Chapter 8

Effluent and serum protein N-glycosylation is associated with inflammation and transport characteristics in peritoneal dialysis patients

Evelina Ferrantelli¹, Frans J. van Ittersum², Agnes L. Hipgrave Ederveen³, Karli R. Reiding³, Karima Farhat², Robert H.J Beelen¹, Manfred Wuhrer¹,³,⁴, and Viktoria Dotz³,⁴.

¹ VU University Medical Center, Department of Molecular Cell Biology and Immunology, Amsterdam, The Netherlands
² VU University Medical Center, Department of Nephrology, Amsterdam, The Netherlands
³ Center for Proteomics and Metabolomics, Leiden University Medical Center, Leiden, The Netherlands
⁴ VU University Amsterdam, Division of BioAnalytical Chemistry, Amsterdam, The Netherlands
In the present study we used glycomic methodology as a novel approach to identify biomarkers in peritoneal dialysis (PD), a life-saving treatment applied in more than 100,000 patients worldwide that are suffering from chronic kidney disease. PD treatment uses the peritoneum as a natural membrane to exchange waste products from blood to a glucose-based solution. Daily exposure of the peritoneal membrane to these solutions may cause complications such as peritonitis, fibrosis and inflammation which in the long term lead to the failure of the treatment. It has been shown in the last years that protein N-glycosylation is related to inflammatory and fibrotic processes. Here, by using a recently developed MALDI-TOF-MS method with linkage-specific sialic acid derivatization, we showed that alpha2,6-sialylation in peritoneal effluents, especially of triantennary N-glycans, is associated with critical clinical outcomes in a prospective cohort of 94 PD patients. Moreover, we found an association between levels of presumably immunoglobulin-G-related glycans as well as galactosylation of diantennary glycans with PD-related complications such as peritonitis and loss of peritoneal mesothelial cell mass. The observed glycomic changes point to changes in protein abundance and protein-specific glycosylation, representing candidate functional biomarkers of PD and associated complications.
INTRODUCTION

Peritoneal dialysis (PD) is a safe treatment modality for patients with end-stage kidney disease. PD uses patients’ peritoneum as a natural membrane to remove waste products and fluid from the blood to the dialysis solution by using mainly glucose as osmotic agent. Currently, PD is used by >100,000 end-stage renal disease patients worldwide and accounts for approximately 11% of the dialysis population. However, long term exposure to PD solutions is associated with functional and structural alterations of the parietal peritoneum which include epithelial to mesenchymal transition (EMT), neovascularization, thickening of the peritoneal membrane as well as peritonitis. Taken together all these events lead to ultrafiltration failure resulting in critical clinical outcomes.

Besides acting as a biological barrier, the peritoneum, and more specifically its mesothelial cell monolayer, also functions as a secretory organ which synthesizes and secretes cytokines responsible for the regulation of peritoneal permeability and local host defense. Alterations of the mesothelial cells lining the peritoneal cavity that progressively lose certain epithelial characteristics to acquire a fibroblast-like phenotype is a process known as EMT. It is represented at the molecular level by an increase of pro-inflammatory factors such as transforming growth factor-β1 (TGFβ-1). Besides inducing EMT, TGFβ-1 has been shown to promote peritoneal fibrosis via various pro-fibrotic events including proliferation of fibroblasts and extracellular matrix deposition. TGFβ-1-induced EMT was shown to affect cellular protein glycosylation in normal mouse mammary gland epithelial cells as well as cancer cells. Loss of mesothelial cells is represented also by a decrease in the levels of cancer antigen 125 (CA-125), a marker of peritoneal cell mass and function, while hyaluronic acid (HA) deposition is characteristic of peritoneal fibrosis subsequent to dialysis treatment. Fibrosis and angiogenesis seem to occur together in the peritoneal tissues. Consequently, an increase in TGFβ-1 levels is often associated to high levels of vascular endothelial growth factor (VEGF), which is known to stimulate angiogenesis via capillary tube formation.

Activated mesothelial cells also produce chemotactic cytokines leading to the recruitment of leukocytes and rapid accumulation of neutrophils, later replaced by monocytes and/or macrophages and lymphocytes, into the peritoneum. This scenario is typical of a peritonitis episode, a common PD-related event driven by cytokines such as interleukin (IL)-6, IL-8, monocytes chemotactic protein 1 (MCP-1) and many others.

In clinical practice effluent markers such as IL-6 and CA-125, are used to assess peritoneal functionality and morphology, but their role as predictors for peritoneal membrane failure is still questioned. Changes in the levels of cytokines detected in peritoneal effluents collected from patients indeed reflect the peritoneal morphological changes only at a very late stage. Thus, relevant predictors for PD technique failure at an earlier stage are still needed.
Protein losses have been considered a major limitation in PD, and have recently been investigated in a few PD proteomics studies. Quantities of protein lost in PD effluent (PDE) range from 5 to 15 g/day, depending on various factors, such as the patients’ clinical status or PD fluid composition. When compared to the overall human proteome, some quantitative differences in PDE have been shown, especially regarding immune-related and vitamin-binding proteins, coagulation factors and apolipoproteins that are known to be locally produced by the peritoneum. Moreover, differences in PD proteome have been shown in patients in relation to, e.g. diabetes, or different peritoneal transport characteristics. However, proteomic studies are scarce and have mainly been conducted in small cohorts. Moreover, little is known about PD-related protein glycosylation, which is a post-translational modification governing protein function such as cell adhesion, signal transduction, receptor activation, molecular trafficking and clearance. Protein N-glycosylation is known to be related to inflammatory and fibrotic processes. Recently, we reported on early N-glycosylation changes in mice with zymosan-induced peritonitis by using linkage-specific derivatization of sialic acids. In the present study we used a similar glycomic methodology as a novel and attractive approach for biomarker identification and demonstrate that changes in the glycosylation profile are associated with PD-related complications such as peritonitis, inflammation and mesothelial cell loss in a prospective cohort of 94 PD patients.

RESULTS AND DISCUSSION

Study design and glycomic profiling
Serum and effluent samples were collected from PD patients in 6-months intervals for up to 24 months (Table 1), and clinical parameters, such as cytokine levels, peritoneal functionality or adverse events were assessed (Table 2). The N-glycomic profiles of the patients’ serum and peritoneal effluent samples were acquired by MALDI-TOF-MS analysis after protein immobilization, enzymatic N-glycan release, and sialic acid stabilization with differentiation of α2,3- and α2,6-linked sialic acids. Data were extracted and integrated. Extracted relative intensities of individual glycans, as well as derived glycan traits, were calculated and statistically analyzed for associations with clinical parameters at baseline (Spearman correlation). Derived glycan traits from the entire longitudinal sample set were further assessed by GEE analysis.

The detected molecular species were assigned to 26 N-glycan compositions in serum and effluent each (Figure 1, Supplementary Tables S-1 and S-2), as based on established knowledge of the human plasma N-glycome and its biosynthetic pathways. No tetraantennary glycans were detected, which may be attributed to the rather limited sensitivity of this method which is most probably caused by...
the limited capacity of the PVDF membrane used for protein immobilization. Of note, the immobilization of proteins on PVDF-membrane was necessary for freeing PDE samples from hexose polymers that would otherwise interfere with N-glycomic analysis (not shown). The signal areas of the 26 detected species were normalized to the total area of these 26 signals and further clustered into 12 derived traits based on structural similarities (Supplementary Tables S-1 and S-2).

**Effluent versus serum protein glycosylation**

At baseline, there were no significant differences in direct or derived glycan traits in either serum or effluent found between PD fluid groups (Dianeal/Physioneal) after Bonferroni-correction for 26 independent Mann-Whitney U tests (not shown). Therefore, we treated all patients as one group for further baseline statistical analyses. For longitudinal data analysis by GEE, we did include treatment group as a confounder (in model 2), since it showed an influence on some associations. Spearman correlation analysis of the 26 glycan species detected in 91 serum and 87 effluent samples at baseline, in total, revealed some discrepancies between the two N-glycomes, recognizable in correlation coefficient of the same detected species of lower than +1 (Supplementary Figure S-1A). This becomes specifically apparent for the glycans 8, 10, 19, and 21 that even lost statistical significance after Bonferroni correction. In contrast, fucosylated, non-sialylated diantennary glycans – both with and without bisecting GlcNAc – correlated well between serum and effluent (peaks 2–6 in Figure 1 and supplementary Table S-1). As for serum, one may assume that these glycans may be largely IgG-derived 34.

When assigning the 26 detected species to 12 derived traits, correlations between serum and effluent glycans increased (Figure 2A). This is in line with previous reports indicating a higher technical robustness and often also biological relevance of derived traits in glycomic comparisons as compared to direct traits 35, 36.

---

**Table 1. Patient numbers and age at baseline and follow-up**

<table>
<thead>
<tr>
<th>Study month</th>
<th>Number of patients a</th>
<th>Mean±SD</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>M</td>
<td>F</td>
</tr>
<tr>
<td>0</td>
<td>63</td>
<td>31</td>
</tr>
<tr>
<td>1 - 6</td>
<td>49</td>
<td>28</td>
</tr>
<tr>
<td>7 - 12</td>
<td>43</td>
<td>23</td>
</tr>
<tr>
<td>13 - 18</td>
<td>27</td>
<td>14</td>
</tr>
<tr>
<td>19 - 24</td>
<td>20</td>
<td>11</td>
</tr>
</tbody>
</table>

a Number of patients (M, male / F, female) left at the respective time periods of sample collection for glycomic profiling and/or determination of clinical parameters as described in the experimental section.
Differences between serum and effluent glycosylation levels for the derived traits M, A2, A3, A2F, A2G, A2E, A3E, and IgG were observed at baseline in paired Wilcoxon signed rank tests upon Bonferroni-correction, whereas A3F, bisection, and α2,3-sialylation were not different (Figure 3, Supplementary Figure S-2A-C). Similar differences were observed during the 24 months follow-up.

In literature, total protein concentration in effluent is reported to remain stable over several months up to years on PD treatment 22, 37. Data on effluent protein composition over time are scarce and, if any, proteomic changes show rather low statistical significance 38. However, pathological and morphological changes of the peritoneum can occur with duration of PD treatment and may be reflected by the

Table 2. Clinical characteristics at baseline

<table>
<thead>
<tr>
<th>Clinical parameter</th>
<th>Number of patients a</th>
<th>Median ± interquartile range</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>M</td>
<td>F</td>
</tr>
<tr>
<td>Diabetes b</td>
<td>10</td>
<td>22</td>
</tr>
<tr>
<td>CAPD/APD b</td>
<td>53/10</td>
<td>27/4</td>
</tr>
<tr>
<td>Dianeal/Physioneal c</td>
<td>55/8</td>
<td>23/8</td>
</tr>
<tr>
<td>Time on PD b</td>
<td>62</td>
<td>31</td>
</tr>
<tr>
<td>DPCrea4</td>
<td>63</td>
<td>31</td>
</tr>
<tr>
<td>Ultrafiltration_PET</td>
<td>63</td>
<td>31</td>
</tr>
<tr>
<td>IL6 (pg/mL)</td>
<td>61</td>
<td>30</td>
</tr>
<tr>
<td>IL8 (pg/mL)</td>
<td>60</td>
<td>30</td>
</tr>
<tr>
<td>MCP1 (pg/mL)</td>
<td>61</td>
<td>30</td>
</tr>
<tr>
<td>TGFβ-1 (pg/mL)</td>
<td>61</td>
<td>30</td>
</tr>
<tr>
<td>VEGF (pg/mL)</td>
<td>60</td>
<td>29</td>
</tr>
<tr>
<td>CA-125 (pg/mL)</td>
<td>61</td>
<td>30</td>
</tr>
<tr>
<td>HA (pg/mL)</td>
<td>61</td>
<td>30</td>
</tr>
</tbody>
</table>

a Number of patients (M, male / F, female) for whom data on the stated parameter was available; see Table 1 for total patient number at study month 0.

b Clinical parameters used as confounders for GEE analysis, next to age and sex; CAPD, Continuous Ambulatory Peritoneal Dialysis; APD, Automated Peritoneal Dialysis; time on PD refers to the total time in months after a patient was first introduced to PD treatment; DPCrea4, Ratio of creatinine in the effluent vs. plasma during 4 h dwell time.

c The Dianeal group was randomized into two treatment groups at baseline, i.e. 27/11 male/female patients continued with Dianeal for 24 months, while 28/12 male/female patients switched to Physioneal for the following 24 months.
N-glycosylation and PD-related inflammation

effluent composition. Accordingly, a few trends could be observed in our glycomic data, in particular, in the effluent towards the end of the study. For instance, the galactosylation of diantennary glycans (A2G) showed a downward trend at 24-months follow-up in the effluent, but not serum (Figure 3A, Supplementary Figure S-2D). This could be mainly attributed to an increase of the agalactosylated glycans H3N4F1 and H3N5F1 (Figure 3C-D), since the relative abundance of the mono- and digalactosylated, presumably IgG-related species remained stable over time in effluent (Supplementary Figure S-2D). A lower galactosylation of IgG is related to a pro-inflammatory state in different pathological conditions. When looking at the dynamic changes in glycosylation in the three treatment groups separately, only group 2 (Dianeal/Physioneal) showed some trends that were, however, not significant after correction for multiple testing (Supplementary Figure S-2E).

Interestingly, in a recent publication high-mannose glycans were reported to be more abundant in PDE as compared to the plasma of healthy mice, whereas our data in PD patients is reverse (Supplementary Figure S-2A). Moreover, the relative α2,6-sialylation of both di- and triantennary glycans (A2E and A3E) was higher in serum than in effluent (Figure 3A), while in healthy mice we did not observe differences in overall or linkage-specific sialylation between plasma and PDE N-glycans.

When correlating glycan features with patient characteristics and clinical parameters, the only correlations remaining significant after Bonferroni correction for multiple comparisons were those of the bisected, agalactosylated glycan H3N5F1 (peak 4) with age and the triantennary, trisialylated glycan H6N5E3 (peak 24) with IL6 in effluent (Figure 2C). Accordingly to the former-mentioned, we observed an area increase in H3N5F1 (peak 4) and a decrease in H5N4F1 (peak 5) with age in both serum and effluent, especially in women (Supplementary Figure S-3). It is known that plasma protein galactosylation decreases with age in both healthy individuals and patients with different pathological conditions, which is mainly related to the galactosylation of IgG-derived glycans, including the two former-mentioned. The crude correlation analyses of direct traits from serum or derived glycan traits in either serum or effluent with clinical parameters were not significant at baseline (Supplementary Figure S-1B-D).

Protein glycosylation in relation to clinical parameters

The association of ten selected clinical parameters, i.e. peritonitis occurrence, ultrafiltration during PET, DPCrea4, the concentrations of TGFβ-1, IL-6, IL-8, MCP-1, VEGF, CA-125 and HA, with 12 derived glycan traits from PD patient serum and effluent was assessed by GEE analysis in longitudinal data. In addition, two presumably IgG-related direct traits were tested for their association with peritonitis based on their trends found for the comparison of baseline vs. 24-months follow-up. After adjustment for confounders, peritonitis occurrence, DPCrea4, TGFβ-1, IL-6, IL-8, MCP-1, VEGF, CA-125 and HA were associated with various glycan traits.
Figure 1. MALDI-TOF mass spectra of a patient’s serum (upper) and peritoneal effluent (lower) protein-derived N-glycans. After enzymatic release of glycans, sialic acids were stabilized in linkage-specific manner and analyzed by positive-ion reflectron mode MALDI-TOF-MS. Blue square, N-acetylglucosamine; yellow circle, galactose; green circle, mannose; red triangle, fucose; purple diamond oriented to the right, α2,6-linked N-acetyleneuraminic acid; purple diamond oriented to the left, α2,3-linked N-acetyleneuraminic acid. Structures are proposed for the 12 most abundant species in the upper mass spectrum, while the lower panel contains 11 additional structures in the two zoomed areas, i.e. peaks 1, 4, 6-8, 10, 12, 15-17, and 21. For the complete list of, in total, 26 detected glycan species, see Supplementary Table 1.
N-glycosylation and PD-related inflammation

(Supplementary Tables S-3A and S-3B), as presented in the following paragraphs and summarized in Table 3. The association of ultrafiltration during PET with derived glycan traits was not significant after adjusting for confounders (Supplementary Table S-3B).

Peritonitis occurrence

Peritonitis occurrence was positively associated with the relative abundance of IgG-derived glycans as well as the relative fucosylation of diantennary glycans (A2F) and α2,6-sialylation of triantennary glycans (A3E) in PD effluent only (excerpt in Table 3 and extensive information in Supplementary Table S-3A). A negative association was found for the relative galactosylation (A2G) and α2,6-sialylation (A2E) of diantennary glycans. These associations are in line with our finding of changes in traits towards study end, i.e. increasing A2F, IgG-glycans, A3E, and decreasing A2G and A2E (see Effluent versus serum protein glycosylation). This strengthens the hypothesis that these N-glycan traits may reflect local peritoneal inflammation in PD patients, in particular, when assessed in PD effluent, since derived glycan traits in serum were not associated with peritonitis (not shown). When analyzing a subselection of direct traits based on our findings for baseline and longitudinal glycan data, we found no association of selected triantennary α2,6-sialylated traits (peaks 16, 21, 23-24, 26) with peritonitis in GEE. However, the two agalactosylated IgG-related structures (peaks 2 and 4), known for reflecting a rather pro-inflammatory state in other disease conditions 39, 40, were positively associated with peritonitis, which might have contributed to the inverse association of galactosylation of diantennary glycans (A2G) with peritonitis (Table 3; Supplementary Table S-3A).

PD effluent contains immunoglobulins, such as IgG and IgM 42, which might reflect local inflammation during peritonitis 43, as indicated by the positive association of peritonitis with effluent but not serum glycans. In addition, protein losses are known to increase during peritonitis, mainly due to enhanced peritoneal membrane permeability caused by inflammation 22, 42. In a proteomic study of PDE collected from twelve patients before and after peritonitis, several proteins were differentially expressed. For example, fibrinogen, ceruloplasmin, zinc-α-2-glycoprotein and α-1-antitrypsin were downregulated during peritonitis, whereas haptoglobin and antithrombin-III were upregulated 44. These are also glycosylated, mainly acute-phase proteins that contribute to the total plasma N-glycome as known from literature 34. Notably, in our global approach we were not able to trace back the origin of the relevant glycan traits to their protein carriers. However, as for the diantennary, fucosylated, non-sialylated glycans in the serum and effluent of PD patients, we assume that these are largely IgG-derived on the basis of literature information revealing IgG as the major contributor to these glycans in the human serum and plasma glycome 34.
Figure 2. Spearman correlation analysis of serum and effluent glycans at baseline. The relative areas of 12 derived effluent vs. serum glycan traits (A), and of 26 effluent glycans vs. selected patient characteristics (B) are shown. Glycan numbers are matching the list in Supplementary Table 1. The ranking of the categorical variables in (B) is matching the listing order of their categories, e.g. for sex, male = 1, female = 2. Dots and crosses refer to significance levels with p<0.05 and p-value corrected for multiple testing, respectively (Bonferroni corrected p-values: p<0.00035 in (A), and p<0.00013 in (B)). Color codes indicate Spearman’s rho-values as depicted in the color scales to the right of each heat map.
Inflammation markers and peritoneal function parameters

All tested inflammatory markers were positively associated with the degree of triantennary α2,6-sialylation of effluent proteins (EfA3E, Table 3). At the same time, the relative abundance of triantennary glycans (A3) and the degree of fucosylation of triantennary glycans (A3F) in serum and/or effluent was positively correlated with the IL6 and IL8 concentration in the effluent. Core and antenna fucosylation of acute-phase proteins has been shown to be increased in various disease conditions, such as chronic pancreatitis 45, rheumatoid arthritis 46 and inflammation related to cancer 47. Alpha-2,6-sialyltransferase expression was induced by different pro-inflammatory cytokines in human endothelial cells 48. This enzyme was furthermore secreted into the medium 48, indicating that α2,6-sialylation of circulating glycoproteins may occur in vivo upon inflammation.

Figure 3. Selected N-glycan traits from serum (Se) and effluent (Ef) at baseline and over time. A-B) Means±SE values of all patients over time classes as defined in Table 1. A) At baseline (study month 0) all p-values were below the Bonferroni-adjusted α = 0.0042 in paired Wilcoxon signed rank tests for serum vs. effluent for A2G, A2E, A3E, A2F, IgG derived traits (N=85 each); A3F was not significantly different at baseline. C-D) Min/Max-values and boxplots with p-values from a paired Wilcoxon signed rank test for IgG-related direct traits at baseline vs. 24 months from 24 patients.
Table 3. Overview of significant associations of clinical parameters and glycans.

<table>
<thead>
<tr>
<th>Glycan trait a</th>
<th>Sample type</th>
<th>Peritonitis OR (95% CI)</th>
<th>IL-6</th>
<th>IL-8</th>
<th>MCP-1</th>
<th>HA</th>
<th>TGFβ-1</th>
<th>VEGF</th>
<th>CA-125</th>
</tr>
</thead>
<tbody>
<tr>
<td>A2 Serum</td>
<td></td>
<td>-0.065 (-0.108; -0.022)</td>
<td>-0.084 (-0.114; -0.054)</td>
<td>-0.003 (-0.071; 0.062)</td>
<td>-0.006 (-0.141; 0.037)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Effluent</td>
<td></td>
<td>-0.008 ^ (-0.015; 0.001)</td>
<td>-0.108 ^ (-0.190; 0.013)</td>
<td>-0.055 ^ (-0.109; 0.030)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A3 Serum</td>
<td></td>
<td>0.077 ^ (0.028; 0.125)</td>
<td>0.073 ^ (0.011; 0.136)</td>
<td>0.039 ^ (0.003; 0.144)</td>
<td>0.093 ^ (0.038; 0.158)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Effluent</td>
<td></td>
<td>0.013 ^ (0.006; 0.022)</td>
<td>0.121 ^ (0.031; 0.210)</td>
<td>0.140 ^ (0.029; 0.252)</td>
<td>0.083 ^ (0.028; 0.148)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A2F Effluent</td>
<td>1.097 ^ (1.009; 1.097)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A3F Serum</td>
<td></td>
<td>0.016 ^ (0.000; 0.032)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Effluent</td>
<td></td>
<td>0.036 ^ (0.012; 0.040)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A2B Serum</td>
<td></td>
<td>0.068 ^ (0.001; 0.135)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Effluent</td>
<td></td>
<td>0.076 ^ (0.002; 0.151)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A2G Serum</td>
<td></td>
<td>0.893 ^ (0.794; 0.996)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.045 ^ (0.016; 0.074)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Effluent</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A2L Effluent</td>
<td>-0.008 ^ (-0.015; -0.004)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.023 ^ (0.009; 0.056)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>A3L Effluent</td>
<td></td>
<td>0.080 ^ (0.015; 0.144)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A2E Effluent</td>
<td></td>
<td>0.894 ^ (0.876; 0.916)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A3E Serum</td>
<td></td>
<td>1.205 ^ (1.004; 1.446)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.045 ^ (0.072; 0.018)</td>
<td></td>
</tr>
<tr>
<td>Effluent</td>
<td></td>
<td>0.005 ^ (0.001; 0.020)</td>
<td>0.113 ^ (0.057; 0.169)</td>
<td>0.062 ^ (0.008; 0.117)</td>
<td>0.041 ^ (0.002; 0.076)</td>
<td>0.049 ^ (0.023; 0.076)</td>
<td>0.052 ^ (0.026; 0.121)</td>
<td>0.082 ^ (0.044; 0.121)</td>
<td>0.045 ^ (0.072; 0.018)</td>
</tr>
<tr>
<td>IgG Effluent</td>
<td></td>
<td>1.116 ^ (1.017; 1.225)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>H3N4F1 Effluent</td>
<td></td>
<td>1.297 ^ (1.098; 1.532)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>H3N5F1 Effluent</td>
<td></td>
<td>2.156 ^ (1.150; 3.898)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

---

a) High-mannose trait (M) was not significant; for calculations and abbreviations of derived traits, see Supporting Information Tables S-1 and S-2.

b) Only significant values (odds ratios for peritonitis and B-values for the others, with 95% confidence intervals) as derived from GEE analysis are shown, in case that the confounder groups (models) used changed the estimate by 10%. The following models were used: 0, crude analysis; 1, model 1 with adjustment for age, sex, diabetes; 2, model 2 with adjustment for CAPD/APD, time on PD and Dia/Physio; 3, model 1+2; see Supporting Information Table S-3 for the complete list of all models used with their respective p-values. IL-6, IL-8, MCP-1, HA, TGFβ-1, VEGF and CA-125 were ln-transformed prior to GEE analysis; relative signal intensities of the derived glycan traits were calculated as %-values, as described in the Experimental section; ---, not tested; inverse associations are labeled gray.

c) No significant associations were found with derived traits A2F, A2L, A3L, A2E and IgG in serum, which are therefore not mentioned here.

d) Association for direct traits with the compositions H3N4F1 and H3N5F1 was only tested for effluent, but not serum.
Previously, various proteins were found to be differentially expressed in PD patients with low and high values of peritoneal transport, including glycoproteins such as haptoglobin, alpha-1 antitrypsin, and immunoglobulins. Moreover, protein losses in PDE were found to be higher in high transport patients expressed by higher DPCrea4 values. Therefore, we analyzed a possible association of serum and effluent protein glycosylation with transport characteristics in PD. Ultrafiltration was not significant after adjustment for confounders (Supplementary Table S-3B), while DPCrea4 was positively associated with triantennary glycans and α2,6-sialylation of those, and inversely associated with diantennary glycans and α2,3-sialylation of those in the effluent but not serum (Table 3). The same glycan traits were associated with TGFβ-1 and VEGF concentrations (Table 3), which have been linked to pathological changes of the peritoneum upon PD. Importantly, TGFβ-1-induced EMT in the context of tumor progression was found to be promoted by an increased expression of α2,6-sialyltransferase 1. A similar mechanism might have led to the predominance of α2,6-sialylation of effluent N-glycans regarding its associations with detrimental PD markers/parameters in our data. Furthermore, galactosylation of diantennary glycans was inversely correlated with effluent TGFβ-1 concentrations, but positively correlated with CA-125 that is considered as a marker of mesothelial cell mass, which is decreasing in PD patients developing complications, such as encapsulating peritoneal sclerosis. Galactosylation of serum diantennary N-glycans was shown to be decreased in liver cirrhosis, a pathological condition preceded by fibrotic tissue transformation that might show similarities to the pathological changes upon long-time PD. Both, a higher α2,6-sialylation of diantennary glycans (A2E) and a higher galactosylation of diantennary glycans in effluent with, at the same time, lower abundance of IgG-glycans seems to reflect a better clinical state, as indicated by their positive associations with CA-125 and inverse associations with peritonitis (Table 3).

In conclusion, as demonstrated by using a recently developed MALDI-TOF-MS method with linkage-specific sialic acid derivatization, α2,6-sialylation of triantennary N-glycans in PD effluent seems to reflect adverse events upon long-term PD. Furthermore, a relative increase in IgG-related glycans and a lower galactosylation of diantennary glycans appeared to be related to peritonitis and the loss of mesothelial cell mass. Thus, glycosylation of effluent proteins in PD patients might bear some potential as future biomarkers of peritoneal functionality. However, future research should include studies of the respective protein carriers, such as IgG, acute-phase proteins originating from the liver, or specific glycoproteins locally produced in the peritoneum, e.g. CA-125.
METHODS

Study population and data collection
The current study is embedded in an open label multi-center prospective randomized clinical trial, which enrolled 94 PD patients (aged over 18 years) from twelve different hospitals in The Netherlands during a period of 24 months. Patients included in the randomized part of the study were treated with standard lactate-buffered PD fluid (Dianeal, Baxter Healthcare, USA), and either continued on Dianeal (group 1, n=38), or switched to a bicarbonate/lactate-buffered PD fluid (Physioneal, Baxter Healthcare, USA; group 2, n=40). A third group of patients (group 3, n=16) was not included in the randomized part of the study and was already treated with Physioneal before the study period. They had never used Dianeal before. Prior to study start all patients had to undergo PD treatment for at least 6 weeks. Episodes of peritonitis or exit site infections that occurred within 6 weeks before entering the study, or use of non-glucose based dialysis solutions, except icodextrin, were considered exclusion criteria. The study was approved by medical ethics committees at all participating centers. All patients gave written informed consent. Block randomization per center was performed centrally at the VU University Medical Center (4 patients per block and 2 patients assigned to each treatment per block).

Sample preparation and measurement
Serum and dialysate collection
Serum and 24-hours (overnight) dialysate were collected at 0, 6, 12, 18 and 24 months and stored at -80°C until laboratory analysis.

Peritonitis events
Peritonitis events were recorded and defined as a dialysate having a cell count higher than 100/μL, of which more than 50% were polymorphonuclear leukocytes. Effluents were cultured to define the microorganism involved and for the diagnosis also symptoms such as abdominal pain, fever and/or cloudy dialysate were taken into account. Peritonitis relapses (peritonitis caused from the same organism or sterile peritonitis occurring within 4 weeks from the previous episode) were considered also as an episode.

Peritoneal Equilibrium Test (PET)
A 4-hour PET using 3.86% Dianeal or Physioneal was performed at 0, 12 and 24 weeks in order to assess peritoneal membrane function. At baseline all PETs were performed using Dianeal. If a patient did not tolerate the 3.86% solution because of hypotension, 2.27% was used. Creatinine levels in effluent were determined using routine laboratory techniques.
**Analysis of effluent cytokines**

Assays for cytokine detection were performed in cell-free supernatants of effluents collected at 0, 6, 12, 18 and 24 months. A multiparameter Bio-Plex 200 chemiluminometric bead assay kit (Biorad, Hercules, California, Texas, USA) was used to measure VEGF, IL-6, IL-8, and MCP-1 levels. HA was determined in an ELISA-based assay according to Fosang et al. using immobilized HA and competition for the binding of biotinylated HA-binding protein by HA-containing samples. TGFβ-1 was measured using a quantitative ELISA (Promega Corporation, Madison, USA, detection limit 32 pg/mL). A two-side sandwich immunoassay using direct chemiluminometric technology (Centaur OV-assay Bayer Diagnostics, Tarrytown, NY, USA) was used to determine CA-125 levels.

**Sample treatment and glycomic analysis**

**PVDF membrane N-glycan release**

N-glycans were released from the protein fraction on a protein-binding hydrophobe Immobilon P Membrane (PVDF) plate (Merck Millipore, Darmstadt, Germany). Throughout the glycomic sample preparation and analysis, ultrapure water (MQ) was used (≥18.2 MΩ at 25°C, Merck Millipore). The membrane was preconditioned by washing 2x with 200 µL 70% ethanol followed by two washes with 200 µL 100 mM NaHCO₃. Biological samples were applied by mixing 72.5 µL 8 M GuHCl with 2.5 µL 200 mM dithiothreitol with 25 µL sample (2.5 times diluted serum and undiluted PDE) on the membrane and washing 4x with 200 µL buffer. Subsequently, 1 mU recombinant peptide-N-glycosidase F (PNGase F; Roche Diagnostics, Mannheim, Germany) was applied to the sample in 50 µL 100 mM NaHCO₃ buffer, followed by overnight incubation at 37°C in a humidity chamber. The released glycans were recovered by centrifugation at 2000 rpm.

**Derivatization for MALDI-TOF-MS**

The released glycans were derivatized for the selective ethyl-esterification of 2,6-linked N-acetylneuraminic acids and lactonization of α2,3-linked N-acetylneuraminic acids. The derivatization mixture was prepared by mixing 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (Fluorochem, Hadfield, UK) with 1-hydroxybenzotriazole (Sigma-Aldrich, Steinheim, Germany), to a final concentration of 0.25 M in ethanol (Merck, Darmstadt, Germany). The reaction was performed in 20 µL of the derivatization mixture with 1 µL released glycans from serum and 2 µL released glycans from PDE. The plate was sealed to prevent evaporation and incubated 1 h at 37°C. To allow protein precipitation 20 µL of acetonitrile (Biosolve, Valkenswaard, The Netherlands) was added, further incubated for 15 min at -20°C before proceeding with glycan enrichment and analysis by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS).
Hydrophilic interaction liquid chromatographic purification for MALDI-TOF-MS

Glycan enrichment was performed by cotton hydrophilic interaction liquid chromatography as previously described. The samples were allowed to return to room temperature before proceeding. The tips with cotton as stationary phase (3 mm cotton thread, ca. 190 µg; Pipos, Utrecht, The Netherlands) were washed and equilibrated with 3x20 µL of MQ and 3x20 µL of 85% acetonitrile; sample loading was achieved by pipetting 20x into the reaction mixture, the washing step consisted of pipetting 3x20 µL 85% acetonitrile 1% trifluoroacetic acid (Merck), 3x20 µL 85% acetonitrile. Samples were finally eluted in 10 µL MQ.

MALDI-TOF-MS measurement

For MALDI-TOF-MS analysis, 1 µL of purified serum glycan mix and 2 µL of purified peritoneal fluid mix was spotted on a MTP AnchorChip 800/384 TF MALDI target (Bruker Daltonics, Bremen, Germany), and 1 µL of matrix (5 mg/mL 2,5-dihydroxybenzoic acid (Bruker Daltonics), 1 mM NaOH in 50% acetonitrile), mixed on plate and left to dry. To achieve uniform crystals, the spot was tapped with 0.2 µL ethanol, causing rapid recrystallization. All analyses were performed on an ultraflexXtreme MALDI-TOF/TOF-MS equipped with a Smartbeam II laser, controlled by proprietary software flexControl 3.4 Build 119 (Bruker Daltonics). The MALDI-TOF instrument was operated in reflectron positive ion mode, calibrated on the known masses of a peptide calibration standard (Bruker Daltonics). For sample measurements 20000 laser shots were accumulated at a laser frequency of 2000 Hz, using a complete sample random walk with 200 shots per raster spot.

Data processing

The mass spectra were extracted and processed for quality control and relative quantification of analytes. The raw spectra were exported from flexAnalysis 3.4 (Build 76; Bruker Daltonics) as text files (x,y), and further processed by MassyTools (version 0.1.8.0). First, internal calibration was performed based on a predefined list of 5 analytes (peaks 2, 3, 5, 9, 18 in Supplementary Table S1). Mass spectra presenting a signal-to-noise ratio (S/N) of 9, based on the MinMax algorithm, or above for the calibration analytes were included for further analysis. Furthermore, spectra (n=10) were excluded if their “fraction of analyte area above S/N 9” was below 3 standard deviations (SD) of the mean.

Structural assignment was based on the putative compositional features (hexose = H, N-acetylhexosamine = N, fucose = F, N-acetylneuraminic acid = E or L for α2,6- and α2,3-linked variants, respectively). After the exclusion of compositions with interfering peaks (n=15), background-subtracted peak areas were normalized to the sum of the area of all the 26 peaks from the final list of 26 glycan compositions. Derived traits were calculated based on the compositional features based on established knowledge of the human plasma N-glycome and its biosynthetic pathways (Supplementary Tables S1 and S2). For example, bisection of...
diantennary glycans was assumed rather than an agalactosylated third antenna, in e.g. H5N5E1F1. Finally, relative intensities were multiplied by the factor of 100 prior to statistical analysis.

**Statistical analysis**
Spearman correlation analysis and the generation of heatmaps for baseline data was carried out by using the ‘rquery.cormat’ function and ‘ggplot2’ and ‘WGCNA’ libraries in R version 3.2.2. Bonferroni method was applied to correct for multiple comparisons. Paired Wilcoxon signed rank tests were used to compare serum vs. effluent glycosylation levels at baseline as well as to compare baseline vs. 24-months levels of glycan traits that showed changing trends towards the end of the study. Analysis of the longitudinal data was further performed by using the Generalized Estimated Equations (GEE) method with exchangeable working correlation matrix in IBM SPSS Statistics 23. A binary logistic GEE model was used for the evaluation of the association of glycan traits with peritonitis occurrence, while a linear model was applied to estimate the association of glycan traits with effluent markers and peritoneal transport parameters, i.e. the concentration of TGFβ-1, IL-6, IL-8, MCP-1, VEGF, CA-125 and HA, as well as ultrafiltration during PET and 4-hour dialysate/plasma creatinine (DPCrea4). Effluent marker levels were ln-transformed prior to statistical analysis due to their wide distribution between patients and their non-normality. Next to performing crude GEE-analysis, we adjusted for confounders in three models: model 1: age, sex and diabetes; model 2: treatment modality (CAPD or APD), time on PD and treatment group; model 3: a combination of all confounders of model 1 and model 2. Age and time on PD were added as continuous variables, whereas sex, diabetes, treatment modality, and treatment group were added as categorical variables. Regression coefficients and odds ratios are given with 95% confidence intervals.

**Acknowledgements**
This work was supported from European Union, Seventh Framework Program “EuTRiPD” under grant agreement Marie Curie ITN-GA-2011-287813 (E.F.).
REFERENCES


Supplementary Figure S-1. Spearman correlation analysis of serum and effluent glycans at baseline. The relative areas of 26 effluent vs. serum glycan traits (A), and selected patient characteristics vs. 26 serum glycan traits (B), as well as 12 derived glycan traits from serum (C) and effluent (D) are shown. For details on glycan structures and derived traits, see Supplementary Table S1 and S2. The ranking of the categorical variables in (B), (C) and (D) is matching the listing order of their categories, e.g. for Sex, male = 1, female = 2. Dots refer to significance levels with $p<0.05$. The analyses did not reach significance after Bonferroni-correction with the following thresholds of $p = 7.4 \times 10^{-5}$ in (A), 0.00013 (B) and 0.00028 for both (C) and (D).
Supplementary Figure S-2. Relative areas of selected N-glycan traits at baseline and over time. A-C) Values of all patients over time classes as defined in Table 1. A) High-mannose type direct traits H6N2 and H9N2 and derived trait M; D) Min/Max-relative-area values and boxplots of 24 patients are shown with p-values from a paired Wilcoxon signed rank test for effluent glycan traits at baseline vs. 24 months, i.e. galactosylation of diantennary glycans (EfA2G), H4N4F1, H5N4F1, and H4N5F1; E) Values of PD fluid group 2 (Dianeal/Physioneal) over time classes as defined in Table 1. The p-values from a paired Wilcoxon signed rank test for the relative area of glycan traits at baseline vs. 24 months are shown.
Supplementary Figure S-3. Age and sex-dependency of N-glycans in serum and peritoneal effluent. Blue square, N-acetylglucosamine; yellow circle, galactose; green circle, mannose; red triangle, fucose. Pearson's correlation analysis from cross-sectional data is depicted from samples collected at baseline (t=0) from 63 male (blue) and 31 female (green) patients. Upper panel, glycan peak 4: in serum, R²=0.042 and 0.390 for males and females, respectively, and in peritoneal effluent R²=0.126 and 0.401 for males and females, respectively. Lower panel, glycan peak 5: in serum, R²=0.001 and 0.332 for males and females, respectively, and in peritoneal effluent R²=0.058 and 0.323 for males and females, respectively.
Supplementary Table S-1. Detected glycan molecular species, their putative structures and related derived traits

<table>
<thead>
<tr>
<th>Peak no.</th>
<th>m/z (M+Na)</th>
<th>Composition</th>
<th>Proposed structure</th>
<th>Derived traits</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>a) Proposed structure</td>
<td>Derived traits</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>b) Derived traits</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>1419.48</td>
<td>H6N2</td>
<td>X</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>1485.53</td>
<td>H3N4F1</td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td>3</td>
<td>1647.59</td>
<td>H4N4F1</td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td>4</td>
<td>1688.61</td>
<td>H3N5F1</td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td>5</td>
<td>1809.64</td>
<td>H5N4F1</td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td>6</td>
<td>1850.67</td>
<td>H4N5F1</td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td>7</td>
<td>1905.63</td>
<td>H9N2</td>
<td>X</td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>1966.71</td>
<td>H4N4E1F1</td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td>9</td>
<td>1982.71</td>
<td>H5N4E1</td>
<td>X</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>2082.72</td>
<td>H5N4L1F1</td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td>11</td>
<td>2128.77</td>
<td>H5N4E1F1</td>
<td>X</td>
<td></td>
</tr>
<tr>
<td>12</td>
<td>2185.79</td>
<td>H5N5E1</td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td>13</td>
<td>2255.79</td>
<td>H5N4L1E1</td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td>14</td>
<td>2301.83</td>
<td>H5N4E2</td>
<td>X</td>
<td></td>
</tr>
<tr>
<td>15</td>
<td>2331.85</td>
<td>H5N5E1F1</td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td>16</td>
<td>2347.84</td>
<td>H6N5E1</td>
<td>X</td>
<td></td>
</tr>
<tr>
<td>17</td>
<td>2401.85</td>
<td>H5N4L1E1F1</td>
<td>X</td>
<td></td>
</tr>
<tr>
<td>18</td>
<td>2447.89</td>
<td>H5N4E2F1</td>
<td>X</td>
<td></td>
</tr>
<tr>
<td>19</td>
<td>2620.93</td>
<td>H6N5L1E1</td>
<td>X</td>
<td></td>
</tr>
<tr>
<td>20</td>
<td>2650.97</td>
<td>H5N5E2F1</td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td>21</td>
<td>2813.02</td>
<td>H6N5E2F1</td>
<td>X</td>
<td></td>
</tr>
<tr>
<td>22</td>
<td>2894.01</td>
<td>H6N5L2E1</td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td>23</td>
<td>2940.05</td>
<td>H6N5L1E2</td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td>24</td>
<td>2986.09</td>
<td>H6N5E3</td>
<td>X</td>
<td></td>
</tr>
<tr>
<td>25</td>
<td>3040.07</td>
<td>H6N5L2E1F1</td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td>26</td>
<td>3086.11</td>
<td>H6N5L1E2F1</td>
<td>X</td>
<td>X</td>
</tr>
</tbody>
</table>
Supplementary Table S-2. Calculation of the relative abundances of 12 glycan traits derived from 26 distinct glycan species detected in MALDI-TOF-MS

<table>
<thead>
<tr>
<th>Derived trait</th>
<th>Calculation</th>
</tr>
</thead>
</table>
| M | high mannose type in spectrum: 

\[
\begin{align*}
&\left(1^* (H6N2 + H9N2) / (1^* (H6N2 + H3N4F1 + H4N4F1 + H3N5F1 + H5N4F1 + H4N5F1 + H9N2 + H4N4E1F1 + H5N4E1F1 + H5N5E1 + H5N4L1E1 + H5N4E2 + H5N5E1F1 + H5N4L1E1F1 + H5N4E2F1 + H5N5E2F1))
\end{align*}
\]

A2 | diantennary species in spectrum: 

\[
\begin{align*}
&\left(1^* (H3N4F1 + H4N4F1 + H3N5F1 + H5N4F1 + H4N4E1F1 + H5N4E1F1 + H5N4E2 + H5N4E2F1 + H5N5E1 + H5N5E1F1 + H5N4L1E1 + 1^* (H6N2 + H3N4F1 + H4N4F1 + H3N5F1 + H5N4F1 + H4N5F1 + H5N4E2 + H5N4E2F1 + H5N5E1 + H5N5E1F1 + H5N4L1E1 + H5N4L1E1F1 + H5N4E2F1 + H5N5E2F1 + H6N5L2E1F1 + H6N5L2E2F1 + H6N5L2E3F1 + H6N5L2E3F2))
\end{align*}
\]

A3 | triantennary species in spectrum: 

\[
\begin{align*}
&\left(1^* (H6N5E1 + H6N5L1E1 + H6N5E2 + H6N5L2E1 + H6N5L2E2 + H6N5L2E3 + H6N5L2E3F1 + H6N5L2E3F2)) / (1^* (H6N2 + H3N4F1 + H4N4F1 + H3N5F1 + H5N4F1 + H4N5F1 + H5N4E2 + H5N4E2F1 + H5N5E2F1))
\end{align*}
\]

A2B | bisection of diantennary glycans: 

\[
\begin{align*}
&\left(1^* (H3N5F1 + H4N4F1 + H3N5F1 + H5N5E1 + H5N5E2F1)) / (1^* (H3N4F1 + H4N4F1 + H3N5F1 + H5N4F1 + H4N5F1 + H5N4E2 + H5N4E2F1 + H5N5E2F1))
\end{align*}
\]

A2G | galactosylation of diantennary glycans: 

\[
\begin{align*}
&\left(1/2^* (H4N4F1 + H4N4F1 + H4N4E1F1 + H5N4E1F1 + H5N4E2F1 + H5N4E2F1)) / (1^* (H3N4F1 + H4N4F1 + H3N5F1 + H5N4F1 + H4N4E1F1 + H5N4E1F1 + H5N4E2F1 + H5N4E2F1))
\end{align*}
\]

A2F | fucosylation of diantennary glycans: 

\[
\begin{align*}
&\left(1^* (H3N4F1 + H4N4F1 + H3N5F1 + H5N4F1 + H4N4F1 + H5N4E1F1 + H5N4E1F1 + H5N4E2F1 + H5N4E2F1)) / (1^* (H3N4F1 + H4N4F1 + H3N5F1 + H5N4F1 + H5N4E1F1 + H5N4E1F1 + H5N4E2F1 + H5N4E2F1))
\end{align*}
\]

A3F | fucosylation of triantennary glycans: 

\[
\begin{align*}
&\left(1^* (H6N5E2F1 + H6N5L2E1F1 + H6N5L2E2F1)) / (1^* (H6N5E1 + H6N5L1E1 + H6N5E2F1))
\end{align*}
\]

IgG | IgG-derived glycans: 

\[
\begin{align*}
&(H5N4F1 + H4N4F1 + H5N4F1 + H4N5F1 + H9N2)
\end{align*}
\]

A2L | 2,3-N-acetyleneuraminic acids per diantennary glycan: 

\[
\begin{align*}
&\left(1/2^* (H5N4L1F1 + H5N4L1E1 + H5N4L1E1F1 + H5N4E2F1 + H5N4E2F1) / (1^* (H3N4F1 + H4N4F1 + H3N5F1 + H5N4F1 + H4N4E1F1 + H5N4E1F1 + H5N4E2F1 + H5N4E2F1))
\end{align*}
\]

A3L | 2,3-N-acetyleneuraminic acids per triantennary glycan: 

\[
\begin{align*}
&(H6N5L1E1 + H6N5L1E2 + H6N5L2E1F1 + H6N5L2E2F1 + H6N5L2E1F1 + H6N5L2E2F1) / (1^* (H5N5E1 + H5N5L1E1 + H5N5L2E1F1 + 1/3^* (H5N5E2F1 + H6N5L1E1 + H6N5L2E1F1 + H6N5L2E2F1)) / (1^* (H5N5E1 + H5N5L1E1 + H5N5L2E1F1 + H6N5L2E2F1) + 1/3^* (H5N5E3))
\end{align*}
\]

A2E | 2,6-N-acetyleneuraminic acids per diantennary glycan: 

\[
\begin{align*}
&\left(1/2^* (H4N4E1F1 + H5N4E1F1 + H5N5E1 + H5N4L1E1 + H5N4L1E1F1 + H5N4E2F1 + H5N4E2F1 + H5N5E2F1)) / (1^* (H3N4F1 + H4N4F1 + H3N5F1 + H5N4F1 + H4N4E1F1 + H5N4E1F1 + H5N4E2F1 + H5N4E2F1))
\end{align*}
\]

A3E | 2,6-N-acetyleneuraminic acids per triantennary glycan: 

\[
\begin{align*}
&(H5N5E1 + H5N5L1E1 + H5N5L2E1F1 + H6N5L2E1F1 + H6N5L2E2F1 + H6N5L2E1F1 + H6N5L2E2F1))
\end{align*}
\]