Immunoglobulin A N-glycosylation Presents Important Body Fluid-specific Variations in Lactating Mothers

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In Brief
We have characterized mature breast milk, saliva, and plasma IgA N-glycan profiles from lactating individuals and studied the potential differences among individuals who delivered infants vaginally or by cesarean section. The comparative analysis obtained by nano-HPLC-chip/TOF-MS reveals a body fluid-specific profile of 50+ N-glycan species some of which can also be influenced by delivery mode.

Highlights
- Comparative N-Glycome analysis of IgA from breast milk, saliva, and plasma of lactating participants revealed 89 glycan compositions that may correspond to over 250 structures.
- Specific N-glycan structures can be used to distinguish IgA from breast milk, saliva, and plasma.
- Delivery mode influenced the maternal IgA N-glycan profile in saliva and plasma.
Immunoglobulin A N-glycosylation Presents Important Body Fluid-specific Variations in Lactating Mothers*

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Secretory Immunoglobulin A (SIgA) is central to mucosal immunity; represents one of the main immunological mechanisms of defense against the potential attack of pathogens. During lactation SIgA is produced by plasmablasts in the mammary gland and is present in breast milk, playing a vital role in the passive immunity of the newborn. Interestingly, the different components of SIgA are highly N-glycosylated, and these N-Glycans have an essential role in health maintenance. In this work, we performed a glycomic study to compare N-glycosylation of SIgA purified from mature breast milk and saliva, and plasma IgA from the same lactating participants. Our results revealed a greater diversity than previously reported, with 89 glycan compositions that may correspond to over 250 structures. Among these glycans, 54 glycan compositions were characterized as body-fluid specific. Most of these unique N-Glycan compositions identified in SIgA from mature milk and IgA from plasma were fucosylated and both fucosylated and sialylated species, whereas in salivary SIgA the unique structures were mainly undecorated complex N-Glycans. In addition, we evaluated the effect of delivery mode on (S)IgA glycosylation. Lactating participants who had given birth by vaginal delivery presented an increased proportion of high mannose and fucosylated glycans in salivary SIgA, and selected high mannose, fucosylated, sialylated, and both fucosylated and sialylated glycans in plasma IgA, indicating that the hormonal changes during vaginal delivery could affect plasma and saliva IgA. These results reveal the structural details that provide a new dimension to the roles of (S)IgA N-Glycans in different tissues, and especially in maternal and new-born protection and infant development. The design of optimal recombinant IgA molecules specifically targeted to protect mucosal surfaces will need to include this dimension of structural detail. Molecular & Cellular Proteomics 18: 2165–2177, 2019. DOI: 10.1074/mcp.RA119.001648.
to hybrid or complex glycans, which can be decorated with fucose or sialic acid residues. IgA is a heavily glycosylated oligomeric complex (4). Both IgA1 and IgA2 contain two N-glycosylation sites in the Fc region of the heavy chain, whereas all the other allotypes of IgA2 carry two additional N-glycosylation sites; moreover, IgA2m(2) and IgA2n contain one additional N-glycosylation site (4). Glycosylation of SlgA is even more complicated, as this glycoprotein assembly comprises one additional N-glycosylation site on the J chain and seven N-glycosylation sites on the secretory component.

Interestingly, removal of N-Glycans from both SlgA and free secretory component drastically decreases the interaction with Gram-positive bacteria highlighting the critical role of IgA glycosylation in bacterial recognition (7), and recently, glycans-lipopolysaccharide interactions were shown to contribute to IgA coating of Gram-negative bacteria independently of antibody-epitope recognition (8). Further, studies have also demonstrated that specific terminal monosaccharides in human milk SlgA N-Glycans including mannose, galactose, fucose, N-Acetylgalactosamine, and sialic acid are potential binding sites for respiratory and gastrointestinal pathogens including Streptococcus pneumoniae, Helicobacter pylori, and Type 1-fimbriated E. coli (9–11). Despite this critical biological role of IgA glycosylation in bacterial attachment, pathogen decay and clearance, structural characterization of SlgA N-glycome has lagged because of its complexity and heterogeneity. However, advances in liquid chromatography and mass spectrometry provided essential tools for glycomics analysis (12, 13). We reported the most extensive site-specific glycosylation analysis of SlgA in human colostrum (14). Ruhaak, Bondt and colleagues characterized plasma IgA N-glycome during pregnancy (15, 16). And more recently, glycopeptides derived from salivary SlgA from men and nonlactating women were reported (17). However, no systematic comparison of IgA glycosylation in multiple body-fluids of the same lactating individual has so far been reported.

Changes in glycosylation during pregnancy have been described in plasma IgA (15, 16), and a recent human milk N-glycome study showed that delivery mode affects the mother’s milk glycome (18). Given that SlgA from breast milk plays a crucial direct role in the passive immunity of the newborn infant during lactation, and at the time that also protects the lactating mother from infections, as well as plasma IgA and salivary SlgA. Thus, there is a fundamental question of whether the IgA in various tissues has identical or different glycosylation for individual lactating mothers. In this study, we characterized the mature breast milk and salivary SlgA N-glycan profile and compared them to the N-glycan profile obtained from plasma IgA purified from the same lactating participants. Moreover, plasma IgA N-glycosylation of nonlactating participants was used as a reference to evaluate the influence of lactation. Additionally, we evaluated whether the N-glycome of SlgA and plasma IgA could be altered by delivery mode.

**EXPERIMENTAL PROCEDURES**

Chemicals and Materials—Staphylococcal super-like protein 7 (SSL7)/Agarose was purchased from InvivoGen (San Diego, CA). Sodium phosphate and glycerine were purchased from EMD Millipore (Billerica, MA). Dialysis tubes (3MWCO) were obtained from Spectrum Lab (Rancho Dominguez, CA). Glyceraldehyde free peptide N-glycosidase F (PNGase F), alpha-2,3-neuraminidase, and alpha-2,3/6/8-neuraminidase were obtained from New England Biolabs (Ipswich, MA). Acetonitrile was purchased from Honeywell (Morristown, NJ) and formic acid was purchased from Fisher Scientific (Hanover Park, IL). Porous graphitized carbon cartridges (PGC) were obtained from Grace Davison Discovery Sciences (Deerfield, IL). MilliQ water was used throughout the experiment. Plasma IgA was purchased from Sigma-Aldrich (StLouis, MO).

**Breast Milk, Saliva, and Plasma Samples**

Lactation Study Participants—Breast milk, saliva, and plasma samples were obtained from a random subset of eight healthy women enrolled in the parent cohort observational study (July 2009 -Feb 2014, Foods for Health Institute Lactation Study) at the University of California, Davis (19). These paired samples were collected on day 71 postpartum from women who delivered healthy full-term infants (>37 weeks gestation) either by vaginal delivery or cesarean C-section. The blood type of all lactating participants was O positive. Additionally, eight fasting plasma samples were collected from gender, aged and blood-type matched nonlactating healthy participants enrolled in a single-blind, randomized-control, dietary fat postprandial pilot study and used as controls for this discovery project. The UC Davis Institutional Review Board approved all aspects of both studies, and informed consent was obtained from all participants. The Davis cohort study was registered on clinicaltrials.gov (ClinicalTrials.gov Identifier: NCT01817127).

Breast Milk Samples—Breast milk samples were collected by each participant in the morning with a protocol based on the previously published method (20), which involved removing milk from one breast using a Harmony Manual Breast Pump (Medela Inc., McHenry, IL) 2–4 h after feeding her infant. Participants fully pumped one breast into a bottle, inverted six times, transferred 12 ml into a 15 ml polypyrrole tube, and subsequently froze the sample in their kitchen freezers (−20 °C). Samples were picked up biweekly, transported to the lab on dry ice, and stored at −80 °C until processing.

Saliva Samples—2 ml samples were collected into a polypyrrole tube by participants. Lactating participants avoided eating, drinking, smoking, and use of oral hygiene products for a minimum of one hour before saliva collection.

Plasma Samples—EDTA-whole blood was drawn after a 12-hour fast. Samples were maintained on ice and centrifuged within 30 min of the collection at 1,300 × g at 4 °C for 10 min. After centrifugation, plasma tubes were kept on ice during liquatung and directly frozen at −80 °C until analyzed.

**Immunoglobulin A Purification**—SlgA from milk and saliva and IgA from plasma were purified by affinity chromatography using SSL7/agarose beads, as described earlier for milk samples and slightly

1 The abbreviations used are: IgA, Immunoglobulin A; SlgA, Secretory Immunoglobulin A; pIgR, polymeric Immunoglobulin Receptor; Hex, hexose; HexNAc, N-acetylhexosamine; GlcNAc, N-acetylgalactosamine; Fuc, fucose; NeuAc, N-acetylneuraminic acid; PGC, porous graphitized carbon; EIC, extracted ion chromatograms; SC, free IgA Secretory Component.
modified for saliva and plasma samples (14). Briefly, milk and saliva samples were centrifuged for 30 min at 13,000 rpm at 4 °C to remove the cream and mucus layer, and the aqueous layer was transferred to a new tube for further analysis. Empty columns were packed with 0.5 ml of SSL7/agarose beads and equilibrated with 10 mM sodium phosphate in 150 mM sodium chloride buffer at pH 7.2 (buffer A). Fractions obtained were loaded onto the column, and the flow-through was reloaded three times. After washing with buffer A, IgA was eluted with 0.1 M glycine (pH 2), and this solution was immediately neutralized by addition of 1 M phosphate buffer (pH 8). Samples were dialyzed against 10 mM ammonium bicarbonate, concentrated, and stored at −80 °C. Purity of SIgA and IgA samples was verified by SDS-PAGE, followed by coomassie-blue staining and, protein concentration was determined by Bradford assay.

N-glycan Release and Enrichment—Aliquots of 50 μg of purified SIgA from milk and saliva and plasma IgA were reduced with dithiothreitol (10 mM DTT in 50 mM NH4HCO3) and alkylated with iodoacetamide (20 mM IAA in 50 mM NH4HCO3, 30 min, RT, in the dark) before deglycosylation. N-Glycans were enzymatically released with PNGase F (2 μl) (37 °C, 16hs) and purified by solid phase extraction using graphitized carbon cartridges as previously described (21). Briefly, cartridges were conditioned sequentially with 80% (v/v) acetonitrile, 0.1% (v/v) trifluoroacetic (TFA) acid and water. Samples were loaded onto the graphitized carbon cartridges (GCC) and washed with water. Finally, N-Glycans were eluted with 40% (v/v) acetonitrile in 0.05% (v/v) TFA. Samples were vacuum dried at room temperature before mass spectrometry (MS) analysis. Similarly, commercial serum IgA N-Glycans were also prepared and used as a standard for quality control for the MS and tandem MS analysis.

Enzymatic Desialylation of N-Glycans—Purified N-Glycans from milk, saliva, and plasma of lactating participants with vaginal delivery and C-section were pooled separately. Glycans were then reduced with 1 M NaBH4 in a 65 °C water bath for 1.5 h and desalted using graphitized carbon cartridges (GCC) and stored at −80 °C. Purification of SIgA and IgA was performed using a new tube for further analysis. Empty columns were packed with 0.5 g of purified SIgA from milk and saliva and plasma IgA were reduced with dithiothreitol (10 mM DTT in 50 mM NH4HCO3) and alkylated with iodoacetamide (20 mM IAA in 50 mM NH4HCO3, 30 min, RT, in the dark) before deglycosylation. N-Glycans were enzymatically released with PNGase F (2 μl) (37 °C, 16hs) and purified by solid phase extraction using graphitized carbon cartridges as previously described (21). Briefly, cartridges were conditioned sequentially with 80% (v/v) acetonitrile, 0.1% (v/v) trifluoroacetic (TFA) acid and water. Samples were loaded onto the graphitized carbon cartridges (GCC) and washed with water. Finally, N-Glycans were eluted with 40% (v/v) acetonitrile in 0.05% (v/v) TFA. Samples were vacuum dried at room temperature before mass spectrometry (MS) analysis. Similarly, commercial serum IgA N-Glycans were also prepared and used as a standard for quality control for the MS and tandem MS analysis.

Nano-LC/MS and LC/MS/MS Analysis of N-Glycans—Purified N-Glycans were reconstituted in 30 μl of water and analyzed by nano-HPLC-chip/TOF Mass Spectrometer (Agilent Technologies) using a Porous Graphite Carbon (PGC) microfluidic chip as previously described (22). Mass spectra were obtained at 1.5 s per spectrum with a mass to charge (m/z) range of 400–2000 in positive ion mode. Glycan masses were calibrated to an internal reference ion mass with m/z 1221.991 value.

Tandem MS data for N-Glycans were obtained from N-glycan samples from milk, saliva, and plasma of all participants using nano-HPLC-chip/TOF Mass Spectrometer (Agilent Technologies). The chromatographic gradient and the column conditions used were identical to those used in the nano-HPLC-chip/TOF analysis. Tandem MS was performed in the positive ion mode using the following parameters optimized for N-glycan analysis: fragmentor 175V, skimmer 60V, and octopole 1 RF 750V. Auto MS/MS mode was used with 0.63 spectra/second to obtain MS and MS/MS spectra. Glycans were observed as singly and multiply charged ions ([M+H]+, [M+2H]+2 and [M+3H]+3). Precursor ions were chosen based on the abundance, and doubly charged ions were selected as priority, singly and triply charged ions were given second and third place whereas all the other charges were given least priority. Collision-induced dissociation (CID) was applied with nitrogen gas depending on the m/z (mass/charge) of the ions according to the following equation:

\[ V_{\text{collision}} = \text{slope}(m/z) + \text{offset} \]  

(Eq. 1)

where slope is (1.8/100 Da) V and offset is 2.4 V.

The fragment ions of 772.3 m/z (HexHexNAc3) and 790 m/z (HexHexNAc2OH), 350.1 m/z (HexNAcFuc), and 292.1 m/z (NeuAc), and 657.2 m/z (HexHexNAc2NeuAc) were used to identify bisecting GlcNAc, fucosylated, and sialylated species, respectively (supplemental Fig. S1). Ion with m/z 790 corresponded to a C fragment, whereas all other ions corresponded to nonreducing end or internal B-type oxonium ions according to Domon & Costello nomenclature (23). Tandem MS spectra were annotated using in-house software previously validated (24).

Data Analysis—N-Glycans were identified and quantified using Agilent MassHunter Qualitative Analysis software (Version B. 06.01, Agilent Technologies) in combination with an in-house retro synthetic N-glycan library of all possible glycan compositions according to accurate mass (25) including complex, hybrid, and high mannose glycan species. Glycan signals with ion counts below signal/noise ratio of 5 were filtered out, and a 20 ppm mass error tolerance was used to correlate the deconvoluted masses to the theoretical masses. Tandem MS confirmation of library matches was previously validated, enabling rapid and accurate assignment of glycan compounds (26, 27).

Relative abundance of individual glycan compositions was calculated by adding integrated peak area (ion counts) for all charge states of a glycan mass and normalizing to the total peak area of all the glycans present in the sample. The N-glycome for a) milk and saliva SIgA, and b) plasma IgA was characterized in individual samples and reported as average values for each glycan composition with relative abundance ≥ 0.05% obtained from all individuals. All MS data and annotated spectra have been stored at the MassIVE repository with an identifier MSV000083029.

Statistical analyses were performed using JMP (SAS Institute Inc), and the abundance of each glycan was log10 transformed before statistical analyses. One-way analysis of variance (ANOVA) was used to compare glycans as the dependent variable among the three sample types as the independent variable: breast milk, plasma, and saliva. Tukey’s HSD test was used for multiple comparisons among the sample types. Similarly, ANOVA and Tukey’s HSD analyses were performed to compare glycan types from plasma IgA of lactating (vaginal and C-section delivery mode) and nonlactating participants. A two-tailed, t test was used to compare glycans between vaginal and C-section delivery mode. All the statistical analyses had an alpha value of 0.05.

RESULTS

We analyzed the N-glycan profiles of SIgA isolated from mature breast milk and saliva samples, and IgA isolated from plasma as well. Nano-LC/MS analysis of IgA from all three body fluids yielded nearly 90 unique glycan compositions, that may correspond to over 250 structures combined (Fig. 1A). Glycans were grouped into five different categories: high mannose, fucosylated-only, sialylated-only, fucosylated+sialylated (simultaneously sialylated and fucosylated), and undecorated (neither fucosylated nor sialylated), depending on their monosaccharide composition and the presence or absence of fucose and sialic acids as decorations. Additionally, glycans containing bisecting GlcNAc and those that are terminally galactosylated are reported separately but may contain several glycan structures of different categories. The relative abundances of bisecting GlcNAc structures were determined by summing the
relative abundances of bisecting GlcNAc species. Similarly, galactosylation was calculated by adding the relative abundances of glycans with terminal galactoses. Mono-, di-, tri-, and tetrafucosylation were calculated by summing the relative abundances of glycans with one to four fucose residues, respectively. Similarly, sialylation was determined depending on the number of sialic acids present.

**Mature Breast Milk SIgA N-Glycome Is Dominated by Fucosylated and Simultaneously Fucosylated and Sialylated Glycans**—Over 70 glycan compositions were identified in SIgA from mature breast milk, listed in the supplemental Table S1. The isomers are provided along with their unique retention times in supplemental Table S2A. Although the isomers are readily identified, the exact structures (mainly linkages) have not been elucidated. For milk, the two most abundant groups were fucosylated-only (32%) and fucosylated + sialylated glycans (28%) (Fig. 1B), whereas undecorated (17%) and sialylated-only (14%) glycans were the next more abundant species. High mannose glycans were the least abundant group (9%).

The most abundant species in milk SIgA N-glycome are shown in the pie chart or glycan wheel in Fig. 2A and supplemental Table S3, with predominant biantennary structures. A smaller fraction of triantennary and high mannose glycans were also observed. Monofucosylation (42%) was a common feature (Table I), whereas 12% of difucosylated glycans and 5% of trifucosylated glycans were also identified. Interestingly, the majority of sialylated glycans were monosialylated (36%). In mature breast milk SIgA, only 25% of the glycans contained bisecting GlcNAc, and a high level of galactosylation was observed (55%).

**Fig. 1.** A, Representative N-glycosylation profiles of SIgA obtained from mature milk and saliva, and plasma IgA from the same donor. High mannose glycans correspond to peaks in red. Complex and hybrid glycans without fucose or sialic acid were categorized as undecorated glycans (orange peaks); glycans containing fucose were selected as fucosylated glycans (green peaks); glycans with sialic acid were categorized as sialylated glycans (pink peaks); and glycans with both fucose and sialic acid were selected as fucosylated + sialylated glycans (blue peaks). B, Relative abundances of N-glycan groups in mature breast milk and saliva SIgA, and plasma IgA. Abundance for each glycan composition in all lactating participants was averaged to observed tissue-specific N-glycan variations.
Fucosylated and Undecorated Glycans Are More Abundant in Maternal Salivary S IgA N-Glycome—In salivary S IgA, similarly over 70 different glycan compositions were identified (supplemental Table S1) and corresponding isomers separated (supplemental Table S2). As in milk S IgA, salivary S IgA also presented fucosylated-only glycans (37%) as the most abundant group; however, undecorated glycans increased (29%), whereas fucosylated/sialylated structures dropped to 13%, an abundance like that found for high mannose glycans (12%). Interestingly, sialylated species were the least abundant (9%).

As with mature milk, S IgA biantennary structures were dominant in saliva, with a lower degree of triantennary, tetraantennary, and high mannose glycans (Fig. 2B and supplemental Table S4). Monofucosylated glycans (39%) were the most abundant fucosylated species (Table I), with di- (5%) and trifucosylated (5%) glycans in lower proportions. Although sialylation was significantly lower than in mature milk S IgA, most of the species were monosialylated (19%). In comparison, glycans containing bisecting GlcNAc were significantly higher (41%), and galactosylation (46%) was significantly lower than in mature breast milk S IgA.

Maternal Plasma IgA N-Glycome Is Dominated by Sialylated and Simultaneously Fucosylated and Sialylated Glycans—In contrast to the N-glycoprofiles described for mature breast milk and salivary S IgA, the plasma IgA N-glycome presented a somewhat lower diversity of glycan species, with over 50 different glycan compositions and isomers (supplemental Table S5). Monofucosylated glycans (39%) were the most abundant fucosylated species (Table I), with di- (5%) and trifucosylated (5%) glycans in lower proportions. Although sialylation was significantly lower than in mature milk S IgA, most of the species were monosialylated (19%). In comparison, glycans containing bisecting GlcNAc were significantly higher (41%), and galactosylation (46%) was significantly lower than in mature breast milk S IgA.
Comparative IgA N-glycans from Milk, Saliva, and Plasma

### Table I

**Comparison of key features of N-Glycans in IgA from lactating participants**

| Glycan group | Composition                     | Milk (mean%, S.D.) | Saliva (mean%, S.D.) | Plasma (mean%, S.D.) | p value<sup>a</sup>, Milk and Saliva | p value<sup>b</sup>, Milk and Plasma | p value<sup>b</sup>, Saliva and Plasma |
|--------------|--------------------------------|--------------------|----------------------|----------------------|--------------------------------------|---------------------------------------|---------------------------------------|
| Galactosylation | 55.40 (8.62) | 45.86 (6.06) | 49.73 (2.08) | 0.0054 | – | – |
| Total Fucosylation | 59.73 (10.05) | 49.98 (6.09) | 38.09 (4.40) | 0.0229 | <0.0001 | 0.0009 |
| Monofucosylation | 41.88 (5.88) | 38.77 (4.55) | 37.37 (4.34) | – | – | – |
| Difucosylation | 11.57 (3.36) | 5.15 (1.23) | 0.49 (0.28) | 0.0009 | <0.0001 | <0.0001 |
| Trifucosylation | 5.07 (1.94) | 5.08 (1.89) | 0.18 (0.12) | – | <0.0001 | <0.0001 |
| Tetrafucosylation | 1.14 (0.68) | 0.98 (0.26) | 0.05 (0.03) | – | <0.0001 | <0.0001 |
| Total Sialylation | 41.96 (16.25) | 22.13 (10.70) | 69.31 (4.20) | 0.0013 | 0.0047 | <0.0001 |
| Monosialylation | 35.78 (12.14) | 18.73 (7.41) | 44.74 (2.88) | 0.0004 | – | <0.0001 |
| Disialylation | 6.07 (4.30) | 3.33 (3.44) | 23.33 (2.46) | 0.0498 | 0.0006 | <0.0001 |
| Trisialylation | 0.11 (0.04) | 0.07 (0.04) | 1.25 (0.63) | – | <0.0001 | <0.0001 |
| Bisecting GlcNAc | 25.26 (13.13) | 41.04 (9.68) | 35.34 (4.66) | 0.0043 | 0.0205 | – |

<sup>a</sup>Values are reported as average relative abundance and standard deviation (S.D.), n = 8.

<sup>b</sup>Statistically significant (p < 0.05), highly significant (p < 0.005).

### Table II

**Significantly more abundant N-Glycans in mature breast milk SlgA**

| Glycan group | Composition | Milk<sup>a</sup> | Saliva<sup>a</sup> | Plasma<sup>a</sup> | p value<sup>b</sup>, Milk and Saliva | p value<sup>b</sup>, Milk and Plasma | p value<sup>b</sup>, Saliva and Plasma |
|--------------|-------------|-----------------|-----------------|-----------------|--------------------------------------|---------------------------------------|---------------------------------------|
| Fucosylated-only | HexHexNAc4Fuc1NeuAc0 | 3.13 | 1.31 | 0.30 | 0.0001 | 0.0001 |
| Fucosylated + sialylated | HexHexNAc4Fuc2NeuAc1 | 8.19 | 1.50 | 3.47 | 0.0001 | 0.0003 |
|                  | HexHexNAc4Fuc2NeuAc1 | 5.80 | 0.90 | ND | 0.0007 | 0.0001 |
|                  | HexHexNAc5Fuc2NeuAc1 | 0.51 | 0.10 | 0.07 | 0.0149 | 0.0002 |

Glycan composition is abbreviated as follows: Hex, Hexosamine, Fuc = Fucose, NeuAc = N-Acetylneuraminic Acid and subscript indicates the number of each monosaccharide residue. ND, not detected.

<sup>a</sup>Values are reported as average relative abundance, n = 8.

<sup>b</sup>Statistically significant (p < 0.05), highly significant (p < 0.005).

ble S1 and [supplemental Table S2C). Plasma IgA yielded a high abundance of sialylated-only glycans (39%), followed by fucosylated + sialylated glycans (32%), Undecorated (17%), fucosylated-only (7%) and high mannose (6%) glycans were the least abundant in plasma IgA.

As in SlgA from mature milk and saliva, the majority of the abundant glycans in plasma IgA were biantennary, with a lower degree of triantennary and high mannose glycans (Fig. 2C and [supplemental Table S5). Monosialylation (45%) was a common feature, and in contrast to what was found in SlgA, disialylated glycans (23%) were also abundant (Table I). Although fucosylation was significantly lower in plasma IgA compared with SlgA from milk and saliva, almost all fucosylated glycans were monofucosylated. Interestingly, in plasma IgA, glycans containing bisecting GlcNAc (35%) were significantly higher than in mature milk SlgA, and galactosylation (50%) was higher than salivary IgA.

**Identification of IgA Body-fluid Specific N-glycan Profiles in Breast Milk, Saliva, and Plasma—**A comparative N-glycan analysis of SlgA obtained from milk and saliva, and plasma IgA allowed us to identify over 50 individual glycan compositions that vary on their abundance in a body-fluid specific manner. In mature milk SlgA, four N-Glycans were found to be significantly more abundant than in salivary SlgA and plasma IgA (Table II), and they corresponded to fucosylated-only, and fucosylated + sialylated N-Glycans. Among them, monosialylated biantennary glycans with one or two fucose residues corresponded to the top two most abundant glycans in milk SlgA. For salivary SlgA, twelve glycans were significantly more abundant and corresponded to Man8, undecorated, fucosylated-only, and fucosylated + sialylated glycans (Table III). Although with low relative abundance, those glycans were selectively present in salivary SlgA. Further, in plasma IgA, we identified seventeen significantly abundant N-Glycans, and they were among the undecorated, sialylated-only, and fucosylated + sialylated glycan groups; eight of these were also the most abundant glycans (Table IV).

Interestingly, we also found twenty-one glycans to be significantly more abundant in SlgA from mature milk and saliva than in circulating plasma IgA. The majority of these glycans were fucosylated-only (71%), followed by fucosylated + sialylated (24%) and undecorated species (5%) (Table V). Remarkably, five glycans corresponding to fucosylated-only and fucosylated + sialylated were the most abundant species observed in mature milk and saliva SlgA.

**Delivery Mode Affects Mucosal and Systemic IgA N-glycosylation—**To evaluate whether the delivery mode affects (SI)gA glycosylation, milk, and saliva SlgA and plasma IgA, the N-glycosylation profiles from each body fluid obtained from lactating participants who delivered their infants vag-
finally were compared with those from participants who delivered their infants by cesarean section (Fig. 3). In salivary SIgA, lactating participants who delivered by c-section showed lower levels of Man5 and bi- and triantennary fucosylated glycans with one and up to four fucosere-
duces (Hex5HexNAc3Fuc0NeuAc0, Hex5HexNAc3Fuc1NeuAc0, Hex5HexNAc3Fuc2NeuAc0, Hex5HexNAc3Fuc3NeuAc0, and Hex5HexNAc3Fuc4NeuAc0; and higher levels of bisecting agalacto-biantennary glycans (Hex3HexNAc5Fuc0NeuAc0) (Fig. 3A). Notably, differences were not restricted only to salivary SIgA, as plasma IgA from lactating participants who delivered their infants by c-section also showed lower levels in Man6, Man7, Man8, and Man 9. Disialylated bi- and triantennary glycans (with compositions Hex5HexNAc5Fuc0NeuAc0 and Hex5HexNAc5Fuc0NeuAc0) were also significantly reduced in participants with c-section. A similar trend was observed among the less abundant trisialylated triantennary glycans (Hex6HexNAc5Fuc1NeuAc3) and monosialylated hybrid glycans (Hex5HexNAc5Fuc2NeuAc0) (Fig. 3B). No significant effects of delivery mode were observed in milk SIgA N-Glycans.

Because sialylated glycans from plasma IgA seemed affected by delivery mode, we also evaluated whether the distribution of sialic acid linkage type was also altered. By ana-

TABLE III

| Glycan group       | Composition                        | Milk     | Saliva   | Plasma    | p value<sub>a</sub>, Saliva and Plasma | p value<sub>b</sub>, Saliva and Plasma |
|--------------------|------------------------------------|----------|----------|-----------|----------------------------------------|----------------------------------------|
| High mannose       | Hex8HexNAc2                        | 1.38     | 1.95     | 1.06      | 0.0181                                 | 0.0011                                 |
| Undecorated        | Hex4HexNAc4Fuc0NeuAc0              | 1.47     | 2.45     | 0.93      | 0.0349                                 | 0.0139                                 |
|                    | Hex7HexNAc6Fuc0NeuAc0              | 0.38     | 2.23     | ND        | 0.0032                                 | 0.0001                                 |
|                    | Hex3HexNAc4Fuc0NeuAc0              | 0.78     | 1.69     | 0.46      | 0.0191                                 | 0.0078                                 |
|                    | Hex5HexNAc3Fuc0NeuAc0              | 0.38     | 0.63     | 0.24      | 0.0202                                 | 0.0007                                 |
| Fucosylated-only   | Hex4HexNAc4Fuc3NeuAc0              | 0.07     | 0.36     | ND        | 0.0013                                 | 0.0007                                 |
|                    | Hex5HexNAc5Fuc4NeuAc0              | ND       | 0.15     | ND        | 0.0001                                 | 0.0001                                 |
|                    | Hex4HexNAc5Fuc2NeuAc0              | ND       | 0.1      | ND        | 0.0001                                 | 0.0044                                 |
| Fucosylated + sialylated | Hex5HexNAc4Fuc2NeuAc2 | 0.05     | 0.24     | ND        | 0.0031                                 | 0.0006                                 |
|                    | Hex6HexNAc5Fuc4NeuAc2              | ND       | 0.20     | ND        | 0.003                                 | 0.0001                                 |
|                    | Hex6HexNAc4Fuc3NeuAc2              | ND       | 0.15     | ND        | 0.0011                                 | 0.0001                                 |
|                    | Hex6HexNAc5Fuc3NeuAc2              | ND       | 0.12     | ND        | 0.0001                                 | 0.0009                                 |

Glycan composition is abbreviated as follows: Hex<sub>n</sub>HexNAc<sub>m</sub>Fuc<sub>p</sub>NeuAc<sub>q</sub> where Hex = Hexose, HexNAc = N-Acetylhexosamine, Fuc = Fucose, NeuAc = N-Acetyleneuraminic Acid and subscript indicates the number of each monosaccharide residue. ND, not detected.

<sup>a</sup>Values are reported as average relative abundance, n = 8.

<sup>b</sup>Statistically significant (p < 0.05), highly significant (p < 0.005).

TABLE IV

| Glycan group       | Composition                        | Milk     | Saliva   | Plasma    | p value<sub>a</sub>, Plasma and Milk | p value<sub>b</sub>, Plasma and Saliva |
|--------------------|------------------------------------|----------|----------|-----------|--------------------------------------|---------------------------------------|
| Undecorated        | Hex5HexNAc5Fuc0NeuAc0              | 0.64     | 1.38     | 3.58      | 0.0001                               | 0.0001                               |
|                    | Hex5HexNAc4Fuc0NeuAc1              | 4.68     | 2.43     | 11.38     | 0.0001                               | 0.0001                               |
|                    | Hex5HexNAc5Fuc0NeuAc1              | 1.99     | 1.69     | 9.77      | 0.0001                               | 0.0001                               |
|                    | Hex4HexNAc4Fuc0NeuAc2              | 1.66     | 0.88     | 4.68      | 0.0058                               | 0.0001                               |
| Sialylated-only    | Hex4HexNAc4Fuc0NeuAc1              | 0.79     | 0.72     | 1.51      | 0.0003                               | 0.0001                               |
|                    | Hex5HexNAc5Fuc0NeuAc2              | 0.22     | 0.14     | 1.37      | 0.0003                               | 0.0001                               |
|                    | Hex6HexNAc5Fuc0NeuAc3              | ND       | ND       | 0.84      | 0.0001                               | 0.0001                               |
|                    | Hex6HexNAc5Fuc0NeuAc1              | ND       | ND       | 0.24      | 0.0002                               | 0.0001                               |
|                    | Hex6HexNAc5Fuc0NeuAc2              | ND       | ND       | 0.16      | 0.0001                               | 0.0001                               |
|                    | Hex5HexNAc5Fuc1NeuAc2              | 1.41     | 0.78     | 9.37      | 0.0005                               | 0.0001                               |
|                    | Hex5HexNAc5Fuc1NeuAc3              | 3.18     | 2.67     | 7.72      | 0.0018                               | 0.0001                               |
|                    | Hex5HexNAc4Fuc1NeuAc2              | 2.56     | 0.8      | 7.14      | 0.0016                               | 0.0001                               |
| Fucosylated + sialylated | Hex6HexNAc5Fuc1NeuAc2 | 0.11     | ND       | 0.59      | 0.0027                               | 0.0001                               |
|                    | Hex6HexNAc5Fuc1NeuAc3              | ND       | ND       | 0.31      | 0.0001                               | 0.0001                               |
|                    | Hex6HexNAc6Fuc1NeuAc1              | ND       | ND       | 0.28      | 0.0001                               | 0.0001                               |
|                    | Hex6HexNAc6Fuc1NeuAc2              | ND       | ND       | 0.15      | 0.0001                               | 0.0001                               |

Glycan composition is abbreviated as follows: Hex<sub>n</sub>HexNAc<sub>m</sub>Fuc<sub>p</sub>NeuAc<sub>q</sub> where Hex = Hexose, HexNAc = N-Acetylhexosamine, Fuc = Fucose, NeuAc = N-Acetyleneuraminic Acid and subscript indicates the number of each monosaccharide residue. ND, not detected.

<sup>a</sup>Values are reported as average relative abundance, n = 8.

<sup>b</sup>Statistically significant (p < 0.05), highly significant (p < 0.005).
lyzing 16 different sialylated glycans after treatment with specific alpha-2,3- and alpha-2,3/6/8-neuraminidase, we found that the distribution of \( /H_9251\)-2,3 and \( /H_9251\)-2,6 linkage is not significantly affected by delivery mode in plasma IgA (supplemental Fig. S2).

Finally, we compared the N-glycosylation profile of plasma IgA from lactating participants with nonlactating female participants. Notably, we found specific variations within high mannose type glycans (Fig. 4), with significantly higher levels of these structures in plasma IgA from lactating participants who had a vaginal delivery, both when compared with nonlactating participants \( (p<0.005) \) and lactating participants who had delivered infants by c-section \( (p<0.05) \).

**DISCUSSION**

IgA is the most abundant immunoglobulin expressed at mucosal surfaces and the second most abundant immunoglobulin in circulation, playing a central role in both the innate and adaptive immune systems. This study characterizes and compares, for the first time, the N-glycosylation profiles of mature breast milk and saliva secretory IgA (SIgA) and plasma IgA obtained from the same lactating individuals using a highly-sensitive nano-HPLC-PGC-chip/TOF MS-based method. The effect of delivery mode on IgA glycosylation was also evaluated by comparing (SI)gA N-glycosylation profiles of mothers that delivered babies vaginally or by c-section with nonlactating women as controls.

The identification of over 70 glycan compositions is reported in SIgA purified from mature breast milk and saliva, respectively. A smaller number, 55 glycans compositions, was obtained from plasma IgA. Interestingly, nearly 50% of all these glycans compositions have not been described in milk, saliva and plasma samples, which may be because of the higher sensitivity of the approach using nano-HPLC-PGC-chip/TOF mass spectrometry platform, thereby offering significant advantages over alternative glycomic technologies (28, 29).

**Table V**

| Glycan group | Composition | Milk\textsuperscript{a} | Saliva\textsuperscript{a} | Plasma\textsuperscript{a} | \( p \) value\textsuperscript{b}, Milk and Plasma | \( p \) value\textsuperscript{b}, Saliva and Plasma |
|-------------|------------|------------------------|------------------------|------------------------|----------------------------------|----------------------------------|
| Undecorated | Hex6HexNAc3Fuc0NeuAc0 | 0.32 | 0.40 | 0.11 | 0.0001 | 0.0001 |
|             | Hex3HexNAc5Fuc1NeuAc0 | 7.83\textsuperscript{c,d} | 13.02\textsuperscript{d} | 1.56 | 0.0001 | 0.0001 |
|             | Hex4HexNAc5Fuc1NeuAc0 | 6.22\textsuperscript{d} | 8.66\textsuperscript{d} | 2.25 | 0.0001 | 0.0001 |
|             | Hex5HexNAc4Fuc2NeuAc0 | 4.06\textsuperscript{e} | 1.85\textsuperscript{d} | ND | 0.0001 | 0.0001 |
|             | Hex5HexNAc4Fuc3NeuAc0 | 3.43\textsuperscript{e} | 2.93\textsuperscript{d} | 0.06 | 0.0001 | 0.0001 |
|             | Hex6HexNAc5Fuc4NeuAc0 | 0.79\textsuperscript{e} | 0.42 | ND | 0.0001 | 0.0001 |
|             | Hex4HexNAc4Fuc1NeuAc0 | 0.69 | 1.02\textsuperscript{a} | 0.27 | 0.0001 | 0.0001 |
|             | Hex6HexNAc5Fuc3NeuAc0 | 0.60\textsuperscript{e} | 0.21 | ND | 0.0001 | 0.0001 |
| Fucosylated-only | Hex4HexNAc3Fuc1NeuAc0 | 0.48\textsuperscript{e} | 0.65 | 0.05 | 0.0001 | 0.0001 |
|             | Hex4HexNAc3Fuc2NeuAc0 | 0.29 | 0.49\textsuperscript{d} | ND | 0.0001 | 0.0001 |
|             | Hex5HexNAc3Fuc1NeuAc0 | 0.22\textsuperscript{d} | 0.49\textsuperscript{d} | ND | 0.0001 | 0.0001 |
|             | Hex5HexNAc3Fuc2NeuAc0 | 0.20 | 0.46\textsuperscript{d} | ND | 0.0001 | 0.0001 |
|             | Hex3HexNAc3Fuc1NeuAc0 | 0.19 | 0.39 | 0.05 | 0.0001 | 0.0001 |
|             | Hex6HexNAc3Fuc1NeuAc0 | 0.15 | 0.22 | ND | 0.0001 | 0.0001 |
|             | Hex4HexNAc4Fuc2NeuAc0 | 0.09 | 0.33\textsuperscript{d} | ND | 0.0001 | 0.0001 |
|             | Hex6HexNAc3Fuc2NeuAc0 | 0.05 | 0.38\textsuperscript{d} | ND | 0.0001 | 0.0001 |
| Fucosylated + sialylated | Hex4HexNAc5Fuc1NeuAc1 | 3.65 | 2.86 | 0.90 | 0.0001 | 0.0001 |
|             | Hex4HexNAc3Fuc1NeuAc1 | 0.79 | 0.55\textsuperscript{e} | 0.10 | 0.0001 | 0.0001 |
|             | Hex5HexNAc3Fuc1NeuAc1 | 0.54 | 0.90\textsuperscript{d} | ND | 0.0003 | 0.0001 |
|             | Hex6HexNAc3Fuc3NeuAc1 | 0.39\textsuperscript{e} | 0.31 | ND | 0.0001 | 0.0001 |
|             | Hex4HexNAc4Fuc1NeuAc1 | 0.34 | 0.28 | 0.09 | 0.0022 | 0.0034 |

Glycan composition is abbreviated as follows: Hex_NexNAc_Fuc_NeuAc where Hex = Hexose, HexNAc = N-Acetylhexosamine, Fuc = Fucose, NeuAc = N-Acetylneuraminic Acid and subscript indicates the number of each monosaccharide residue. ND, not detected.\textsuperscript{a}Values are reported as average relative abundance, \( n = 8 \).\textsuperscript{b}Statistically significant \( (p<0.05) \), highly significant \( (p<0.005) \).\textsuperscript{c}Reported in the SC and the J chain by Royle et al, 2003 using N-glycan analysis.\textsuperscript{d}Reported in the SC and the J chain by Huang et al, 2015 using glycopeptide analysis.\textsuperscript{e}Reported in the SC and the J chain by Plomp et al, 2018 using glycopeptide analysis.
be reflecting a common trend in the maturation of the human milk N-glycome during lactation, and toward potentially protecting neonates from infectious diseases. The changes found further suggest a specific enhanced pathogen decoy activity in mature breast SigA to sequester bacteria and viruses, preventing subsequent infection on the growing breastfeeding infant. This notion is further supported by the increased production of functionally relevant fucosylated and sialylated structures on mature breast milk SigA including Lewis and sialyl-Lewis epitopes which have been shown to serve as targets for bacterial lectins and adhesins (1) such as enteropathogenic *Escherichia coli* (31), *Campylobacter jejuni* (32), *Helicobacter pylori* (11) and *Noroviruses* (33). Further, this altered glycosylation can, in turn, influence the composition of

**Fig. 3.** Effect of delivery mode on N-glycosylation of (A) salivary SigA and (B) plasma IgA. Relative abundance of each IgA N-glycan was compared between cesarean delivery and vaginal delivery. Asterisks indicate the statistical significance in the two groups, \( p < 0.05 \). Numbers represent glycan compositions and each given value represents the number of each monosaccharide residue in the following order Hex-HexNAc-Fuc-NeuAc.
Comparative IgA N-glycans from Milk, Saliva, and Plasma

FIG. 4. Comparison of high mannose glycans observed in plasma IgA of lactating participants according to the delivery mode and nonlactating participants. *p < 0.05 is considered as statistically significant.

The gut microbial community. Pioneering studies showed that N-Glycans present on the SC of colostrum SIgA mediates interactions with beneficial intestinal Gram-positive bacteria (7, 8, 34). Recently, it was shown that human commensal Gram-negative bacteria remodel their surface capsular polysaccharides to bind to SIgA N-Glycans, thereby enhancing adhesion to intestinal epithelial cells and enabling stable mucosal colonization. The bacteria produce mucus-associated functional factors that facilitate symbiosis with members of the Firmicutes phylum, protecting against colitis (8, 34, 35). In this context, the role of individual milk SIgA N-Glycans in shaping the composition of the beneficial gut microbiome and maintaining intestinal homeostasis remains mainly unexplored. The identification of mature breast milk SIgA N-Glycans presented in this work provides a valuable framework for future studies exploring the specific SIgA glycan types that drive host-microbe symbiosis and intestinal neonate well-being.

In this investigation, we also report the most comprehensive N-glycoprofile of whole salivary SIgA and the first such study using samples from lactating women, revealing a highly diverse array of over 70 glycan compositions. To our knowledge, only one recent publication in literature describes the site-specific occupancy of salivary SIgA N-Glycans from men and nonlactating women samples (17). Compared with Plomp and colleagues, we observed higher levels of fucosylation, galactosylation, sialylation, and bisecting GlcNAc, which is likely because of sex differences as previously identified in plasma IgA N-glycoprofiles (36). Likewise, methodological aspects may also account for these differences in key glycosylation features, as we reported the relative abundance of each modification in the complete N-glycome derived from whole salivary SIgA, and Plomp reported each modification as the number of fucose, galactose, and sialic acid residues per glycan identified in a site-specific manner (17). Besides, we have described a higher number of glycans species possibly because of a difference in sample preparation, the higher sensitivity of our LC-MS method of analysis, and type of samples analyzed.

In this study, the comparison of the two mucosal SIgA N-glycoprofiles (from mature breast milk and saliva samples) obtained from the same participants unveiled significant qualitative similarities and quantitative differences. Interestingly, the 30 most abundant glycans compositions were common to both samples (Fig. 2A and 2B), but their abundances significantly varied in a fluid-specific manner, suggesting that similarities between milk and salivary SIgA could arise from their mucosal origin, and as a result of certain biophysical requirements for SIgA stability and functionality. It has been shown that selective removal of IgA heavy chain N-glycans alters the proper protein conformation, resulting in degradation and reduced secretion, with marked reduced IgA dimer assembly (37, 38). Remarkably, differences in crucial glycosylation features were also observed: salivary SIgA displayed significantly lower levels of sialylation, galactosylation, and fucosylation, but higher levels of bisecting glycans than mature breast milk SIgA (Table I). This decreased levels of sialylated glycans, including sialylated-only and sialylated+fucosylated species, in conjunction with higher levels of undecorated glycans observed in salivary SIgA glycans, may result from the interaction with glycosidases from oral cavity microbes such as Fusobacterium, Neisseria, Porphyromonas, and Tannerella, which have been described as sialic acid scavengers (39–41).

Similarly, fucosylated glycans are the targets of the oral yeast Candida albicans (42). Notably, we also identified body-fluid specific glycans for milk and saliva samples, being saliva-specific glycans more diverse than in milk (Table II and III). We could infer that differences between salivary and mature milk SIgA N-glycans arise not only from a tissue-specific mucosal N-glycosylation machinery but also result from an evolutionary pressure toward the production of specific glycans that will protect the mother from oral mucosal pathogens and the newborn from gastrointestinal pathogens, respectively. Potential functional differences of individual glycans still need to be determined in SIgA from both mature breast milk and saliva, and thus, further research is warranted.

This study characterized the most comprehensive plasma IgA N-glycome reported thus far. N-Glycosylation analysis of plasma IgA has received considerably more attention than other body fluids given its potential biomarker role for a wide range of human pathologies, including autoimmune diseases and cancer. Altered IgA O-glycosylation including a decrease in sialylation levels has been identified as a potential biomarker for IgA nephropathy (43, 44), and aberrant IgA N-glycosylation has been reported in aging (36) and ovarian cancer studies (45, 46). However, fewer studies have been conducted on plasma IgA N-glycosylation in normal physiological states such as pregnancy and lactation (15, 16). In agreement with these reports, the most abundant glycoforms in plasma IgA from lactating woman corresponded to fully processed N-glycans (17). The identification of milk SIgA N-Glycans in plasma IgA of lactating participants according to the delivery mode and nonlactating participants.
results from a complex and dynamic sequence of endocrine
progesterone is critical for the maintenance of pregnancy, the
evidence a potential role of hormonal regulation of IgA glycosylation. The hormonal balance between estrogens and androgens, the plasma IgA and salivary SIgA in a body-fluid specific manner, may also arise from the almost complete pre-meric SIgA, and precisely the reduced diversity of glycans in secretory SIgA, sixteen of them were mapped in the secretory component and J chain in plasma IgA. This likely also reflects the component and J chain (14, 17). This finding indicates that glycosylation differences between circulating IgA and mucosal dimeric SIgA, and precisely the reduced diversity of glycans in plasma IgA, may also arise from the almost complete predominance of monomeric IgA and the absence of secretory component and J chain in plasma IgA. This likely also reflects an evolutionary adaptation to the mainly aseptic circulating environment in contrast to the oral mucosal cavity and gastrointestinal tract environments that harbor complex microbial communities of symbionts and pathobiont bacteria, fungi, and viruses.

We also observed that delivery mode affected N-glycans on plasma IgA and salivary SIgA in a body-fluid specific manner, evidencing a potential role of hormonal regulation of IgA glycosylation. The hormonal balance between estrogens and progesterone is critical for the maintenance of pregnancy, the onset of labor, and lactation. In humans, vaginal delivery results from a complex and dynamic sequence of endocrine events that involves a decreased production of progesterone, increase production of estrogens and prostaglandins and increase levels of oxytocin and relaxin (48). Such a cascade of events and shift in maternal circulating hormone levels at the end of pregnancy may not equally take place during delivery by c-section. Therefore, it is possible that circulating estrogen levels may be altered in women who delivered a baby by c-section. Interestingly, estrogens have been shown to regulate sialylation and corresponding functional properties of circulating gonadotrophins (LH, FSH, and CG) (49). Thus, our results of lower level of sialylated glycans in plasma IgA in women that delivered by c-section may result from a potentially lower level of estrogen at least at the time of delivery. Further research is needed to elucidate the impact and biological significance of differences in IgA glycosylation and maternal health.

In conclusion, this study has characterized and compared for the first time the secretory IgA N-glycoprofile of mature breast milk and saliva with plasma IgA of the same lactating individuals who delivered their infants vaginally or by c-section. The comparative analysis obtained by nano-HPLC-chip/TOF-MS revealed a greater diversity of glycans than reported thus far and over 50 body-fluid specific N-glycan compositions, some of which are also influenced by delivery mode. This study could be necessary to better understand the maternal control of (S)IgA glycosylation and for unraveling the functional role of mature milk SIgA N-Glycans that drives the assembly of the gut microbiota and promotes neonate well-being. Similarly, the design of optimal recombinant IgA molecules specifically targeted to protect mucosal surfaces will need to include this dimension of structural detail. We expect that the current study may also guide salivary SIgA-glycan based-biomarker research efforts for the diagnostic of oral cavity diseases such as periodontitis, Sjogren’s syndrome, and systemic conditions such as pulmonary and kidney diseases, B-cells pathologies (e.g. rheumatoid arthritis, systemic lupus erythematosus, multiple sclerosis), cancer or infectious diseases.

DATA AVAILABILITY

All raw mass spectrometry data and annotated spectra have been deposited at the MassIVE repository (https://massive.ucsd.edu/ProteoSAFe/static/massive.jsp) with an identifier MSV000083029.

* This work was supported by the National Institute of Health, National Institute of Diabetes, Digestion and Kidney Diseases (R21 DK118379–0) and National Center for Complementary and Integrative Health (AT007079). The content of this work is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health.

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