EXPERIMENTAL SECTION

Detailed description of N-glycomics analysis.

N-glycan release from plasma samples

The N-glycan release of the discovery set was performed in microcentrifuge tubes, while the glycan release of the testing set was performed in 96-well plates. Both methods were shown to perform similarly (**Supplementary Figure 3**). The procedures are the same and similar to previously published methods (1, 2). Briefly, 25 μL of a 200mM ammonium bicarbonate (Sigma-Aldrich, St. Louis, MO) solution with 10 mM dithiothreitol (DTT, Promega, Madison, WI) was added to 25 μL of plasma. For the discovery set, this was performed in microcentrifuge tubes; for the testing set, this was performed in 96-well plates. Proteins in the samples were denatured using six cycles alternating between 100°C and room temperature (RT) for 10 seconds each. One μL of PNGaseF (New England Biolabs, Ipswich, MA, corresponding to 1000 NEB units or 15 IUB mU) was added to the samples, and enzymatic glycan release was performed for the discovery set in a CEM (Matthews, NC) microwave at 20W for 10 min. Enzymatic release was performed in batches of 23 samples and one standard serum, to allow quality control. For the testing set, glycan release was performed overnight (16h) at 37°C and a standard serum sample was included in the plate after every ten samples to allow quality control. Upon glycan release, deglycosylated proteins were precipitated using 200 μL of ice-cold ethanol, and the samples were chilled at -80°C for 1 hour. Upon centrifugation, the supernatant was transferred, and dried *in vacuo*.

N-glycan purification using graphitized carbon SPE

Oligosaccharides released by PNGaseF were purified using graphitized carbon SPE cartridges (Discovery set, Grace, Deerfield, IL) or porous graphitized carbon 96-wells SPE plates (Testing set, Glygen, Columbia, MD) For the discovery set SPE was performed on a Gilson liquid handler (Middleton, WI) in batches of 24 (23 study samples and 1 quality control), cartridges were conditioned using 4 mL of 80% ACN containing 0.05% TFA (EMD chemicals, Gibbstown, NJ), followed by 4 mL of water containing 0.05% TFA. Released N-glycan samples were reconstituted in 500 μL of water and subsequently loaded onto the cartridges. Cartridges were washed using 3 x 4 mL of water and N-glycans were eluted using 4 mL of 40% ACN containing 0.05% TFA. For the testing set, all samples were processed simultaneously and after each step solvent was removed by centrifugation at 1000g for 30 sec. Wells of 96-wells PGC SPE plates were conditioned using 2 x 200 μL of 80% ACN containing 0.05% TFA followed by 3 x 200 μL of water containing 0.05% TFA. Released N-glycan samples were reconstituted in 200 μL of water and subsequently loaded onto the wells. SPE plates were washed using 5 x 200 μL of water and N-glycans were eluted using 2 x 200 μL of 40% ACN containing 0.05% TFA. Eluates from both the discover set and the testing set were dried *in vacuo* prior to analysis.

nHPLC-chip-TOF-MS analysis

N-glycans were analyzed using an Agilent (Santa Clara, CA) 6200 series nanoHPLC-chip-TOF-MS, consisting of an autosampler, which was maintained at 8°C, a capillary loading pump, a nanopump, HPLC-chip-MS interface and an Agilent 6210 time of flight (TOF) mass spectrometer. The microfluidic chip (glycan chip II, Agilent) contained a 9 x

0.075 mm i.d. enrichment column coupled to a 43 x 0.075 mm i.d. analytical column; both packed with 5 μm porous graphitized carbon (PGC). N-glycans were reconstituted in 100 μL of water prior to analysis; 1 μL of sample was used for injection. Upon injection, the sample was loaded onto the enrichment column using 3% ACN containing 0.1% Formic acid (FA, Fluka, St. Louis, MO). After the analytical column was switched in-line, the nano-pump delivered a gradient of 3%ACN with 0.1% FA (solvent A) and 90% ACN with 0.1% FA (solvent B).). The gradient increased from 0%B to 33 %B over 13 minutes, followed by an increase to 36%B at 16.5 minutes and 100%B at 17 minutes. The column was then washed at 100%B for 5 minutes, followed by 10 minutes reequilibration at 0%B. Positive ions were generated and mass spectra were acquired over a mass window of 400 *m/z* to 3000 *m/z*. The method has previously been shown to be highly stable and suitable for biomarker discovery (3).

Data processing

Data processing was performed using Masshunter® qualitative analysis (version B.03.01, Agilent) and Microsoft® Excel® for Mac 2011 (version 14.1.3, Microsoft), similar to previous publications (2, 4) with modifications. Data was loaded into Masshunter qualitative analysis, and glycan features were identified and integrated using the Molecular Feature Extractor algorithm. First, signals above a signal to noise threshold of 5.0 were considered. Then, signals were deconvoluted using a tolerance of 0.0025 *m/z* ± 10 ppm. The resulting deconvoluted masses were subsequently annotated using a retrosynthetic theoretical glycan library, where a 15 ppm mass error was allowed. Glycan

compositions and peak areas were exported to csv-format for further statistical evaluation.

REFERENCES

1. Kronewitter SR, An HJ, de Leoz ML, Lebrilla CB, Miyamoto S, Leiserowitz GS. The development of retrosynthetic glycan libraries to profile and classify the human serum N-linked glycome. Proteomics. 2009;9:2986-94.
2. Ruhaak LR, Nguyen UT, Stroble C, Taylor SL, Taguchi A, Hanash SM, et al. Enrichment strategies in glycomics based lung cancer biomarker development. Proteomics Clinical applications. 2013.
3. Ruhaak LR, Taylor SL, Miyamoto S, Kelly K, Leiserowitz GS, Gandara D, et al. Chip-based nLC-TOF-MS is a highly stable technology for large-scale high-throughput analyses. Anal Bioanal Chem. 2013;405:4953-8.
4. Hua S, An HJ, Ozcan S, Ro GS, Soares S, DeVere-White R, et al. Comprehensive native glycan profiling with isomer separation and quantitation for the discovery of cancer biomarkers. Analyst. 2011;136:3663-71.