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RESEARCH PAPER

Mouse milk immunoglobulin G Fc-linked N-glycosylation nano-LC–MS analysis in a model of vancomycin exposure during pregnancy

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Abstract

Breast milk immunoglobulin G (IgG) plays an important role in the transfer of passive immunity in early life and in shaping the neonatal immune system through N-glycan-mediated effector functions. Currently, there are no protocols available to analyze breast milk IgG-Fc glycosylation in mouse models. Therefore, we developed and validated a glycoproteomic workflow for the medium-throughput subclass-specific nano-LC–MS analysis of IgG enriched from small milk volumes of lactating mice. With the established methods, the IgG glycopatterns in a mouse model of antibiotic use during pregnancy and increased asthma susceptibility in the offspring were analyzed. Pregnant BALB/c mice were treated with vancomycin during gestation days 8–17 and IgG1F, IgG2, and IgG3-Fc glycosylation was subsequently analyzed in maternal serum, maternal breast milk, and offspring serum on postnatal day 15. The IgG glycosylation profiles of mouse maternal milk and serum revealed no significant differences within the glycoforms quantified across subclasses. However, vancomycin use during pregnancy was associated with changes in IgG-Fc glycosylation in offspring serum, shown by the decreased relative abundance of the IgG1F-G1 and IgG3-G0 glycoforms, together with the increased relative abundance of the IgG3-G2 and S1 glycoforms. The workflow presented will aid in the emerging integrative multi-omics- and glycomics-oriented milk analyses both in rodent models and human cohorts for a better understanding of mother–infant immunological interactions.

Keywords Immunoglobulins · Antibiotics · Lactation · N-glycans · Mass spectrometry

Abbreviations

Abs. Int.	Summed absolute intensities of doubly and triply charged glycopeptide ions per IgG subclass.
ACN	Acetonitrile
CGE-LIF	Capillary gel electrophoresis with a laser-induced fluorescence
Fc	Fragment crystallizable
FcRn	Neonatal Fc receptor
G	Gestation day
GlcNAc	N-acetylglucosamine
HPLC	High-performance liquid chromatography
Ig	Immunoglobulins
IgG	Immunoglobulin G
IPQ	Isotopic pattern quality
nano-LC–MS	Nano-liquid chromatography coupled to mass spectrometry
PBS	Phosphate-buffered saline
PN	Postnatal day
sIgA	Secretory immunoglobulin A
S/N	Signal-to-noise ratio
TFA	Trifluoroacetic acid
UPLC-FLR	Ultra-performance liquid chromatography with fluorescence detector

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Introduction

Breastfeeding is important for growth and development during infancy, and breast milk also contributes to the microbial colonization of the infant's intestine and subsequent immune system development via its constituent nutrients, bioactive compounds, and immune components [1]. Considering immune components, breast milk is a major source of maternal immunoglobulins (Ig), which participate in passive immunity and protect the infant against diseases during early life [2]. Before birth, the fetus acquires IgG transplacentally via the neonatal Fc receptor (FcRn) [3], whereas in neonates, the same receptor transfers maternal milk IgG from the neonatal gut to the circulation (Fig. 1). Though secretory immunoglobulin A (sIgA) is the most abundant immunoglobulin in human breast milk [4], studies have also shown potential important roles for milk IgG antibodies in shaping the neonatal microbiota [5], protection against bacterial or viral enteric infection [6], and the development of allergies and asthma [7], shown in Fig. 1a–e. Though these findings demonstrate an important potential role for IgG in the neonate [8], there is a paucity of studies examining breast milk IgG [9]. In particular, the influence of breast milk IgG glycosylation (Fig. 1f), as well as antibody subclasses, stability, and half-life (Fig. 1g) on the aforementioned pathways, remains unexplored.

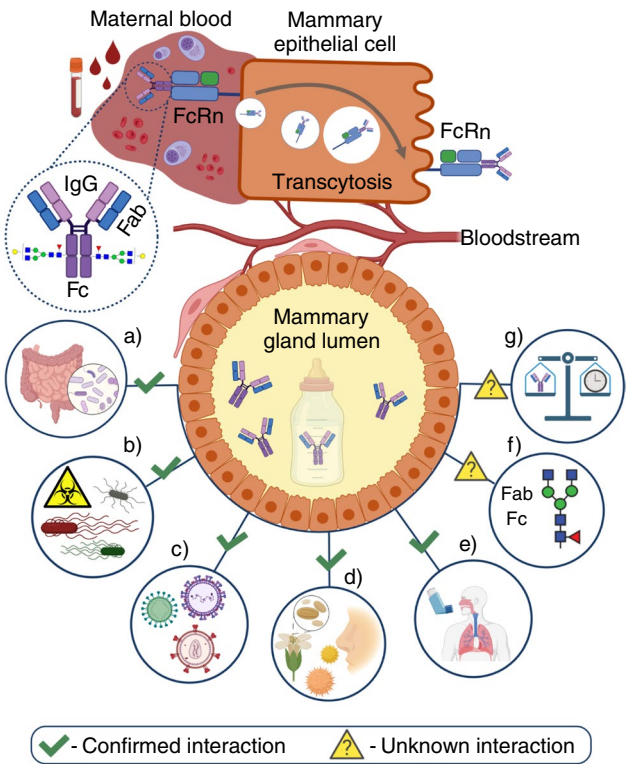

Fig. 1 Diverse roles of breast milk IgG in the neonate. IgG is transferred by FcRn-mediated transcytosis from the maternal bloodstream to the breast milk and then via the breast milk to the neonate. Functions in the neonate include **a** shaping the gut microbiota, **b**, **c** suppressing bacterial and viral enteric infections, and **d**, **e** contributing to allergy and/or asthma development. Unknown aspects include the influence of **f** IgG-Fc and Fab N-glycosylation, as well as **g** antibody subclasses, stability, and half-life on these pathways

IgG antibodies exhibit a wide range of effector functions, and N-glycans attached to the fragment crystallizable (Fc) region can directly influence the inflammatory capacity of this molecule [10]. The backbone of an N-glycan consists of *N*-acetylglucosamine (GlcNAc), mannose with or without fucose, galactose, and sialic acid, and these molecules can theoretically form 36 possible combinations in each subclass of IgG. Each particular combination determines the pro- or anti-inflammatory properties of the antibody, with the addition of galactose or sialic acid generating an anti-inflammatory effector function [11]. Though mouse serum IgG-Fc glycosylation has been well studied, revealing age-, sex-, and strain-dependent differences [12], to our knowledge, there is currently no method established to measure IgG glycosylation patterns in mouse breast milk. Given the potential impact of breast milk IgG glycosylation on the neonate, a protocol for analyzing IgG-Fc glycosylation in mouse breast milk will be of great benefit.

To enable the study of subclass-specific IgG-Fc N-glycosylation in mouse breast milk, we developed and validated a method using nano-liquid chromatography coupled to mass spectrometry (nano-LC–MS). Using this model, we performed site-specific N-glycosylation profiling of mouse IgG1f, IgG2a/b/c, and IgG3 enriched from minimal volumes of milk. The suitability of the newly developed workflow was tested on our recently established mouse model of antibiotic use during pregnancy [13]. Here, we use our method to assess how antibiotic-induced microbial dysbiosis during pregnancy influences IgG glycosylation in both maternal breast milk and serum and the serum of the progeny.

Materials and methods

Animals

Ten-week-old BALB/c mice were obtained from Janvier Labs (Le Genest-Saint-Isle). Mice were maintained under a 12/12 h light/dark cycle under specific pathogen-free conditions and housed in groups of five per cage, and food was provided ad libitum. All animal experiments were approved by local authorities (Landesamt für Gesundheit und Soziales; LAGeSo) and were performed in accordance with German and international guidelines.

After mating, the formation of a vaginal plug was denoted gestation day (G)0 of pregnancy. From G8 to G17, pregnant mice were treated once daily, orally via micropipette, with 25 µL of vancomycin (20 mg/kg, Sigma-Aldrich) mixed with 15 µL of Ora-Sweet syrup (Paddock Laboratories). Control pregnant mice were treated with distilled water plus Ora-Sweet. Offspring were kept with their mothers until postnatal day (PN)15 and then euthanized for sample collection. The experimental design is shown in Fig. 2a.

Sample collection

After 4 h of separation from their pups (to allow for milk accumulation), maternal milk was collected from the PN15 mothers using a protocol specially adapted from [14]. In short, PN15 lactating mice were anaesthetized with ketamine and xylazine, placed on a heating pad, and intraperitoneally injected with 1 IU oxytocin (Sigma-Aldrich) in 50 µL of phosphate-buffered saline (PBS) to stimulate milk secretion. Milk was collected over a period of 20–30 min using a specially constructed, air-tight milk collector connected to a human breast milk pump. After collection, milk was flash-frozen in liquid nitrogen and stored at − 80 °C until analysis. For blood collection, both mothers and offspring were terminally anaesthetized, and blood was collected from the orbital sinus. Serum was collected by centrifugation, and samples were stored at − 80 °C.

Mouse milk skimming and IgG immunoaffinity enrichment

IgG was enriched from 25 to 100 µL of milk. Thawed samples were diluted to 250 µL with 1 × PBS (pH 7.4) and vortexed and centrifuged at 10 000 × *g* at + 4 °C for 15 min to separate the fat and cells. The 130 µL of clear skimmed milk layer (infranatant) was transferred to a clean 2-mL microtube, and the centrifugation step was repeated with another 250 µL 1 × PBS. Combined infranatants (260 µL) were diluted to 1 mL final volume with 1 × PBS (Fig. 2b). Four standards (100 µL) prepared by pooling the cohort milk samples were included in the analysis. The same volume of ultrapure water was included as a single blank sample.

IgG was enriched on FastFlow protein G agarose beads (EMD Millipore Corp.). The required volume of the slurry was prewashed three times with 5 mL of 1 × PBS and then 30 µL of agarose (packed volume) was added to each skimmed milk sample. The samples were shaken for 1 h at 75 rpm on a rocking shaker (IKA®-Werke GmbH & Co.) and spun down, and the supernatant was discarded. Next, the beads were washed three times with 200 µL of 1 × PBS and once with 100 µL of ultrapure water to remove the unbound proteins. The IgG was eluted from the beads by adding 200 µL of 0.1 M formic acid (Merck KgaA) and incubated with shaking for 10 min at room temperature. The eluted IgG was transferred to a 0.5 mL microtube containing 25 µL 1 M ammonium bicarbonate (Acros Organics) to neutralize the

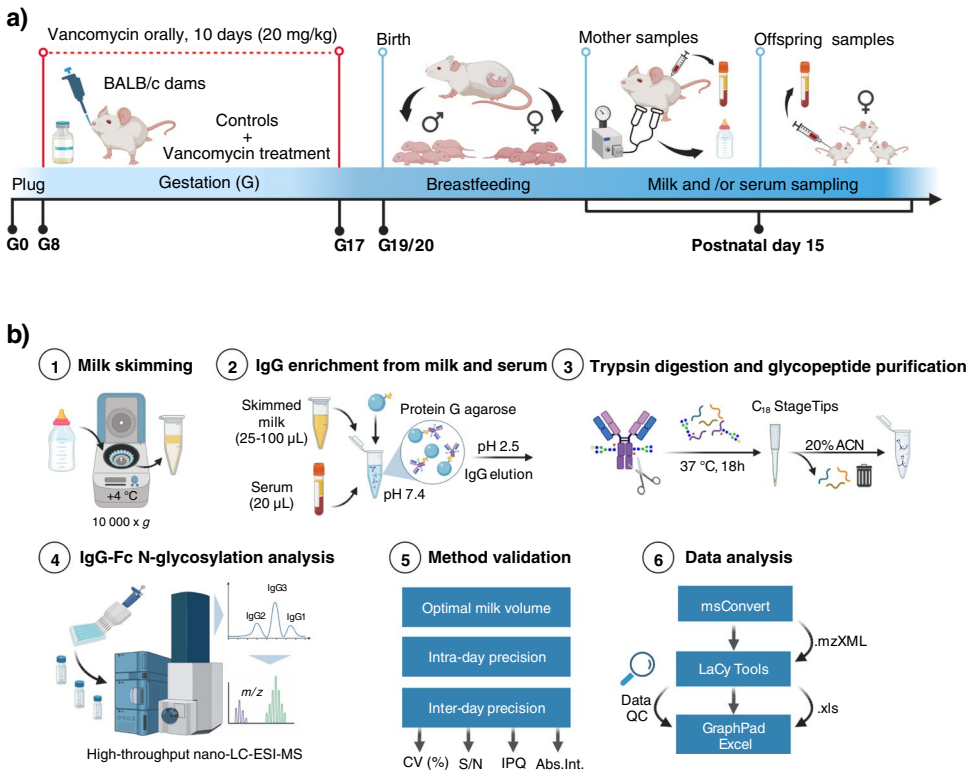

Fig. 2 Experimental design and subclass-specific IgG N-glycosylation analysis workflow. **a** Experimental design of vancomycin-induced maternal gut dysbiosis. Pregnant mice were treated orally, once daily from G8-G17, with vancomycin 20 mg/kg or water as control. Serum and milk samples from mothers as well as serum samples from offspring were collected. **b** Mouse milk and serum immunoaffinity IgG enrichment on protein G agarose, nano-LC-ESI-QqTOF tryptic N-glycopeptide analysis, method validation, and data analysis workflow

acid and then dried down in a SpeedVac™ vacuum centrifuge (Thermo Scientific) at 40 °C.

IgG enrichment from mouse serum

Twenty µL of mouse serum was diluted with 980 µL of 1 × PBS (pH 7.4), and IgG enrichment was performed as described for milk samples. Four standards (20 µL), prepared by pooling the cohort serum samples, were included in the analysis. The same volume of ultrapure water was included as a single blank sample.

Trypsin digestion and glycopeptide desalting

The dried IgG from milk and serum was redissolved in 25 µL of 25 mM ammonium bicarbonate and digested with a 0.2 µg of sequencing grade trypsin (Promega Corp.) for 18 h at 37 °C. The tryptic glycopeptides were desalted on 200 µL StageTips made by punching Empore™ C_{18} solid-phase extraction disks (3 M Company) with a 16-gauge blunt needle. The medium-throughput workflow was facilitated by stacking the StageTips onto a 2-mL 96-well collection plate (Pall Corp.) for centrifugation (Fig. S1a, b).

The trypsin digestion was stopped by adding 75 µL of 0.1% (*v*/*v*) trifluoroacetic acid (TFA). The tips were first washed with 100 µL of 80% (*v*/*v*) acetonitrile (ACN) and equilibrated with 100 µL 0.1% TFA by centrifugation using an Eppendorf® deep-well plate swing rotor (2 min per step, 1027 × *g*). Diluted samples were applied to the pre-conditioned StageTips (4 min, 657 × *g*) and washed 2 times with 100 µL of 0.1% TFA. Flowthrough was then discarded, tips were restacked directly onto high-performance liquid chromatography (HPLC) vials (Fig. S1c, d), and glycopeptides were eluted with 50 µL of 20% ACN (v/v) by centrifugation and dried down in a vacuum centrifuge. The dry samples were stored at − 20 °C.

Nano-LC-ESI-QqTOF analysis

The site-specific N-glycosylation analysis of mouse IgG was performed as previously described using an ACQUITY UPLC M-class nano-LC system (Waters, Milford) equipped with a binary analytical and auxiliary pump, autosampler, column heater, and trap valve manager. The LC was coupled to a Compact™ QqTOF mass spectrometer via a CaptiveSpray™ ESI ion source supported with nanoBooster™ gaseous dopant addition technology (Bruker Daltonik GmbH). Dried glycopeptides were redissolved in 50 µL of ultrapure water, and 7 µL was injected into a C_{18} Acclaim™ PepMap™ trapping column (5 × 0.3 mm, 5 µm, 100 Å, Thermo Scientific). The sample was desalted for 2 min with a 40 µL/min flow rate of 0.1% TFA (*v*/*v*) from the auxiliary pump and separated on a SunShell C_{18} column (150 × 0.1 mm, 2.6 µm, 90 Å, ChromaNik Technologies Inc.) heated to 30 °C. A fast exponential 7-min gradient (curve 10), starting from 15% solvent B (80% ACN in 0.1% TFA, *v*/*v*) and 80% solvent A (0.1% TFA, *v*/*v*), and ending in 27% solvent B, was used for high-throughput nano-LC–MS analysis. The mass spectrometer was operated in a reflectron positive ion mode. The profile spectra were acquired at 2 × 0.5 Hz within the m/z range 600–2000.

Glycoproteomic data processing

The expected most abundant mouse IgG1, IgG2m and IgG3 N-glycopeptide compositions (Fig. 3a) were assigned based on doubly, $[M + 2H]^{2+}$, and triply, $[M + 3H]^{3+}$, charged ions for each subclass. Allelic variants IgG1F and IgG1I as well as IgG2a, IgG2b, and IgG2c were analyzed as total IgG1 or IgG2, respectively. LaCy Tools data processing software (v.1.0.1, build 5) was used for automated chromatogram alignment and data extraction [15]. Its design and usage for high-throughput mouse IgG glycoproteomics have been described elsewhere [16]. Raw MS1 files were converted to mzXML format using ProteoWizard MSConvert (v. 3.0), and extracted ion chromatograms were aligned based on three calibrant m/z values for each cluster. At least 95% of the theoretical isotopic distribution was quantified for each glycopeptide. Peak areas were normalized to the total integrated area of each IgG subclass. The software automatically calculates the signal-to-noise (S/N), isotopic pattern quality (IPQ), and relative mass accuracy (in ppm) for output data quality control. Analytes with mass error > 35 ppm, S/N < 9, and IPQ > 0.35 were not quantified. Complete LaCy Tools search, alignment, and extraction settings are listed in Table S1.

Method validation

The milk volume collected from the mouse mothers depended on the litter size. Thus, we first validated the newly developed protocol feasibility with 25, 50, 100, and 200 µL of a mouse milk pool collected independently from healthy lactating females of the same strain. The analysis was done in triplicate, and the performance was assessed based on summed absolute intensities of doubly and triply charged protonated ions, S/N, and IPQ of each IgG-Fc glycoform detected. Next, intra- and inter-day precision were determined by analyzing four replicates of 30 and 100 µL of mouse milk by a single analyst for

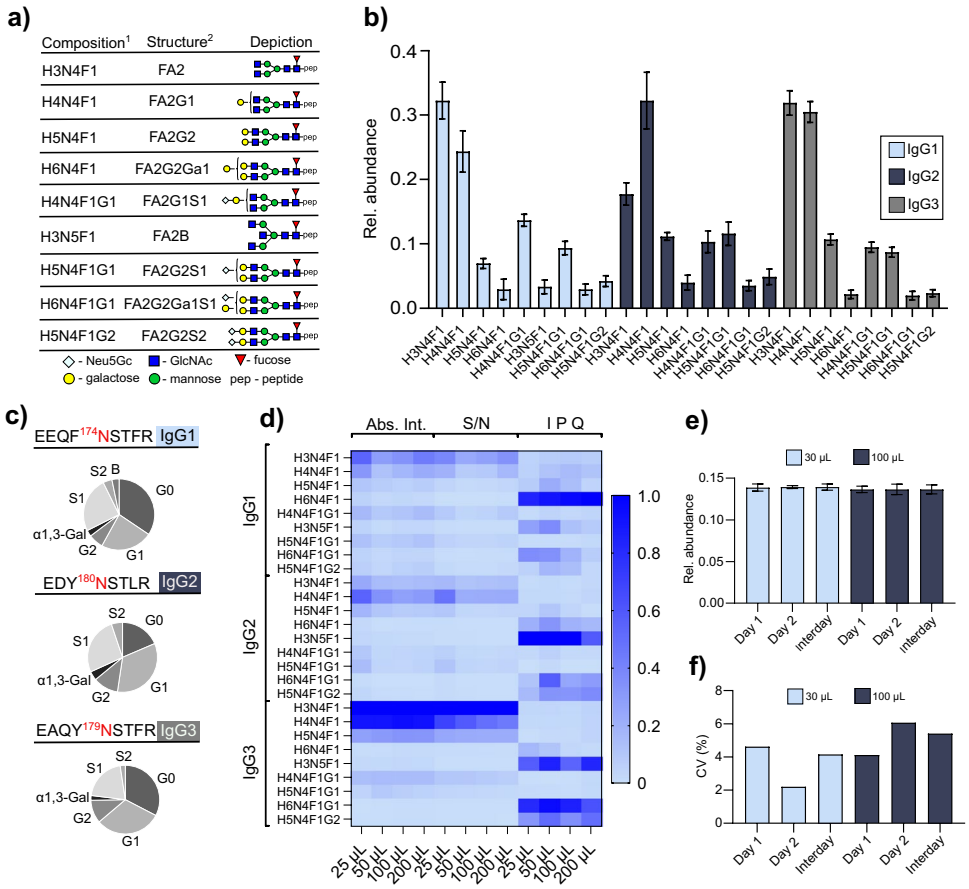

Fig. 3 **a** Structure and composition of glycan structures. The depictions are given according to the Symbol Nomenclature for Glycans (SNFG). [1]Composition: H, hexose; N, GlcNAc; F, fucose; G, Neu5Gc. [2]Structure: F, core fucose; A*n*, numbers of antennas; G*n*, number of galactoses; S*n*, number of sialic acids (Neu5Gc). **b** Relative abundances of mouse milk IgG-Fc N-glycoforms per subclass. **c** Derived glycosylation traits are calculated as the percentage of the corresponding glycoform(s) relative abundance in the total glycoproteome of each IgG subclass. B, bisection; G0, agalactosylation; G1, monogalactosylation; G2, digalactosylation; α1,3-Gal, α1,3-linked galactosylation; S1, monosialylation; S2, disialylation. The tryptic glycopeptide sequences and their N-glycosylation site position (marked in red) are given above each pie chart. **d** Milk volume optimization results are summarized in a heatmap (25–100 µL, $n=3$ for each volume tested). Abs. Int., summed absolute intensities of doubly and triply charged glycopeptide ions per IgG subclass; S/N, signal-to-noise ratio; IPQ, isotopic pattern quality. The values are min–max normalized. **e**, **f** Inter- and intra-day method precision was assessed for 30 µL and 100 µL milk ($n=4$ for each volume)

2 consecutive days using the same LC–MS setup. The subclass-specific IgG N-glycosylation analysis workflow is detailed in Fig. 2b.

Statistical analysis

Data are presented as the mean ± standard error or SEM where indicated. Normality of the data was assessed by the Shapiro–Wilk test. Parametric/nonparametric data were analyzed by *t* test/Mann–Whitney *U* test. All the statistical tests and calculations were performed with the software GraphPad Prism 9.

Results and discussion

Milk IgG enrichment workflow and nano-LC–MS glycopeptide analysis

Although studies have described IgA glycosylation [17] and IgG sialylation [9] in human breast milk and mouse studies report the total breast milk glycoproteome [18], to our knowledge, no study has provided a detailed profile of subclass-specific IgG glycosylation in the breast milk of humans or mice. Mouse milk immunoglobulins analysis is limited by

small sample volume and high-fat content, complicating downstream sample processing and antibody enrichment. Here, we demonstrate a medium-throughput glycoproteomic workflow for mouse milk IgG-Fc N-glycosylation profiling using as little as 25–100 µL milk volume. This procedure included milk skimming, immunoaffinity IgG enrichment, and glycopeptide preparation followed by nano-LC-ESI-QTOF analysis. In short, a modified breast milk pump was used to collect milk from lactating mice, samples were skimmed by high-speed centrifugation, and IgG was enriched from the remaining defatted infranatant on protein G agarose beads. Tryptic N-glycopeptides were prepared from the enriched IgG by digestion with trypsin and desalted on C_{18} StageTips instead of 96-well filter plates to minimize analyte loss (Fig. S1a, b). Seamless StageTip handling and centrifugation for enhanced throughput were achieved by stacking the tips onto the 96-well collection plates instead of handling them individually (Fig. S1c, d). Nano-LC–MS glycoproteomic analysis allowed for fast subclass-specific IgG-Fc N-glycosylation analysis (Fig. S2), and we have successfully quantified nine (for IgG1) and eight (for IgG2 and IgG3) of the most abundant IgG glycoforms enriched from mouse milk (Fig. 3b).

Subclass-specific IgG-Fc N-glycosylation of mouse milk

In this study, we analyzed the subclass-specific N-glycosylation of breast milk and serum IgG enriched from 15 BALB/c mothers (9 control and 6 vancomycin treated) and 22 of their female offspring. Mouse serum IgG glycosylation has been analyzed extensively both on released glycan and glycopeptide levels using ultra-performance liquid chromatography with a fluorescence detector (UPLC-FLR), capillary gel electrophoresis with laser-induced fluorescence (CGE-LIF), and nano-liquid chromatography-mass spectrometry (nano-LC–MS(/MS)) techniques [19]. However, the glycosylation patterns of mouse milk IgG have not been reported thus far. Our experiments using mouse breast milk show that for all three IgG subclasses, the most prominent glycosylation feature was the presence of agalactosylated (H3N4F1), monogalactosylated (H4N4F1), and diglalactosylated (H5N4F1) core-fucosylated glycans (Fig. 3b). This was also prominent within derived glycosylation traits G0, G1, and G2 (Fig. 3c) and is in line with the literature data on mouse serum IgG glycosylation [19]. On all sialylated glycoforms, either one (H4N4F1G1, H5N4F1G1) or two (H5N4F1G2) Neu5Gc residues were present. Two structures bearing α1,3-galactose were also present, with one of them being Neu5Gc-monosialylated (H6N4F1 and H6N4F1G1). The α1,3-galactosylation was previously reported in mouse tissues [19], and our analysis showed that its presence in the mouse milk IgG-Fc glycome was represented by two N-glycan structures (H6N4F1 and H6N4F1G1). Although present on all mouse IgG subclasses, a single biantennary structure of low abundance bearing the bisecting GlcNAc (H3N5F1) was quantified reliably only on IgG1. Afucosylated high-mannose glycoforms were not detected due to their generally very low occurrence on mouse IgG-Fc [19].

Method validation

As the milk production rate is dependent on the mouse litter size, the protocol for mouse milk IgG-Fc N-glycosylation analysis must perform successfully with a limited sample quantity. Hence, we first validated the minimal required milk volume. Tryptic N-glycopeptides prepared from IgG enriched from four different volumes of pooled mouse milk (25, 50, 100, and 200 µL) were analyzed in triplicate, and the results were assessed based on summed absolute intensities of doubly and triply charged ions for each glycoform, S/N ratio, and IPQ. The most abundant glycoforms for all three IgG subclasses (H3N4F1, H4N4F1, and H5N4F1) yielded the highest absolute signal intensities (Fig. S3) and showed the highest S/N ratios and lowest IPQ scores for all starting volumes of milk tested (Fig. 3d). The percentage of all quantified analytes with mean IPQ < 0.1 and S/N > 27 was the highest when IgG enrichment was performed from 100 and 200 µL of milk. All low abundant IgG glycoforms such as bisected and α1,3-galactosylated structures (e.g., H6N4F1 and H3N5F1) were characterized by low S/N and high IPQ values due to the ionization suppression from coeluting analytes (Figs. S4 and S5).

For the longitudinal and follow-up cohort studies, the stability of the analytical system and the repeatability and reproducibility of the protocols performed is of primary importance. Hence, we validated the established glycoproteomic protocol for intra- and inter-day precision for the minimal (30 µL) and maximal (100 µL) volume of milk obtained from the lactating mouse mothers. The relative abundances of all quantified glycoforms showed excellent stability when the analysis was performed on 2 consecutive days (Fig. 3e, Fig. S6). For both intra- and inter-day precision, the percentage of analytes with CVs < 5% was 50% (for 30 µl milk) and 75% (for 100 µL of milk) (Fig. 3f, Fig. S7). In short, the minimal volume of mouse milk for successful IgG-Fc glycoprofiling can be as low as 25 µL. However, larger volumes are preferred, if available, to obtain good S/N ratios and IPQ of lower-abundance N-glycopeptides.

Treatment of pregnant mice with vancomycin affects offspring serum IgG-Fc glycosylation

The successfully developed nano-LC–MS method for mouse breast milk IgG-Fc N-glycosylation analysis was next tested in an experimental setting using a model

of maternal vancomycin treatment during pregnancy that results in increased asthma susceptibility in the offspring [13]. We analyzed IgG-Fc glycosylation in breast milk collected from PN15 mother mice, as well as maternal and offspring serum at the same time point. Though we did not observe any differences in the maternal breast milk or serum between control and treated animals, significant differences were seen in the serum of offspring from vancomycin treated versus control mothers. Prenatal vancomycin-exposed offspring serum had a lower percentage of IgG1 monogalactosylation (H4N4F1) and a lower percentage of the IgG3 agalactosylated form (H3N4F1) compared to prenatal control offspring (Fig. 4a, b). Conversely, serum IgG3 of prenatal vancomycin-exposed offspring had significantly higher percentages of digalactosylation (H5N4F1) and monosialylation (H5N4F1G1), as shown in Fig. 4c and d.

Our analysis indicates that the IgG-Fc glycosylation profile in the serum of prenatal vancomycin-exposed offspring is shifted towards an anti-inflammatory phenotype at PN15. Though we cannot yet answer why this is the case, several possibilities may have contributed to this phenomenon. Our previous study of this mouse model showed that maternal antibiotic treatment during pregnancy was associated with gut microbial dysbiosis in both mothers and their offspring [13]. While it is tempting to hypothesize that microbial dysbiosis may be involved in the observed changes in offspring serum IgG glycosylation, this can be ruled out as a primary factor since the serum and breast milk of antibiotic-treated mothers (who also had a dysbiotic gut microbiome) were not affected. At PN15, offspring IgG is mainly obtained from the mother through the breast milk [6], and since breast milk IgG glycosylation was not altered in our study, we hypothesize that the observed differences in offspring serum may be related to the altered transfer of IgG across the offspring intestinal epithelium. Alteration of IgG transfer across the

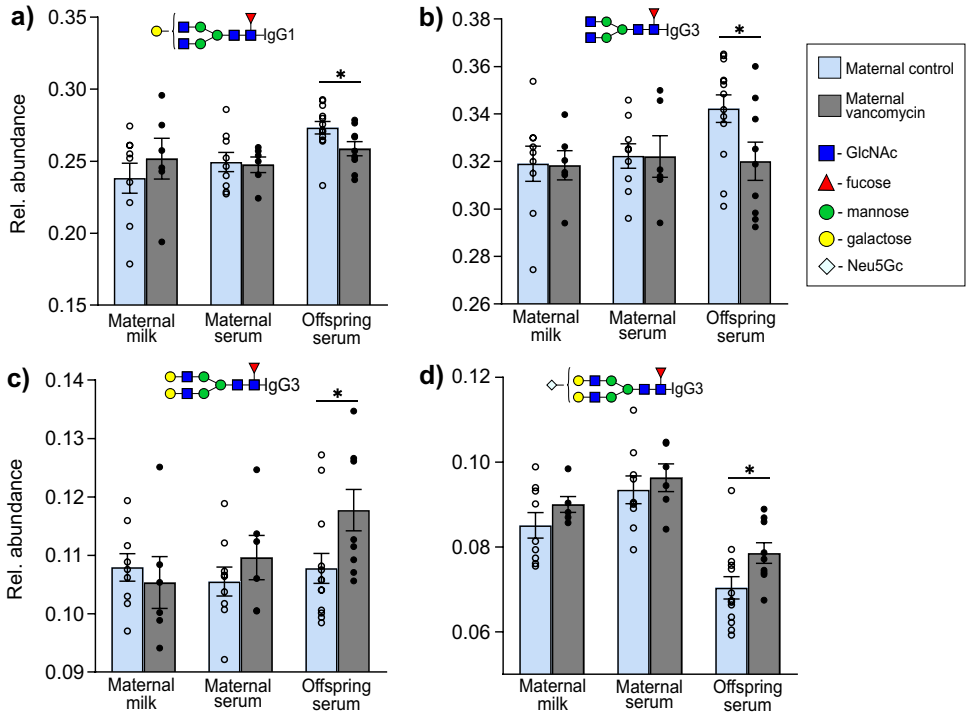

Fig. 4 Effect of maternal vancomycin administration during pregnancy on relative abundances of IgG-Fc N-glycoforms in three different samples: maternal milk, maternal serum, and offspring serum. Only significantly altered relative abundances are shown. **a** Relative abundances of the H4N4F1 (G1) glycoform in the IgG1 subclass. **b** Relative abundances of the H3N4F1 (G0) glycoform in the IgG3 subclass. **c** Relative abundances of the H5N4F1 (G2) glycoform in the IgG3 subclass. **d** Relative abundances of the H5N4F1G1 (S1) glycoform in the IgG3 subclass. Data information: results represent two independently performed experiments. Means ± SEM are shown. Maternal control ($n = 9$), maternal vancomycin treated ($n = 6$), offspring of maternal control ($n = 13$), and offspring of maternal vancomycin treated ($n = 9$). Significance is represented by $*P < 0.05$, either *t* test or Mann–Whitney *U* test compared against the control

neonatal intestinal epithelium could be a secondary effect of gut microbial dysbiosis in early life. These data call for future studies assessing microbial dysbiosis and IgG transport in the neonatal intestine.

Conclusions

With our validated glycoproteomic workflow, we were able to identify and relatively quantify the most abundant N-glycoforms of IgG1F, IgG2, and IgG3 subclasses enriched from small volumes of mouse breast milk. Further, we used this method to analyze milk and serum IgG-Fc N-glycosylation in a cohort of mouse mothers and offspring with prenatal exposure to vancomycin. The changes in offspring serum IgG1 and IgG3 glycosylation indicate the possible link between antibiotic-induced gut dysbiosis and IgG-Fc glycoprofile in early life.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s00216-023-04635-5.

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Data availability The mass spectrometry glycoproteomics data (raw MS1 files) and LaCy Tools search results for cohort analysis and method validation have been deposited to the Mendeley Data online repository (https://data.mendeley.com/) with the data set identifier https://doi.org/10.17632/zhrhgvgnym.1.

Declarations

Ethics approval All animal experiments were approved by the local authorities (Landesamt für Gesundheit und Soziales; LAGeSo) and were performed in accordance with the German and international guidelines.

Source of biological material BALB/c mice were obtained from Janvier Labs (53940 Le Genest-Saint-Isle, France).

Statement on animal welfare The authors declare that the standardized institutional and international procedures for the care and use of laboratory animals compliant with the highest ethical standards were followed in this study.

Conflict of interest Gordan Lauc is the founder and CEO of Genos Ltd., a company specializing in high-throughput glycomics and glycoproteomics. Siniša Habazin and Mislav Novokmet are employees of Genos Ltd. The other authors declare no competing interests.

References

1. Andreas NJ, Kampmann B, Mehring Le-Doare K. Human breast milk: a review on its composition and bioactivity. Early Hum Dev. 2015;91:629–35. https://doi.org/10.1016/j.earlhumdev.2015.08.013.
2. Hanson LÅ. Breastfeeding provides passive and likely long-lasting active immunity. Ann Allergy Asthma Immunol. 1998;81:523–34. https://doi.org/10.1016/S1081-1206(10)62704-4.
3. Palmeira P, Quinello C, Silveira-Lessa AL, Zago CA, Carneiro-Sampaio M. IgG placental transfer in healthy and pathological pregnancies. Clin Dev Immunol. 2012;985646. https://doi.org/10.1155/2012/985646.
4. Rio-Aige K, Azagra-Boronat I, Castell M, Selma-Royo M, Collado MC, Rodríguez-Lagunas MJ, Pérez-Cano FJ. The breast milk immunoglobulinome. Nutrients. 2021;13(6):1810. https://doi.org/10.3390/nu13061810.
5. Gopalakrishna KP, Hand TW. Influence of maternal milk on the neonatal intestinal microbiome. Nutrients. 2020;12(3):823. https://doi.org/10.3390/nu12030823.
6. Zheng W, Zhao W, Wu M, Song X, Caro F, Sun X, Gazzaniga F, Stefanetti G, Oh S, Mekalanos JJ, Kasper DL. Microbiota-targeted maternal antibodies protect neonates from enteric infection. Nature. 2020;577:543–8. https://doi.org/10.1038/s41586-019-1898-4.
7. Mosconi E, Rekima A, Seitz-Polski B, Kanda A, Fleury S, Tissandie E, Monteiro R, Dombrowicz DD, Julia V, Glaichenhaus N, Verhasselt V. Breast milk immune complexes are potent inducers of oral tolerance in neonates and prevent asthma development. Mucosal Immunol. 2010;3:461–74. https://doi.org/10.1038/mi.2010.23.
8. Caballero-Flores G, Sakamoto K, Zeng MY, Wang Y, Hakim J, Matus-Acuña V, Inohara N, Núñez G. Maternal immunization confers protection to the offspring against an attaching and effacing pathogen through delivery of IgG in breast milk. Cell Host Microbe. 2019;25:313-323.e4.
9. Lis-Kuberka J, Królak-Olejnik B, Berghausen-Mazur M, Orczyk-Pawiłowicz M. Lectin-based method for deciphering human milk IgG sialylation. Molecules. 2019;24:1–16. https://doi.org/10.3390/molecules24203797.
10. van de Bovenkamp FS, Hafkenscheid L, Rispens T, Rombouts Y. The emerging importance of IgG Fab glycosylation in immunity. J Immunol. 2016;196:1435–41. https://doi.org/10.4049/jimmunol.1502136.
11. Jennewein MF, Alter G. The immunoregulatory roles of antibody glycosylation. Trends Immunol. 2017;38:358–72. https://doi.org/10.1016/j.it.2017.02.004.
12. De HN, Falck D, Wuhrer M. Monitoring of immunoglobulin N- and O-glycosylation in health and disease. Glycobiology. 2020;30(4):226–40. https://doi.org/10.1093/glycob/cwz048.
13. Alhasan MM, Cait AM, Heimesaat MM, Blaut M, Klopfleisch R, Wedel A, Conlon TM, Yildirim A, Sodemann EB, Mohn WW, Bereswill S, Conrad ML. Antibiotic use during pregnancy increases offspring asthma severity in a dose-dependent manner. Allergy Eur J Allergy Clin Immunol. 2020;75:1975–86. https://doi.org/10.1111/all.14234.
14. Gómez-Gallego C, Ilo T, Ulla-Marjut J, Salminen S, Periago MJ, Ros G, Frias R. A method to collect high volumes of milk from mice. An Vet Murcia. 2013;29:55–61.
15. Jansen BC, Falck D, De Haan N, Hipgrave Ederveen AL, Razdorov G, Lauc G, Wuhrer M. LaCyTools: a targeted liquid chromatography-mass spectrometry data processing package for relative quantitation of glycopeptides. J Proteome Res. 2016;15:2198–210. https://doi.org/10.1021/acs.jproteome.6b00171.

16. Zaytseva OO, Jansen BC, Hanić M, Mrčela M, Razdorov G, Stojković R, Erhardt J, Brizić I, Jonjić S, Pezer M, Lauc G. MIgGGly (mouse IgG glycosylation analysis) - a high-throughput method for studying Fc-linked IgG N-glycosylation in mice with nanoUPLC-ESI-MS. Sci Rep. 2018;8:13688. https://doi.org/10.1038/s41598-018-31844-1.
17. Smilowitz JT, Totten SM, Huang J, Grapov D, Durham HA, Lammi-Keefe CJ, Lebrilla C, German JB. Human milk secretory immunoglobulin A and lactoferrin N-glycans are altered in women with gestational diabetes mellitus. J Nutr. 2013;143:1906–12. https://doi.org/10.3945/jn.113.180695.
18. Zhou J, Wang Y, Fan Q, Liu Y, Liu H, Yan J, Li M, Dong W, Li W. High levels of fucosylation and sialylation of milk N-glycans from mothers with gestational diabetes mellitus alter the offspring gut microbiome and immune balance in mice. FASEB J. 2020;34(3):3715–31. https://doi.org/10.1096/fj.201901674R.
19. de Haan N, Reiding KR, Krištić J, Ederveen ALH, Lauc G, Wuhrer M. The N-glycosylation of mouse immunoglobulin G (IgG)-fragment crystallizable differs between IgG subclasses and strains. Front Immunol. 2017;8:1–14. https://doi.org/10.3389/fimmu.2017.00608.