NST (G3) with possible DCIS of right breast & metastatic carcinoma, advanced left breast cancer	D	06/01/2005	3		55.879
08PM1472	57	III	Invasive ductal carcinoma (G3) with associated DCIS (high grade) of right breast & metastatic carcinoma					28.967
09PM1880	55	III	Invasive ductal carcinoma (G3) (basal type differentiation) with associated DCIS (high grade) of	A	N/A			41.572

			left breast & metastatic carcinoma					
11PM0575	33	III	Invasive mucinous carcinoma (G1) of left breast & metastatic carcinoma	A	N/A			33.724
11PM1136	42	III	Invasive ductal carcinoma (G3) with associated DCIS (high grade) of right breast & metastatic carcinoma	D	31/08/2013	2.17		37.683
14PM1036	83	III	Invasive ductal carcinoma NST (G3) with associated DCIS (high grade) of right breast & metastatic carcinoma	A	N/A			32.856
15PM0518	53	III	Invasive ductal adenocarcinoma (G3) with associated LCIS (high grade) of left breast & metastatic carcinoma	A	N/A			52.059
15PM0965	56	III	Invasive carcinoma (basal) (G3) with associated DCIS (high grade) of left breast & metastatic carcinoma	A	N/A			73.658
11MH0137	43	III	Invasive ductal carcinoma NST (G3) with associated DCIS (high grade) of right breast & metastatic carcinoma	A	N/A			30.078
09RMH727	45	III	Poorly differentiated invasive ductal carcinoma (G3) & metastatic carcinoma	A	N/A			53.378
10RMH275	69	III	Invasive ductal carcinoma NTS (G1) with DCIS (intermediate to high grade) & metastatic carcinoma					42.857
12MH0211	48	III	Invasive ductal carcinoma (G2) with metastatic ductal carcinoma	A	N/A			41.259
12MH0483	56	III	Micropapillary invasive ductal carcinoma (G3)	A	N/A		Yes	44.142
13MH0077	56	III	Invasive lobular carcinoma (G1) & metastatic lobular carcinoma	A	N/A			53.031
18MH0981	36	III	Invasive ductal carcinoma NTS (G3) with DCIS (low and high grade)	A	N/A			26.363
01PM0213 BLD	66	III	Invasive ductal carcinoma (moderately differentiated) (G2/3) with DICS (intermediate) & metastatic carcinoma					31.120
01PM0463	81	III	Invasive ductal carcinoma (G2) & metastatic carcinoma	D	12/09/2003	2	Yes	38.968
01PM0443	76	III	Invasive ductal carcinoma (G3) with some lobular pattern & metastatic carcinoma	D	20/11/2002	1.17		19.001

02PM1143	39	III	Invasive ductal carcinoma (G2) with associated DCIS (high grade) & metastatic carcinoma	D	05/01/2011	8.08		26.883
03PM0227 BLD	57	III	Invasive ductal carcinoma NTS (G3) with DCIS (high grade)					40.808
08PM2010 BLD	50	III	Invasive ductal carcinoma (G3)	A	N/A		Yes	27.890
17MH0909bl	49	IV	Invasive ductal carcinoma (G3) with associated DCIS (high grade) of left breast with foci of LVSI & metastatic carcinoma	D	06/05/2018	0.83		181.200
17MH0907bl	76	IV	Metastatic breast carcinoma (stable stage IV for 7 years, primary lung cancer)					203.389
15MH1848bl	54	IV	Metastatic adenoid cystic carcinoma of right breast (G3) (also adenoid cystic carcinoma of brain consistent with primary breast cancer)					134.566
15MH1826bl	45	IV	Invasive adenocarcinoma NST (right breast, G3; left breast, G2) with associated DCIS (high grade) & metastatic carcinoma (clavicular head; 6/7 right lymph nodes; 7/7 left lymph nodes), history of Non-Hodgkin lymphoma	A	N/A			138.211
15MH1733bl	27	IV	Invasive ductal carcinoma (G3) with associated DCIS (high grade) of left breast & metastatic carcinoma (4/5 sentinel nodes; 10/11 lymph nodes with extravascular spread)	A	N/A			92.548
13MH1140bl	62	IV	Invasive lobular carcinoma of left breast (G2); Invasive lobular carcinoma (G2) and invasive ductal carcinoma NST (G3) of right breast & metastatic carcinoma (3/18 right axillary lymph nodes)	A	N/A			74.595
13MH0914bl	42	IV	Invasive ductal carcinoma (G3) of left breast; recurrent disease; metastasis to the vertebrae	D	07/12/2013	0.25	Yes	59.108
13MH0767bl	48	IV	Invasive ductal carcinoma (G2) of left breast & metastatic carcinoma (bone metastasis; 1/1 lymph nodes)	D	03/02/2016	2.5		62.962
13MH0529bl	62	IV	Invasive ductal adenocarcinoma (G3) of right breast & metastatic carcinoma (pulmonary metastasis, 2/5 lymph nodes)	D	02/03/2016	2.75		50.774

13MH0217bl	61	IV	Invasive ductal adenocarcinoma (G3) of right breast & metastatic adenocarcinoma (9/12 lymph nodes with extravascular extension; bone metastasis)	A	N/A			36.572
12MH1318	41	IV	Invasive ductal carcinoma (G2) of left breast with associated DCIS (high grade) & metastatic carcinoma (1/13 lymph nodes; pulmonary metastasis)	A	N/A			67.650
12MH0226	66	IV	Invasive ductal carcinoma (G3) of right breast & metastatic carcinoma (19/21 lymph nodes; pulmonary metastasis)	D	12/12/2016	3.75		55.635
02PM0246	73	IV	Longstanding metastatic breast carcinoma (bone, pulmonary and adrenal metastasis)					176.060
02PM0320	64	IV	Invasive primary breast adenocarcinoma (uterus, ovary, fallopian tube and omentum metastasis)					130.884
05PM1349	48	IV	Invasive lobular carcinoma (G2) with minor mucinous component & DCIS (intermediate and high grade) of left breast	D	28/09/2005	0.25		126.856
06PM0159	54	IV	Primary breast carcinoma & metastatic carcinoma (oesophagus)	D	26/12/2006	0.83		88.416
08PM1819	57	IV	Invasive ductal carcinoma of breast (metastasis to left shoulder/chest wall)					77.338
08PM1958	47	IV	Metastatic primary breast carcinoma (left femoral head)					39.002
10PM1401	60	IV	Metastatic primary breast adenocarcinoma (metastasis to brain and spine)					62.476
11PM0548	51	IV	Metastatic primary breast carcinoma (metastasis to brain)					46.607
11PM1336	39	IV	Metastatic breast carcinoma (adenocarcinoma of ovary and fallopian tubes)	A	N/A			29.661
11PM1339	39	IV	Invasive ductal carcinoma NST (G3) with associated DCIS (intermediate to high grade) of both breasts & metastatic breast adenocarcinoma (metastasis to ovaries)	D	29/06/2014	2.83		53.760
13PM0154	72	IV	Metastatic lobular carcinoma (15/18 lymph nodes of left axillary; 10/36 lymph nodes of neck)	A	N/A			35.877

13PM0931	73	IV	Metastatic mucinous carcinoma (metastasis to lung)	A	N/A		Yes	30.148

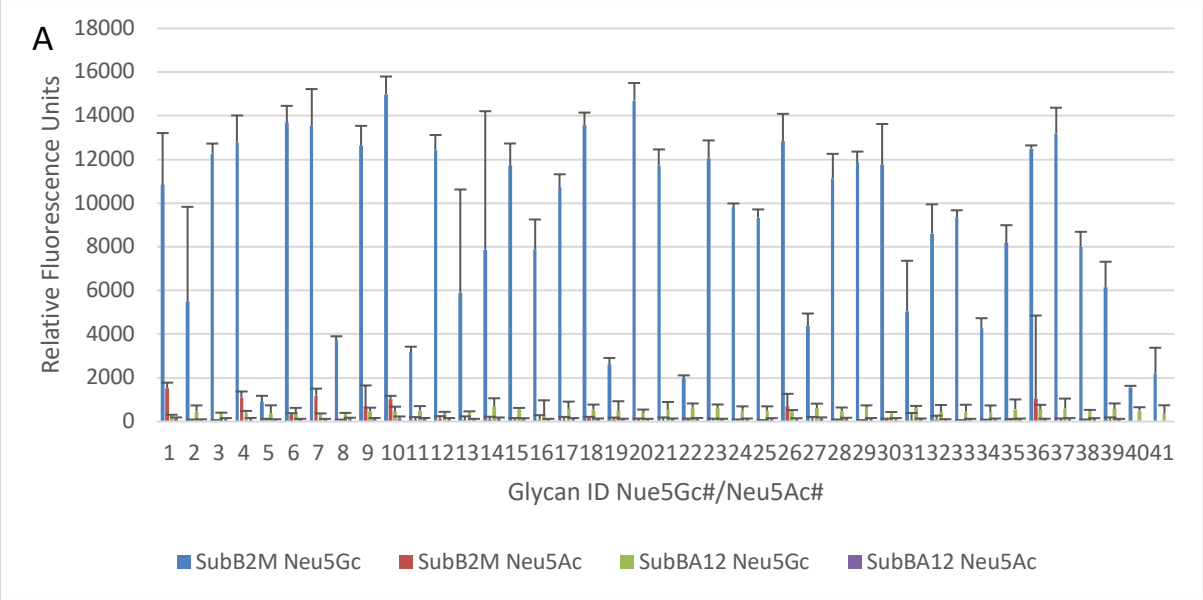
*From date serum sample was taken to date of death

Supplementary Table S3. Details for each of the breast cancer patients from the Circ.BR cohort used in this study. Patient details were provided by the Brisbane Breast Bank with written informed consent from all patients.

Specimen no.	Age	Breast cancer stage	Type of breast cancer	Vital status	Date of death	Survival time (years)*	Recurrence
10-14-183	51	T3N3a	Invasive ductal carcinoma with micropapillary carcinoma	D	25/10/2016	2.2	Yes
10-13-139	74	N/A	Inflammatory breast cancer	D	22/04/2015	4.3	Yes
10-14-093	56	T2N3a	Invasive ductal carcinoma	D	05/08/2016	2.3	Yes
10-14-193	72	T3N2a	Invasive ductal carcinoma	D	04/01/2018	3.4	Yes
10-14-162	51	T2N3a	Invasive ductal carcinoma	D	17/11/2015	1.3	Yes
10-14-092	62	T3Nx	Invasive ductal carcinoma	D	07/12/2015	1.6	Yes
10-14-131	43	T3N3a	Invasive lobular carcinoma	A	N/A		Yes
10-14-062	50	T2N3a	Mixed invasive ductal carcinoma/micropapillary	A	N/A		Yes
10-16-028	69	T2N1	Mixed invasive ductal carcinoma/invasive lobular carcinoma	A	N/A		Yes
10-14-017	45	T3N2	Invasive ductal carcinoma	A	N/A		No
10-14-142	58	T3N1a	Invasive ductal carcinoma	A	N/A		No
10-13-204	39	T3N1MX	Invasive ductal carcinoma	A	N/A		No
10-13-186	53	T1N1c	Invasive ductal carcinoma	A	N/A		No
10-13-157	36	T3N1miM0	Invasive ductal carcinoma	A	N/A		No
10-14-122	37	T2N1a	Mixed metaplastic	A	N/A		No

*Time to death from date of diagnosis

Figure S1


A
Relative Fluorescence Units
18000
16000
14000
12000
10000
8000
6000
4000
2000
0
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30 31 32 33 34 35 36 37 38 39 40 41
Glycan ID Nue5Gc#/Neu5Ac#
SubB2M Neu5Gc
SubB2M Neu5Ac
SubBA12 Neu5Gc
SubBA12 Neu5Ac

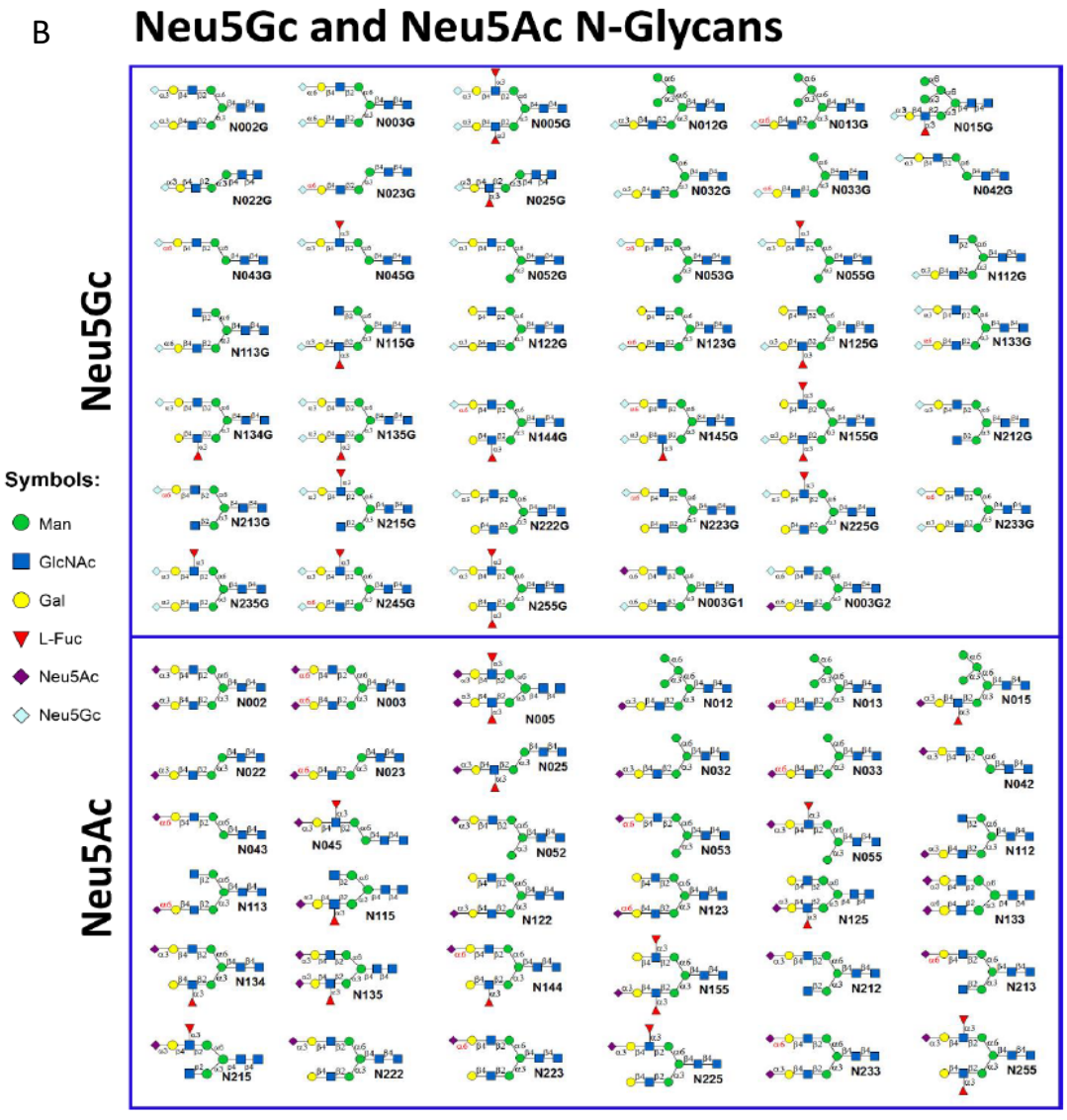

B
Neu5Gc and Neu5Ac N-Glycans
Neu5Gc
Neu5Ac
Symbols:
Man
GlcNAc
Gal
L-Fuc
Neu5Ac
Neu5Gc

Figure S2

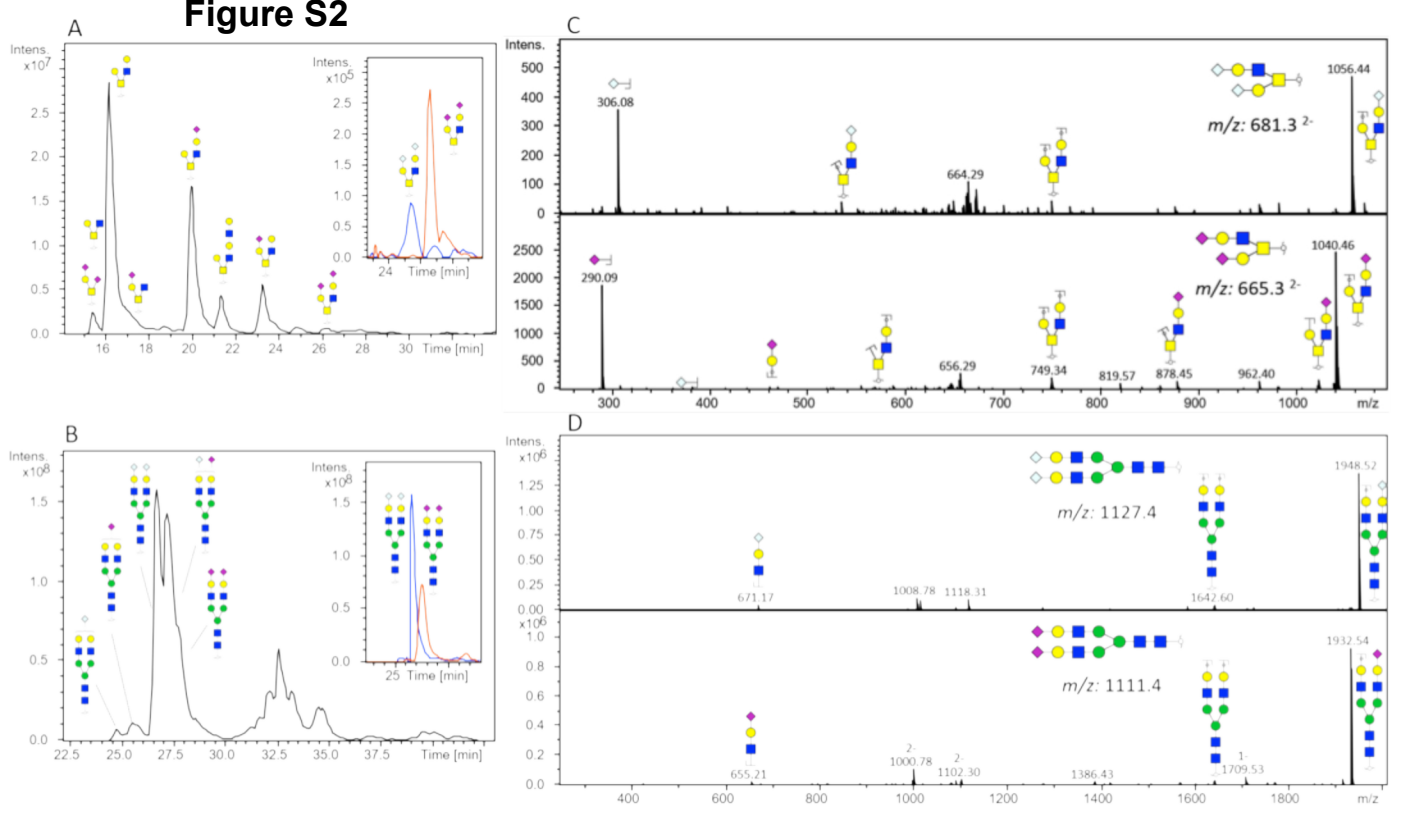

A
Intens.
x10^7
Time [min]
B
Intens.
x10^8
Time [min]
C
Intens.
306.08
664.29
1056.44
m/z: 681.3 2-
290.09
656.29
749.34
819.57
878.45
962.40
1040.46
m/z: 665.3 2-
m/z
D
Intens.
671.17
1008.78
1118.31
1642.60
1948.52
m/z: 1127.4
655.21
2- 1000.78
2- 1102.30
1386.43
1- 1709.53
1932.54
m/z: 1111.4
m/z

Figure S3

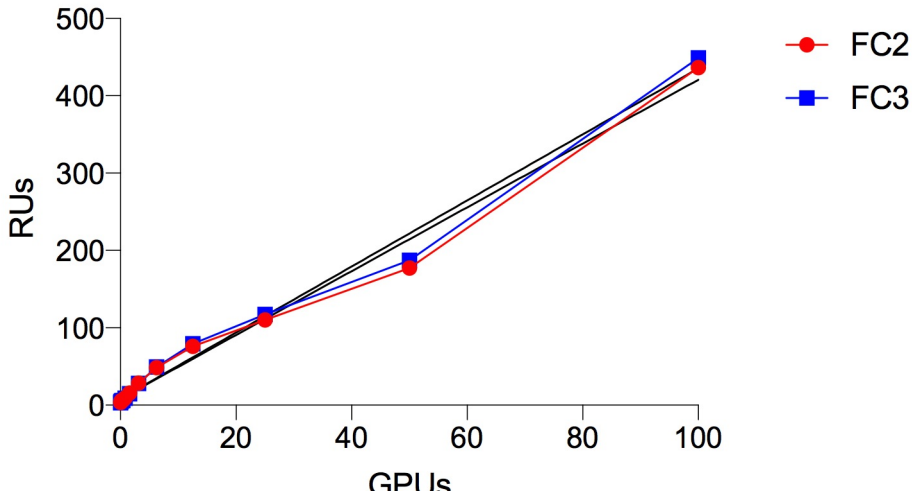


Figure S4

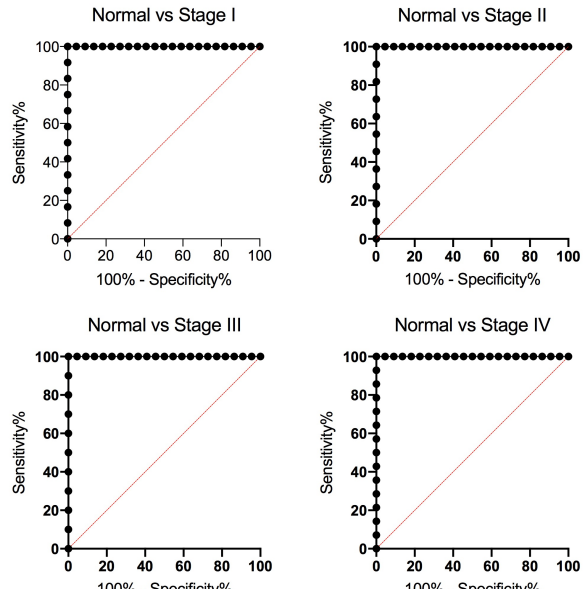

Normal vs Stage I
Normal vs Stage II
Normal vs Stage III
Normal vs Stage IV
Sensitivity%
100% - Specificity%
0
20
40
60
80
100

Figure S5

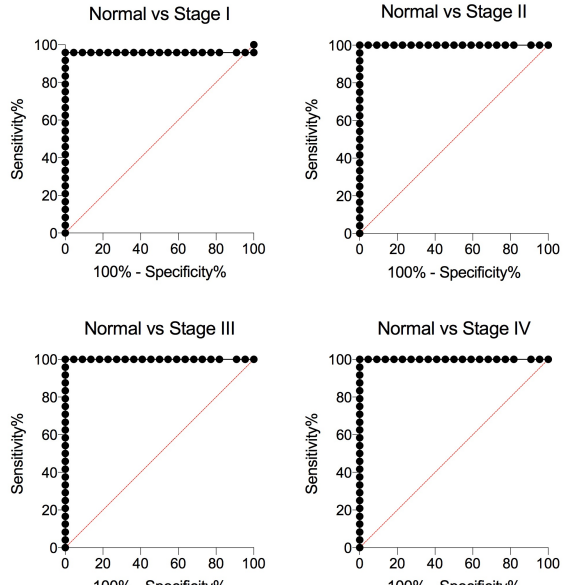

Normal vs Stage I
Normal vs Stage II
Normal vs Stage III
Normal vs Stage IV
Sensitivity%
100% - Specificity%
0
20
40
60
80
100

Figure S6A

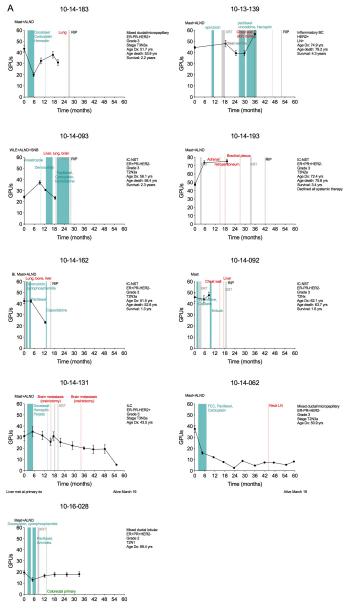
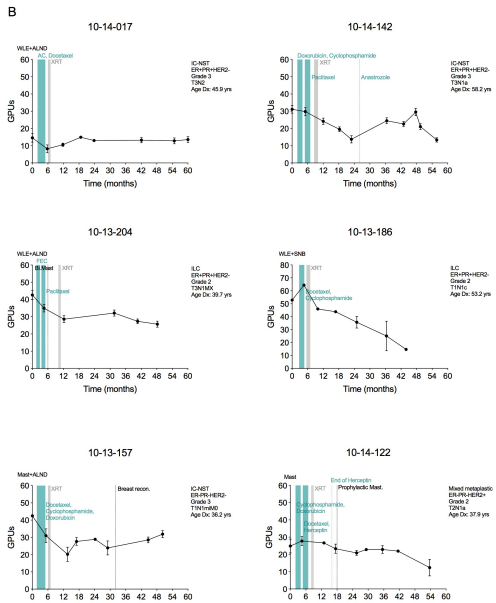

A
10-14-183
10-13-139
10-14-093
10-14-193
10-14-162
10-14-092
10-14-131
10-14-062
10-16-028
Time (months)
GPNs

Figure S6B


B
10-14-017
WLE+ALND
AC, Docetaxel
XRT
IC-NST
ER+/PR+/HER2-
Grade 3
T3N2
Age Dx: 45.9 yrs
GPUs
Time (months)
10-14-142
Doxorubicin, Cyclophosphamide
XRT
Paclitaxel
Anastrozole
IC-NST
ER+/PR+/HER2-
Grade 3
T2N1a
Age Dx: 58.2 yrs
10-13-204
WLE+ALND
XRT
BI-Mast
XRT
Paclitaxel
ILC
ER+/PR+/HER2-
Grade 3
T3N1MX
Age Dx: 39.7 yrs
10-13-186
WLE+SNB
XRT
Docetaxel,
Cyclophosphamide
ILC
ER+/PR+/HER2-
Grade 2
T1N1c
Age Dx: 53.2 yrs
10-13-157
Mast+ALND
XRT
Breast recon.
Docetaxel,
Cyclophosphamide,
Doxorubicin
IC-NST
ER-/PR-/HER2-
Grade 3
T1N1mM0
Age Dx: 36.2 yrs
10-14-122
Mast
End of Herceptin
XRT
Prophylactic Mast.
Cyclophosphamide,
Doxorubicin
Docetaxel,
Herceptin
Mixed metaplastic
ER-/PR-/HER2+
Grade 2
T2N1a
Age Dx: 37.9 yrs