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Immunoglobulin G Fc N-glycan profiling in patients with gastric cancer by LC-ESI-MS: relation to tumor progression and survival

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Abstract The IgG Fc glycans strongly influence the Fcγ receptor interactions and Fc-mediated effector mechanisms. Changes in the structure of IgG glycans are associated with various diseases, such as infections and autoimmunity. However, the possible role of Fc glycans in tumor immunity is not yet fully understood. The aim of this study was to profile the Fc N-glycans of IgG samples from patients with gastric cancer (n=80) and controls (n=51) using LC-ESI-MS method to correlate the findings with stage of cancer and patients survival. Analysis of 32 different IgG N-glycans revealed significant increase of agalactosylated (GnGnF, GnGn(bi)F), and decrease of galactosylated (AGn(bi), AGn (bi)F, AA(bi), AAF) and monosialylated IgG glycoforms (NaAF, NaA(bi)) in cancer patients. A statistically significant increase of Fc fucosylation was observed in tumor stage II and III whereas reverse changes were found for the presence of bisecting GlcNAc. Higher level of fully sialylated glycans and elevated expression of glycans with bisecting GlcNAc were associated with better survival rate. Our findings provide the first evidence that the changes in Fc glycan profile may predict the survival of patients with gastric cancer. Cancer stage-dependent changes in Fc fucosylation and the bisecting *N*-acteylglucosamine expression as well as an association of several IgG glycoforms with the survival suggest that IgG glycosylation is related to pathogenesis of cancer and progression of the disease.

Keywords IgG glycosylation · Fc · Cancer · Survival · Mass spectrometry

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Introduction

Immunoglobulins (Igs) are glycosylated molecules of the humoral immune system, which display an inherited set of glycoforms that differ by number, type and site of oligosaccharide attachment [1]. Changes in the glycosylation patterns of Igs alter their respective functions, including affinity, complement fixation, the formation of immune complexes, complement-dependent cytotoxicity, activation of macrophages, elimination of antigens, and antibody-dependent cellular cytotoxic activity [2–8].

Immunoglobulin G (IgG), further sub-divided into four different subclasses (IgG_1, IgG_2, IgG_3 and IgG_4), is the most prevalent serum immunoglobulin with concentrations of approximately 10–15 mg/mL [1, 9]. Each IgG molecule is bi-functional: while the variable region of the antigen-binding fragment (Fab) recognizes the respective antigen targets and provides the structural basis for the tremendous immunological diversity of antibodies, the crystallizable fragment (Fc) allows antibodies to interact with the Fc receptors on effector cells of the immune system [9]. The Fc fragment bears two oligosaccharides, N-linked to the conserved Asn-297 on both heavy chain-derived polypeptides [1]. The N-glycans found on IgG are of the complex biantennary type, differing in the levels of the terminal sialic acid, galactose (G0, G1, G2), core fucose and bisecting

N-acetylglucosamine (GlcNAc) [1, 10]. Additionally, 15–20% of serum-derived IgG molecules also have oligosaccharides attached to the Fab region [11].

As the presence of N-linked sugar chains was found to play a crucial role in IgG effector functions [1, 2, 8], there has been an increasing interest in the analysis of the N-glycosylation profile(s) of human IgG in health and a number of disease states, such as infections, inflammation and autoimmunity [10–18]. However, less is known about the potential role of the Fc glycans in malignancy and tumor immunity.

Gastric cancer is still associated with a poor prognosis and a low survival rate due to the asymptomatic nature of the disease and usually relatively late diagnosis. Until now, there are no reliable serologic markers available, which would allow early diagnosis, monitoring and prognosis of patients.

Using lectin-ELISA, we recently showed that the glycosylation profiles of both, total serum IgG as well as of IgG specific to tumor-associated Thomsen-Friedenreich glycotope (Galβ1-3GalNAc), exhibited significant differences between gastric cancer patients and healthy controls. Furthermore, we were also able to show association of the respective glycosylation profiles with the survival of patients [19, 20], suggesting that IgG glycoforms are functionally different and therefore potentially clinically relevant.

The objectives of the present study were: 1) to investigate the IgG Fc fragment glycosylation pattern in healthy controls, patients with benign stomach diseases and gastric cancer by liquid chromatography—electospray ionization—mass spectrometry (LC-ESI-MS), in order 2) to determine if the glycosylation changes are IgG subclass specific and cancer related, and 3) to find out whether specific IgG glycoforms are associated with the survival rate of patients with gastric cancer.

Materials and methods

Subjects

Serum samples were obtained from healthy blood donors, patients with benign stomach diseases and patients with histologically verified gastric carcinoma (Table 1). The investigation was carried out in accordance with the ICH GCP Standards and was approved by the Tallinn Medical Research Ethics Committee. Tumor staging was based on the histopathological (pTNM) classification of malignant tumors. The serum samples were stored in aliquots at -20°C until use.

Table 1 Characteristics of the subjects tested

Group	n	Males	Females	Median age (range)
Donors	37	11	26	53 (24–69)
Patients with benign stomach diseases[a]	14	12	2	63 (56–76)
Non-cancer group (a combined group of donors and patients with benign stomach disease)	51	23	28	59 (24–76)
Cancer patients Stage I-IV	80	47	33	67 (28–87)
Stage I	16	7	9	67 (46–84)
Stage II	13	10	3	68 (46–83)
Stage III	46	25	21	66 (28–87)
Stage IV	5	5	0	71 (49–74)

[a] peptic ulcer of the stomach, duodenal ulcer and chronic gastritis

Serum IgG purification on protein G Sepharose

To analyze N-glycans of the IgG Fc fragment a preliminary purification of serum total IgG was performed on Protein G HP Spin Trap column as described by the manufacturer (GE Healthcare). About 8.5 mg of IgG was obtained from 1 ml of serum applied onto the Protein G Sepharose column. The samples were immediately neutralized, dialyzed against PBS—0.1% NaN_3 and stored at +4 ˚C until tested.

Glycopeptide preparation

Purified total IgG (4 μg) was subjected to standard SDS-PAGE under reducing conditions using 12% (w/v) gel with 1% crosslinker. Coomassie-stained gel band of the IgG heavy chain were excised. *S*-carbamidomethylation, tryptic digestion, and extraction were performed using routine methods [21, 22].

LC-ESI-MS of glycopeptides

IgG Fc fragment N-glycans were analyzed as tryptic glycopeptides by LC-ESI-MS as described elsewhere [23], except that the sample was applied directly to the analytical column as in Stadlmann *et al*. [24].

Data were evaluated using MassLynx 4.0 software and herein, notably, the MaxEnt3 deconvolution/deisotoping feature [25]. Thirty two different glycoforms of IgG_1 and IgG_2 (IgG_2 and IgG_3 have an isomeric/isobaric tryptic glycopeptides, therefore IgG_2 results also include results of IgG_3) subclass were studied (Fig. 1 and Supplementary Material Table 1): the method used did not allow to distinguish between structural isomers such as AGnF or GnAF or A(bi)MF. Furthermore, glycoforms with truncated core structure were included. Considering the tryptic digestion of human IgG, one miss-cleavage was allowed and the final result represents the sum of the relative intensities of the two glycopeptides. For each glycoform the % of frequency was

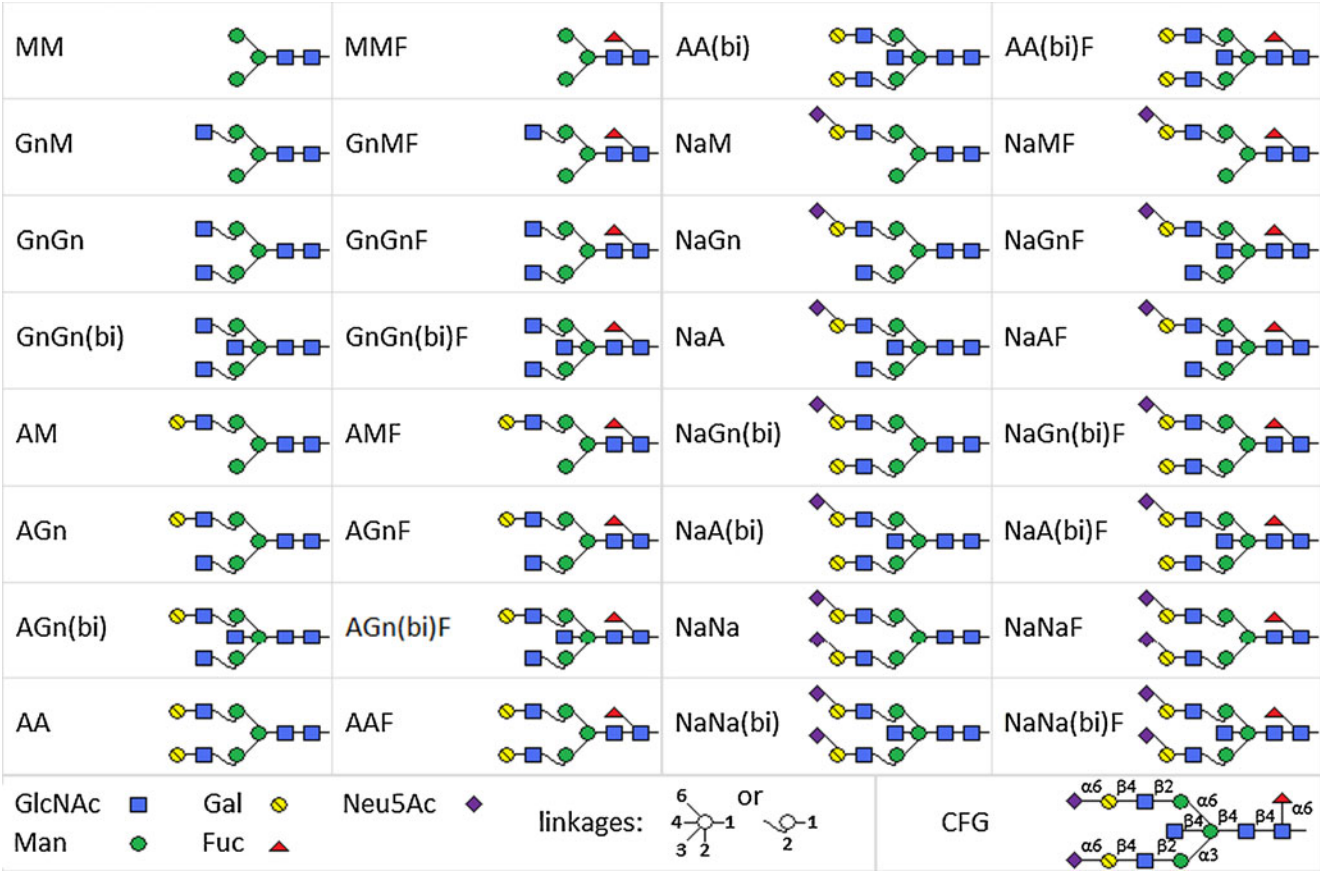

Fig. 1 Structures of human IgG Fc N-glycans dealt with in this study. Glycoforms are named according to the proglycan system [26] and monosaccharide symbols are used as suggested by the Consortium of Functional Glycomics (CFG, www.functionalglycomics.com). In short, as each N-glycan of IgG Fc fragment has a pentasaccharide core structure (except truncated glycoforms) only terminal residues are represented in glycoform name: M—mannose, Gn—*N*-acetylglucosamine, A—galactose, Na—*N*-acetylneuraminic acid (sialic acid), F—fucose, (bi)—bisecting *N*-acetylglucosamine. In the bottom right corner, IgG *N*-glycan is shown in true CFG style. Structures of potential structural isomers (for example AGnF, GnAF or A(bi)MF) are not represented

calculated, the sum of relative intensities of all glycoforms was set to 100%.

IgG subclass analysis

The analysis of subclasses and the respective glycosylation profiles was performed simultaneously by LC-ESI-MS of tryptic peptides as described in Stadlmann *et al.* [24]. To identify IgG_1+IgG_3 (isomeric tryptic peptides), IgG_2 and IgG_4, subclass-specific reporter peptides of Ig gamma CH2 regions were used: ALPAPIEK, GLPAPIEK, GLPSSIEK (from The UniProt database, http://www.expasy.org/uniprot), respectively.

Statistical methods

For statistical analysis, glycoforms were grouped by 1) fucosylation, 2) galactosylation (G0—glycoforms with no galactose, G1—with one galactose, G2—with two galactoses), 3) sialylation (0Na—glycoforms with no sialic acid, 1Na—with one sialic acid, 2Na—with two sialic acids) and 4) presence of bisecting GlcNAc. In each statistic group % of frequency was calculated, the sum of relative intensities of all glycoforms in the group set to 100%.

Results were analyzed for normality of distribution and expressed as group mean % ± SD. Comparisons between the study groups were performed using the Student's t test or Mann-Whitney U test, where appropriate. One- and two-way ANOVA with the Bonferroni test for multiple comparisons was applied for further analysis, including the possible association of the Fc glycosylation profile with the age and gender. For correlations Pearson r was calculated and the survival analysis was performed by Kaplan-Meier method using the median level of IgG glycoform as a cut-off limit. A difference between the groups was considered to be statistically significant when $P \leq 0.05$. All calculations were performed by the GraphPad Prism 5 software.

Results

N-glycan profile of total IgG Fc fragment

The N-glycosylation profiles of human IgG Fc fragments, derived from healthy donors, patients with benign stomach diseases and gastric cancer are shown in Table 2 and Fig. 2. Among the 32 IgG-typical glycan structures analyzed, the glycoforms GnGnF, GnGn(bi)F showed significant ($P \leq 0.05$) increase in patients with cancer, when compared to healthy donors. In contrast, the AGn(bi), AGn(bi)F, AA (bi), AAF, NaAF, and NaA(bi) glycoforms were significantly decreased. These results clearly imply a significant agalactosylation of Fc-derived N-glycans of gastric cancer patients compared to healthy donors: there was an 22% increase in the G0 ($P<0.0001$) and 4% in the non-sialylated (0Na) glycoforms ($P<0.0001$), 25% ($P<0.0001$) and 22% ($P<0.0001$) decrease in the G2 and the monosialylated glycoforms (1Na), respectively (Fig. 2 and Supplementary Material Table 2 and 3). There is a strong negative correlation ($r>-0.9$, $P<0.001$) between agalactosylated and monosialylated glycoforms in all study groups since sialic acid can be added only when the base-structure is already galactosylated. An evident positive correlation ($r>0.76$, $P<0.001$) between G2 and 1Na glycoforms indicates IgG N-glycans to be at least monosialylated if prior doubly galactosylated.

IgG Fc N-glycan galactosylation and sialylation results of patients with benign gastric disease exhibit intermediate values between cancer and donor group (Fig. 2). Agalactosylation was observed in both, the cancer and the benign gastric disease group, asialylation seemed to be more cancer specific. Although a decrease of the G2 glycoform appeared to be related to both pathologies, the additional decrease of the G1 and a concomitant increase of the G0 glycoform were exclusively detected in cancer-patient derived samples. It seemed that, in the benign group, the G2 glycoform changed mostly into the G1 glycoform, whereas in cancer patients, the G0 glycoform was more prevalent. However, these findings need to be supported by a further study of the patients with benign stomach disease.

An additional control group was created by combining donors and patients with benign stomach diseases, *i.e.* non-cancer group. This group is clinically more meaningful given that the discrimination between cancer and non-cancer is known to be much more difficult to achieve than between cancer and healthy individuals. In multiple testing by ANOVA the highly significant differences in G0, G2, 0Na and 1Na glycoforms were confirmed, whereas only slight, though significant ($P=0.04$—0.05) changes of fucosylation and the presence of the bisecting GlcNAc were found in cancer patients compared to the non-cancer group (Fig. 2 and Supplementary Material Table 3).

A strong negative correlation between the level of fucosylation of IgG Fc and the presence of bisecting GlcNAc ($r=-0.81$, $P<0.001$, $n=132$) is demonstrated, while no difference in the level of these glycans between cancer patient and donors was observed (Fig. 2, Supplementary Material Table 2 and 3).

Similar changes were observed in the glycosylation pattern of IgG_1 and IgG_2 of all three study groups, thus being not IgG subclass specific albeit IgG_2 Fc glycans were more fucosylated, less galactosylated and more sialylated compared to IgG_1 (Table 2, Supplementary Material Table 2).

A significant correlation between the levels of IgG G0, G2, glycoforms and age was found in donor and cancer group (G0: $r=0.55$, $r=0.39$; G2: $r=-0.55$, $r=-0.43$, respectively; $P<0.02$). After the stratification of cancer patients and non-cancer group into subgroups by age—below and above 60 years, the differences in Fc glycosylation between the groups were analyzed by two-way ANOVA (Supplementary material Table 4). In cancer *versus* non-cancer group comparisons, the differences found in G0, G2, 0Na and 1Na glycoform incidence remained significant in both age subgroups. In contrast, a significant increase of fucosylation ($P=0.005$) and a decrease of the bisecting GlcNAc expression ($P=0.007$) were found only in the older group of cancer patients compared to the controls. It appears that the increase of bisecting GlcNAc with age described recently (2011) by Pucic *et. al* is mostly characteristic of a younger age. In the non-cancer group, the significantly higher level of G0, 0Na and the presence of bisecting GlcNAc glycoforms were revealed in males, while no association of any IgG glycoform level with gender in both age subgroups of cancer patients was observed (data not shown). Thus, the differences found in the cancer *versus* non-cancer group were not closely related to age and gender.

Subclass distribution of IgG

The relative amount of four subclasses of IgG was evaluated simultaneously with glycosylation measurements. For $IgG_{1(+3)}$, IgG_2 and IgG_4 tryptic reporter peptides were used [24]. IgG subclass analysis showed a significant increase of $IgG_{1(+3)}$ subclass (68.98% and 61.41%, $P=0.002$) and decrease of IgG_2 in cancer group (27.94% and 35.39%, $P=0.001$, respectively) compared to donors.

Glycosylation of IgG from gastric cancer patients with different disease stage (I-IV)

There was no significant difference in IgG fucosylation or presence of bisecting GlcNAc between cancer patients and donors, but a low level of significance between cancer and

Table 2 Immunoglobulin G Fc N-glycan profiles. Glycosylation of human IgG_1 and IgG_2 in healthy donors, patients with benign stomach diseases and gastric cancer (group mean %±SD)

Glycoform	Donor			Benign			Cancer		
	IgG_1	IgG_2	Combined	IgG_1	IgG_2	Combined	IgG_1	IgG_2	Combined
MM	0.05±0.10	0.01±0.06	0.03±0.06	0.19±0.18	0.01±0.02	0.06±0.06	0.04±0.14	0.02±0.09	0.02±0.09
MMF	0.04±0.10	0.10±0.23	0.08±0.17	0.12±0.17	0.00±0.00	0.03±0.06	0.03±0.09	0.13±0.31	0.13±0.31
GnM	0.07±0.12	0.03±0.09	0.05±0.09	0.20±0.30	0.00±0.00	0.05±0.05	0.13±0.17	0.03±0.10	0.05±0.10
GnMF	1.28±1.31	1.58±1.30	1.48±1.30	0.79±0.66	1.01±0.76	0.96±0.65	1.75±1.36	2.11±1.54	2.04±1.57
AM	0.11±0.20	0.01±0.02	0.04±0.07	0.08±0.13	0.00±0.00	0.02±0.03	0.08±0.17	0.02±0.08	0.03±0.08
GnGn	1.31±0.70	0.45±0.35	0.76±0.47	2.05±0.92	0.68±0.55	1.14±0.59	1.94±0.85	0.63±0.42	0.91±0.63
AMF	0.87±0.63	0.58±0.57	0.67±0.58	0.52±0.38	0.24±0.32	0.34±0.34	0.90±0.68	0.64±0.59	0.70±0.58
GnGnF[a]	19.10±6.68	26.97±8.41	23.99±7.33	20.34±4.31	29.48±3.43	25.99±3.54	24.17±6.20	33.62±6.60	31.67±7.50
AGn	2.57±0.99	0.83±0.50	1.43±0.65	3.11±0.93	1.01±0.66	1.76±0.73	2.88±1.23	1.00±0.51	1.37±0.75
GnGn(bi)	2.22±1.27	3.36±1.81	3.01±1.58	2.76±0.75	5.15±1.90	4.38±1.58	2.76±1.35	3.27±1.63	3.09±1.44
NaM	0.09±0.29	0.00±0.00	0.04±0.16	0.08±0.13	0.00±0.00	0.02±0.03	0.30±0.70	0.02±0.07	0.12±0.30
AGnF	25.53±2.91	24.27±3.31	24.72±3.00	26.14±1.46	23.71±1.73	24.51±1.43	25.37±4.00	23.05±3.49	23.48±3.54
AA	1.70±1.04	0.49±0.44	0.90±0.52	1.54±0.66	0.29±0.32	0.72±0.38	1.40±0.86	0.68±0.76	0.88±0.76
GnGn(bi)F[a]	4.13±1.62	5.40±1.88	4.97±1.75	4.40±1.11	6.17±1.04	5.52±1.01	5.75±2.03	6.89±1.73	6.57±1.81
AGn(bi)[b]	3.89±1.41	3.37±1.58	3.59±1.46	4.40±0.90	4.50±1.23	0.45±1.07	3.51±1.28	2.65±1.11	2.83±1.25
NaMF	0.12±0.20	0.07±0.12	0.08±0.07	0.15±0.22	0.02±0.05	0.05±0.07	0.05±0.11	0.07±0.14	0.08±0.13
NaGn	0.18±0.19	0.03±0.07	0.08±0.08	0.28±0.23	0.03±0.07	0.11±0.08	0.12±0.17	0.05±0.09	0.06±0.10
AAF[b]	14.07±4.30	10.55±3.43	11.79±3.48	11.63±2.22	8.32±1.46	9.60±1.74	10.49±2.99	7.74±2.46	8.36±2.66
AGn(bi)F[b]	6.25±1.44	4.11±1.15	4.84±1.12	6.30±0.96	3.94±0.86	4.75±0.89	5.98±1.88	3.63±1.03	4.12±1.23
AA(bi)[b]	1.88±1.13	1.43±1.16	1.62±1.11	1.88±0.69	1.30±0.58	1.53±0.50	1.14±0.74	0.77±0.55	0.88±0.63
NaGnF	1.39±0.57	4.13±0.86	3.22±0.68	1.51±0.32	3.84±0.67	3.01±0.51	1.54±0.46	3.88±0.85	3.42±0.95
NaA	0.63±0.51	0.11±0.16	0.29±0.25	0.68±0.51	0.04±0.12	0.30±0.28	0.44±0.38	0.09±0.14	0.19±0.23
NaGn(bi)	0.59±0.59	1.57±0.92	1.24±0.76	0.83±0.35	2.17±0.75	1.76±0.65	0.62±0.62	1.45±1.06	1.18±0.82
AA(bi)F	1.17±0.62	0.98±0.50	1.05±0.48	0.98±0.42	0.56±0.41	0.71±0.34	0.91±0.44	0.65±0.41	0.70±0.39
NaAF[b]	8.34±2.55	6.83±2.74	7.37±2.42	6.65±1.90	4.87±1.22	5.58±1.47	5.85±1.96	4.72±1.81	4.93±1.91
NaGn(bi)F	0.17±0.19	0.03±0.08	0.09±0.11	0.16±0.21	0.01±0.02	0.07±0.12	0.16±0.19	0.03±0.08	0.06±0.10
NaA(bi)	1.84±1.01	2.04±1.31	2.02±1.22	1.95±0.63	2.18±0.37	2.16±0.42	1.40±1.03	1.38±0.94	1.41±0.97
NaNa	0.04±0.09	0.00±0.01	0.02±0.05	0.03±0.08	0.00±0.00	0.01±0.03	0.01±0.03	0.01±0.03	0.01±0.02
NaA(bi)F	0.18±0.22	0.55±0.55	0.41±0.34	0.10±0.11	0.40±0.45	0.27±0.27	0.09±0.13	0.63±0.74	0.53±0.68
NaNaF	0.04±0.07	0.00±0.01	0.02±0.03	0.08±0.14	0.00±0.00	0.02±0.04	0.03±0.09	0.01±0.03	0.02±0.05
NaNa(bi)	0.12±0.18	0.10±0.13	0.10±0.10	0.06±0.09	0.08±0.12	0.07±0.09	0.14±0.19	0.11±0.17	0.11±0.14
NaNa(bi)F	0.02±0.05	0.01±0.02	0.01±0.02	0.03±0.07	0.00±0.00	0.01±0.01	0.02±0.06	0.02±0.05	0.03±0.06

Significant [a] increase or [b] decrease in cancer group compared to donor's group

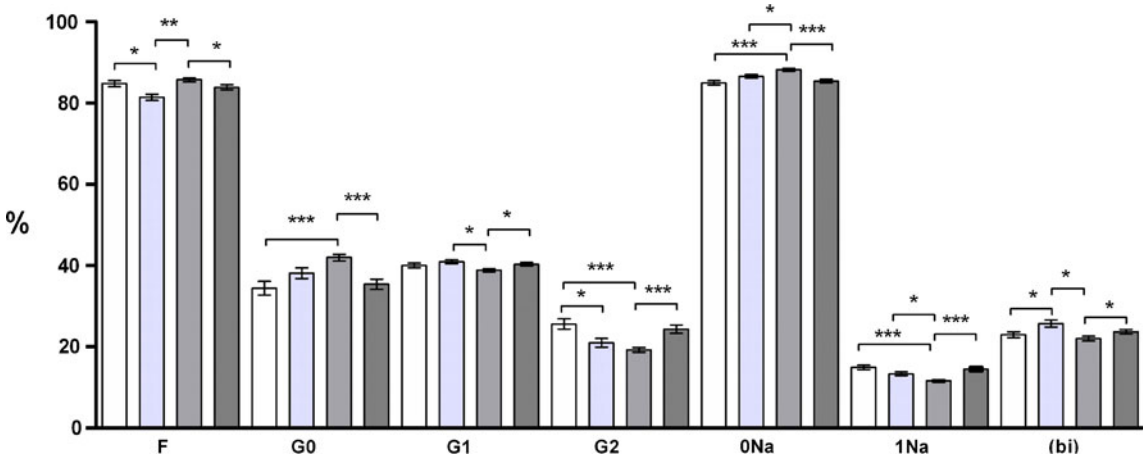

Fig. 2 Glycosylation differences in total IgG Fc fragment between gastric cancer patients and controls. Shown are glycosylation differences between healthy donors (white), patients with benign stomach diseases (light grey), gastric cancer patients (medium grey) and non-cancer group (dark grey) (group mean %±SD). * $P \leq 0.05$, ** $P \leq 0.001$, *** $P \leq 0.0001$. F—fucosylation, G—galactosylation (G0, G1 and G2 glycovariants), Na—sialylation (0Na, 1Na glycovariants), (bi)—the presence of bisecting GlcNAc

non-cancer group was found (P=0.02). However, the distribution of cancer patients by stage of the disease revealed significant stage-dependent changes. Compared to both control groups, a significant increase of IgG fucosylation was demonstrated in stage II and III whereas quite opposite changes were found for the presence of bisecting GlcNAc (Fig. 3a and b). Patients with chronic gastric diseases showed values similar to stage I cancer patients, and big variations for both parameters were found in advanced cancer (stage IV).

Fc glycoforms and survival of gastric cancer patients

Better survival was observed in patients with higher level of G2 glycoforms (Hazard Ratio (HR)=2.06; 95% CI=0.90 to 4.71, P=0.08) (Fig. 4a). Notably this effect was mostly accounted for IgG_2 (HR=2.05; 95% CI=0.90 to 4.68, P=0.09) though a similar slight trend was also observed for IgG_1 (HR=1.51; 95% CI=0.66 to 3.44, P=0.33). In contrast, higher level of IgG G0 glycoforms was associated with a lower survival rate (HR=0.52; 95% CI=0.23 to 1.19, P=0.12) (Fig. 4b). The level of Fc N-glycans with single galactose showed intermediate position between G0 and G2 results.

Cancer patients with higher level of disialylated IgG glycoform showed better survival rate (HR=2.24; 95% CI=0.98 to 5.16, P=0.06) (Fig. 4c) irrespective of IgG subclass. Higher expression of bisecting GlcNAc was also associated with better outcome of patients with cancer (Fig. 4d) (HR=2.14; 95% CI=0.93 to 4.91, P=0.07), mostly of IgG_2 isotype (HR=2.12; 95% CI=0.92 to 4.88, P=0.08). Another significant association found was better survival of cancer patients with high-level of IgG_1 NaGnF glycoform (Fig. 4f) (HR=2.38; 95% CI=1.04 to 5.46, P=0.04). The level of total IgG fucosylation alone was not related to the survival of cancer patients.

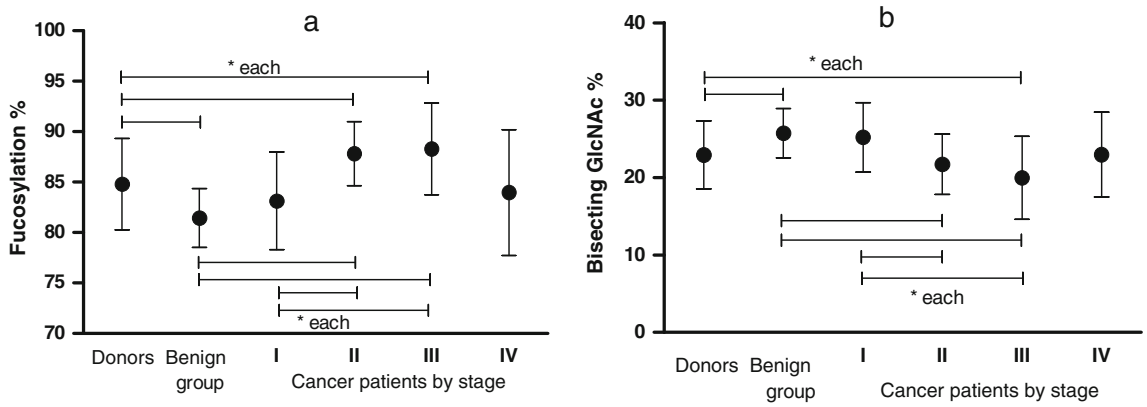

Fig. 3 Differences of IgG N-glycan fucosylation **a** and presence of bisecting GlcNAc **b**. Shown are differences between donors, patients with benign stomach diseases and gastric cancer patients in different stage of the disease (group mean %±SD). * $P \leq 0.05$

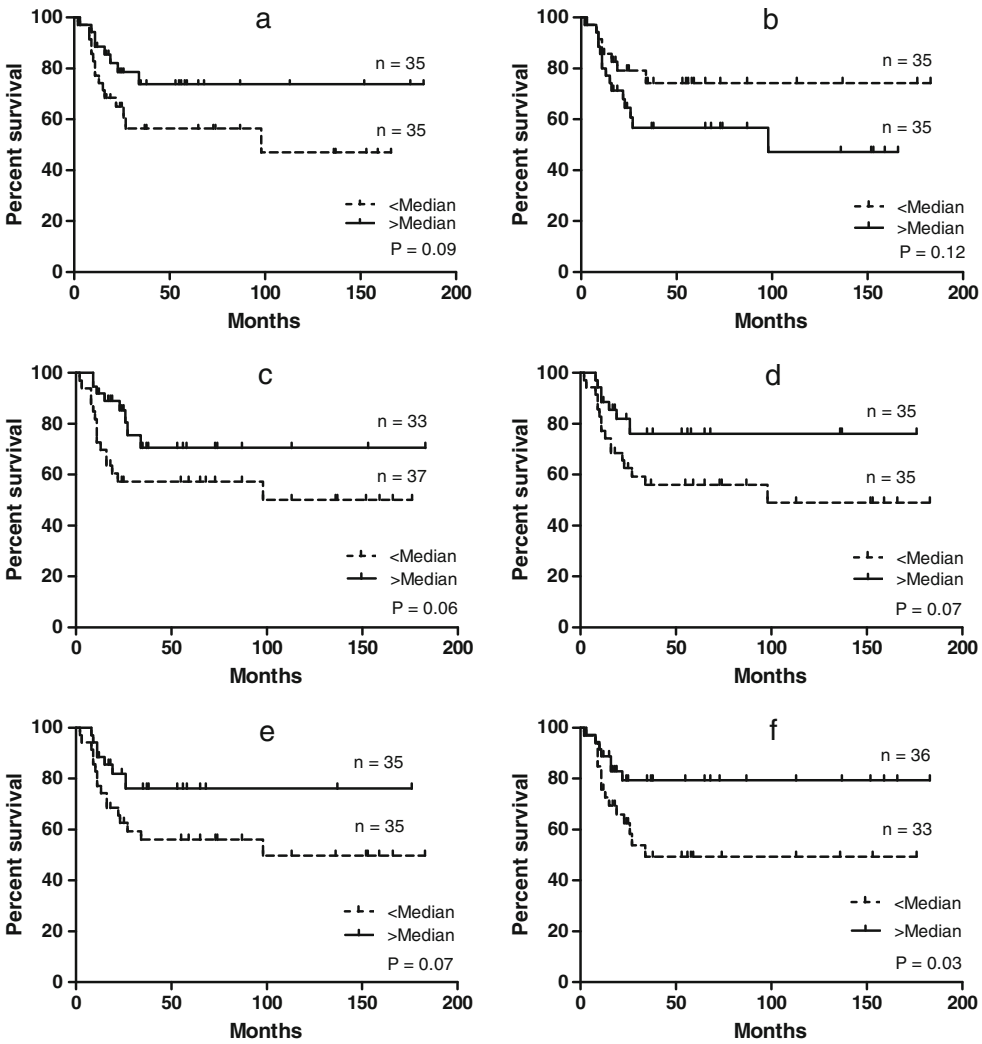

Fig. 4 Probability of survival (Kaplan-Meier) of gastric cancer patients in relation to IgG glycoform levels. Patients with different glycoform levels, which were lower, equal to or higher than the median, are compared. **a**—G2 level, **b**—G0 level, **c**—2Na level, **d**—(bi) level, **e**—level of G2 glycoforms with (bi), **f**—NaGnF IgG1 level. Dark line—values above the median, dotted line—values below or equal to the median

Thus, the degree of IgG galactosylation/sialylation and the presence of IgG glycoforms with bisecting GlcNAc may predict outcome of patients with gastric cancer.

Discussion

During the last decade, it became apparent that the N-glycans of the Fc-fragment strongly influence IgG—Fcγ receptor interactions and thus the Fc—mediated effector mechanisms [8, 27–31]. While many serum glycoproteins exhibit carbohydrate changes in malignancy [32–35], comparably little is known about IgG glycosylation in patients with cancer.

Gercel-Taylor *et al.* [36] demonstrated that patients with ovarian cancer exhibited higher levels of Concanavalin A positive IgG in the serum and, in tumor derived IgG in particular. This suggests that aberrantly glycosylated serum IgG may be either of tumor origin, or accumulated in tumor tissue. In another recent study, Bones *et al.* [38] reported on increased levels of agalactosylated IgG in patients with stomach cancer, to be accompanied by complement activation. The authors considered this an indication of humoral immune response to the tumor. Interestingly, higher levels of agalactosylated IgG oligosaccharides, which increase with tumor progression, were also reported for patients with prostate cancer [38], and ovarian cancer [36].

Aberrant IgG glycosylation was also demonstrated in multiple myeloma [40]. Notable alterations in glycosylation of the IgG Fc region have also been described in other diseases such as rheumatoid arthritis, inflammatory bowel disease, periodontal disease, Lambert-Eaton Myasthenic Syndrome and infection with HIV [1, 16–18, 35, 40–42], suggesting that these modifications are not exclusively specific for cancer. The factors contributing to the changes observed, as well as their putative clinical relevance in patients with cancer, remain to be elucidated.

In this study, altogether 32 Fc glycan structures from the individual serum IgG samples of healthy controls, patients with benign stomach diseases and patients with gastric

cancer were analyzed. Heavy chains of purified IgG were electrophoretically separated, digested with trypsin, and the Fc glycosylation profiles of IgG_1 and IgG_2 were analysed using a recently described LC-ESI-MS method [23, 24]. This approach allowed the glycoprotein- and subclass-specific relative quantification of both neutral and sialylated glycan structures, including those occurring in small quantities [43]. Due to their extremely low abundance, however, we were not able to quantitatively analyze the N-glycosylation profiles of IgG_3 and IgG_4.

A significant decrease in galactosylation and sialylation of the IgG Fc oligosaccharide (*i.e.* increase of G0 and decrease of 1Na glycoforms, respectively) was found in patients with cancer compared to healthy controls and non-cancer group (Fig. 2 and Supplementary Material Table 3). These changes were not related to the stage of the disease. The presence of cancer-related, yet disease-stage-independent changes in Fc galactosylation/sialylation makes it reasonable to suppose that these alterations are actually not a result of tumor growth (*i.e.* secondary phenomenon) but rather precede tumor development and may be considered a risk factor for cancer. This might provide useful information for predicting a disposition to malignancy based upon a change in the IgG Fc glycosylation. Similar, but less pronounced changes were observed in patients with chronic gastric diseases.

Only moderate differences in total fucosylation of IgG Fc were revealed between cancer and non-cancer group. However, significant stage-dependent changes were found, namely a higher degree of IgG fucosylation and lower level of bisecting GlcNAc in stage II and III of cancer (Fig. 3). This implies that these changes are related to tumor progression. A significant negative correlation between both parameters was observed. This was not unexpected, given that IgG oligosaccharide bisecting GlcNAc modification results in the suppression of further processing and elongation of N-glycans including core fucosylation [44].

Significantly higher level of $IgG_{1(+3)}$ and lower level of IgG_2 subclass were found in patients with cancer compared to donors. Benign group results were intermediate showing no significant difference from donor or cancer group. At present, we cannot give any explanation for these changes.

Age and gender have been factors that influence IgG glycosylation with decreasing of galactosylation and sialylation with higher lifetime while the incidence of bisecting GlcNAc was found to increase with age for both sexes [45, 46]. However, the differences between genders are minor compared to the influence of age [45, 46]. Since the changes in Fc galactosylation/sialylation have been shown to reach a plateau at the age of 60 years [47], we stratified patients and controls into subgroups by age—below and above 60 years. In both subgroups the significant differences described above (Fig. 2) remained significant (Supplementary Material Table 4), indicating that the cancer-related changes can not be explained by age-associated changes only. In the older group the changes were less pronounced, possibly because the age-dependent changes in Fc glycosylation partially mask those associated with cancer. The gender-related differences found in the non-cancer group disappeared in patients with cancer, may be because these are hidden by the cancer-associated changes observed. It has to be noted that the reported data on the association of IgG glycosylation with age and sex exclusively apply to healthy individuals [46].

To our knowledge no data have been published so far about possible relation of IgG glycan modifications to the survival of cancer patients. We found that high level of IgG G2 and fully sialylated (2Na) glycoforms as well as the presence of bisecting GlcNAc was associated with a benefit in survival of cancer patients (Fig. 4a, c, d, e, respectively). In contrast, lower survival rate was observed in patients with high-level of agalactosylated IgG glycoforms (Fig. 4b). No relation of IgG fucosylation to the survival of cancer patients was found except significantly better survival rate of patients with higher level of IgG_1 NaGnF glycoform (Fig. 4f). However, possibly it is rather a result of N-glycan sialylation than the presence of the core fucose.

Given that recently a similar glycosylation pattern of IgG was found for purified IgG specific to tumor-associated Thomsen-Friedenreich antigen (unpublished data) we speculate that alterations observed in total IgG of patients with gastric cancer might modulate antibody-mediated immune response to tumor. It has been shown that the level of anti-TF IgG antibodies is significantly associated with survival of cancer patients being higher in long-term survivors [48, 49]. However, the glycoforms of anti-TF IgG responsible for this effect remain to be determined. Substantial inter-individual variations in glycosylation profile of IgG are present in three study groups, including glycoforms related to the cancer patients survival. Therefore, using the pooled IgG samples for such studies is inappropriate and has to be reconsidered in further investigations of antibody glycans in cancer.

It has been reported that the sialic acid-containing IgG displays an anti-inflammatory effect by enhancing the expression of the inhibitory IgG Fc receptor IIB [27]. In this study, the lower level of Fc sialylation in patients with cancer was observed. Since the pro-inflammatory micro-environment may promote tumor growth [50, 51] it is possible that selective removal of pro-inflammatory IgG G0 or transfusion of anti-inflammatory sialylated G2 glycoform [27, 52] could be effective in optimizing of anti-tumor humoral immunity. The beneficial effect of intravenous immune globulin transfusion in some cancer patients [52] may also be related to changes in IgG glycosylation profile thus influencing antibody-based tumor immunity.

In conclusion, this study provides a comprehensive analysis of serum IgG Fc fragment-derived N-glycans in patients with gastric cancer and controls. Our findings are the first evidence that changes in the N-glycosylation profile of Fc are associated with the survival of cancer patients. It appears that higher levels of the IgG G2 glycoform and the presence of the bisecting GlcNAc on Fc glycans may predict a better survival of patients with gastric cancer. Cancer stage-dependent changes in fucosylation of Fc and the bisecting GlcNAc expression as well as an association of several IgG glycans with the survival strongly suggest that glycosylation of IgG is related to pathogenesis of cancer and progression of the disease. Aberrantly glycosylated glycans of Fc and changes in their proportion may be responsible for the efficacy of antibody-dependent tumour immunity especially for elimination of circulating tumor cells and micro-metastases after surgery. Further MS-based analysis of the IgG antibody specific to tumour-associated antigens may provide clearer understanding of the possible impact of glycosylation of IgG on tumour progression and evaluation of Fc glycans as a potentially useful (predictive) bio-marker for monitoring of patients with cancer.

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