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Immunoglobulin G Glycosylation Profiling in Patients with Gastric Cancer

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Declaration:
Hereby I declare that this doctoral thesis, my original investigation and achievement, submitted for the doctoral degree at Tallinn University of Technology has not been submitted for any academic degree.

Kristel Kodar

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KRISTEL KODAR

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To life, and all the science therein

CONTENTS

INTRODUCTION

Cancer is the second most frequent cause of death worldwide, including Estonia. Gastric cancer is still associated with poor prognosis and 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 cancer.

Over the last few decades, there has been an increasing interest in determining the glycosylation of glycoproteins because of the importance of glycosylation in affecting a wide range of biologically important parameters, such as activity, stability, solubility and biological half-life. The oligosaccharides attached to glycoproteins help orient binding faces, provide protease protection and restrict nonspecific interactions. However, the biological role of many glycoconjugates and the diversity of their glycans have no obvious functional relevance yet. In the humoral immune system, all immunoglobulins (Ig) are glycosylated. Recent findings about the presence of differently glycosylated Igs of various subclasses and the pathogenetic role of the aberrantly glycosylated antibody (Ab) in different diseases (for example rheumatoid arthritis (RA), acquired immune deficiency syndrome) implies that Ab glycosylation patterns strongly influence the effector functions of Abs, including affinity, tissue distribution and Ab-mediated cellular reactivities such as antibody-dependent cellular cytotoxic activity, phagocytosis, and interaction with Fc receptors, thus modulating the innate and adaptive immune response.

As the main function of the mammalian immune system is to monitor tissue homeostasis, to protect against invading or infectious pathogens and to eliminate damaged cells, it is surprising that cancer occurs with such a high frequency in humans. The presence of natural Abs to many self, crypt-self and tumor-associated (TA) antigens is a well-established fact. The immune system may not only protect the host against tumor development, but can also promote tumor development and progression by selecting variants with reduced immunogenicity. Since the immune response occurs in the early stage of tumor development, an autoantibody to the TA antigen is a sensitive indicator of an abnormal cellular metabolism in cancer patients already in the early stages of carcinogenesis. While many glycoproteins exhibit carbohydrate changes in cancer, little is known about the glycosylation of IgG in patients with cancer, especially of IgG specific to TA autoimmunogenic antigens such as the Thomsen-Friedenreich glycotope (TF) which is expressed in a majority of carcinomas. It has been reported that naturally-occurring IgG antibody to TF may predict a long-term survival of patients with gastric cancer. We hypothesize that alterations in the glycosylation profile of the total and antigen-

specific IgG in patients with cancer may influence tumor immunity, the disease outcome, and can be of clinical importance for cancer diagnostics and prognostics. In an attempt to evaluate potential biomarkers for stomach cancer diagnosis and patients prognosis, the glycosylation of total and the TF antigen specific IgG was investigated using lectins of various sugar specificities and liquid chromatography electrospray ionisation mass spectrometry (LC-ESI-MS).

ORIGINAL PUBLICATIONS

The thesis in based on the following publications and a manuscript which are referred to in the text by their Roman numerals.

I Klaamas, K., **Kodar, K.**, Kurtenkov, O. (2008). An increased level of the Concanavalin A - positive IgG in the serum of patients with gastric cancer as evaluated by a lectin enzyme-linked immunosorbent assay. Neoplasma, 55, 143–150.

II **Kodar, K.**, Kurtenkov, O., Klaamas, K. (2009). The Thomsen-Friedenreich antigen and αGal- specific human IgG glycoforms: Concanavalin A reactivity and relation to survival of cancer patients. Immunological Investigations, 38, 704–717.

III **Kodar, K**., Stadlmann, J., Klaamas, K., Sergeyev, B., Kurtenkov, O. (2012). Immunoglobulin G Fc N-glycan profiling in patients with gastric cancer by LC-ESI-MS: relation to tumor progression and survival. Glycoconjugate Journal, 29, 57–66.

IV **Kodar**, **K.**, Izotova, J., Kurtenkov, O., Klaamas, K., Järvekülg, L., Sergeyev, B. An aberrant glycosylation of the Thomsen-Friedenreich glycotope specific IgG in patients with stomach cancer: the diagnostic and prognostic relevance. Manuscript (submitted to World Journal of Gastroenterology)

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ABBREVIATIONS

AAL	*Aleuria aurantia* lectin
Ab	Antibody
ADCC	Antibody-dependent cellular cytotoxicity
Asn	Asparagine
CI	Confidence interval
ConA	Concanavalin A lectin
ELISA	Enzyme-linked immunosorbent assay
Fab	Antigen-binding fragment
Fc	Crystallizable fragment
FcγR	Fcγ receptor
GlcNAc	N-acetylglucosamine
Ig	Immunoglobulin
IgG	Immunoglobulin G
LC-ESI-MS	Liquid chromatography electrospray ionisation mass spectrometry
MBL	Mannan-binding lectin
PtA	Protein A
PtG	Protein G
RA	Rheumatoid arthritis
ROC	Receiver operator characteristic
RU	Relative unit
SD	Standard deviation
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SE	Standard error
SEM	Standard error of mean
SNA	*Sambucus nigra* lectin
TA	Tumor-associated
TF	Thomsen-Friedenreich antigen

1. REVIEW OF THE LITERAUTURE

1.1 Protein glycosylation

It has been estimated that human integrity depends on the action of ~10^6 protein molecules. Protein diversity arises from alternative RNA splicing and post-translational modifications *etc*. Among phosphorylation, acetylation and many others, protein glycosylation is the most complex post-translational modification process. Furthermore, ~50% of proteins are glycosylated. Oligosaccharides may be added through the amide side-chain nitrogen of an asparagine (Asn) residue (N-linked) or the oxygen side chain of serine or threonine (O-linked). N-linked oligosaccharides are initiated by the addition of a common oligosaccharide structure co-translationally. However, processing during passage through the Golgi apparatus results in the generation of site specific glycoforms exhibiting heterogeneity. The structure of N-linked oligosaccharides is determined, in part, by the conformation of the polypeptide chain in the immediate vicinity of the Asn residue being processed and availability of different glycosyltransferases to complete the glycan structure (Wong, 2005; Holland *et al*., 2006; Schwarz and Aebi, 2011).
Over the last decade, there has been an increasing interest in determining the glycosylation of glycoproteins because of the importance of the process in affecting a wide range of biologically important parameters, such as activity, stability, solubility and biological half-life. The oligosaccharides attached to glycoproteins help orient binding faces, provide protease protection, restrict nonspecific interactions and play an important role in cell-cell interactions (Gagneux and Varki, 1999; Rudd *et al*., 2002; Raju and Scallon, 2006; Schwarz and Aebi, 2011). However, the biological role of many glycoconjugates and the diversity of their glycans have no obvious functional relevance yet.

1.2 Immunoglobulin G

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 (Arnold *et al*., 2007; Schroeder and Cavacini, 2010). 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 Fc receptors on the effector cells (such as mast cells, basophils, monocytes, macrophages and neutrophils) of the immune system (Alberts *et al.*, 2002; Schroeder and Cavacini, 2010) (Figure 1).

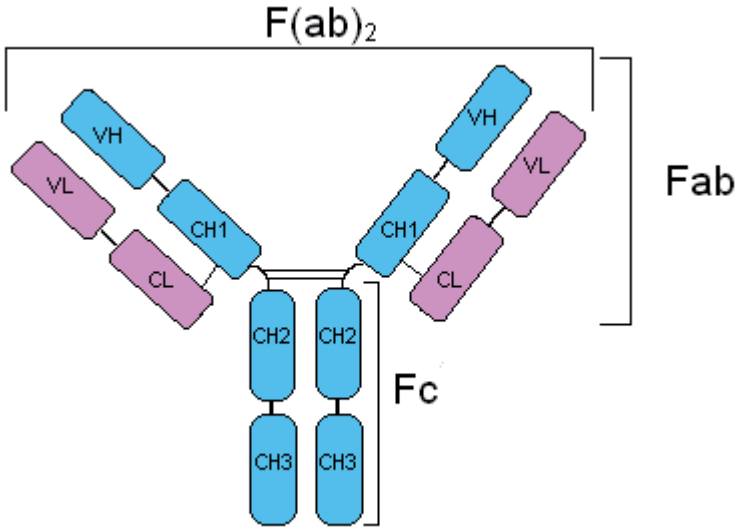

Figure 1. A schematic view of an antibody (immunoglobulin) molecule
The IgG molecule is bi-functional: the variable region of the Fab fragment recognizes the respective antigen targets (see above) and the Fc fragment interacts with Fc receptors on the effector cells of the immune system. (C – constant, V – variable, H – heavy, L – light. Fc – crystallizable fragment, Fab – antibody binding fragment)

IgG is responsible for the detection and destruction of pathogens or their noxious products and is involved in pathogen clearance, tissue destruction and inflammation during autoimmune disease (Alberts *et al*., 2002; Anthony and Nimmerjahn, 2011). IgG molecules bind to Fcγ receptors (FcγR) which are widely expressed on the cells of the innate immune system, via the Fc fragment and thereby provide a link between the specificity of the adaptive immune system (Fab) and the potent effector function triggered by innate immune effector cells (Anthony and Nimmerjahn, 2011). There are two major types of FcγRs: immune reaction activating (FcgRIa, FcgRIIa and FcgRIIIa) and inhibiting receptors (FcgRIIb) (Lux and Nimmerjahn, 2011).

1.2.1 IgG glycosylation

In the humoral immune system, all immunoglobulins as most immune system proteins are glycosylated, each with an inhered set of glycoforms, which differ in number, type and oligosaccharide location as seen in Figure 2 (Arnold *et al*., 2007). Changes in the glycosylation patterns of Igs alter their respective functions, including affinity, the formation of immune complexes, complement-dependent cytotoxicity, activation of macrophages, elimination of antigens, and antibody-dependent cellular cytotoxic (ADCC) activity (Nose and Wigzell, 1983; Jefferis *et al*., 1998; Margni and Malan Borel, 1998; Shields *et al*., 2002; Shinkawa *et al*., 2003; Barbin *et al*., 2006; Raju, 2008).
IgG is less glycosylated than other antibodies: the Fc fragment bears two oligosaccharides, N-linked to the conserved Asn297 on both heavy chain-derived polypeptides (Arnold *et al*., 2007). In contrast to the other Ig isotypes, the IgG-associated sugar domain is not exposed on the IgG surface but rather buried within the hydrophobic core between the two heavy chains of the CH2-domain forming multiple non-covalent bonds with polypeptide chains and impacting Fc structure (Lund *et al*., 1996; Lux and Nimmerjahn, 2011). The N-glycans found on IgG are of a complex biantennary type, differing in the level of the terminal sialic acid, galactose (G0, G1 and G2 glycoforms), core fucose and bisecting N-acetylglucosamine (GlcNAc) (Arnold *et al*., 2007, Wuhrer *et al*., 2007) (Figure 3). Over 30 different glycoforms have been described (Arnold *et al*., 2007). Unusually, only ~24% of the sugar chains attached to IgG are sialylated as most of the N-linked sugar chains of other serum glycoproteins are highly sialylated (Kobata, 2008). Further, in a single IgG molecule, the two Asn297 sites may be differently glycosylated (Arnold *et al*., 2007).
In addition, 15–20% of serum-derived IgG molecules also have oligosaccharides attached to the variable region of the Fab fragment, where they can significantly influence antigen binding (Holland *et al*., 2002, Arnold *et al*., 2007). Compared with the oligosaccharides attached to the Fc fragment, Fab glycans are more sialylated, particularly more bi-sialylated (Holland *et al*., 2006, Stadlmann *et al*., 2008).

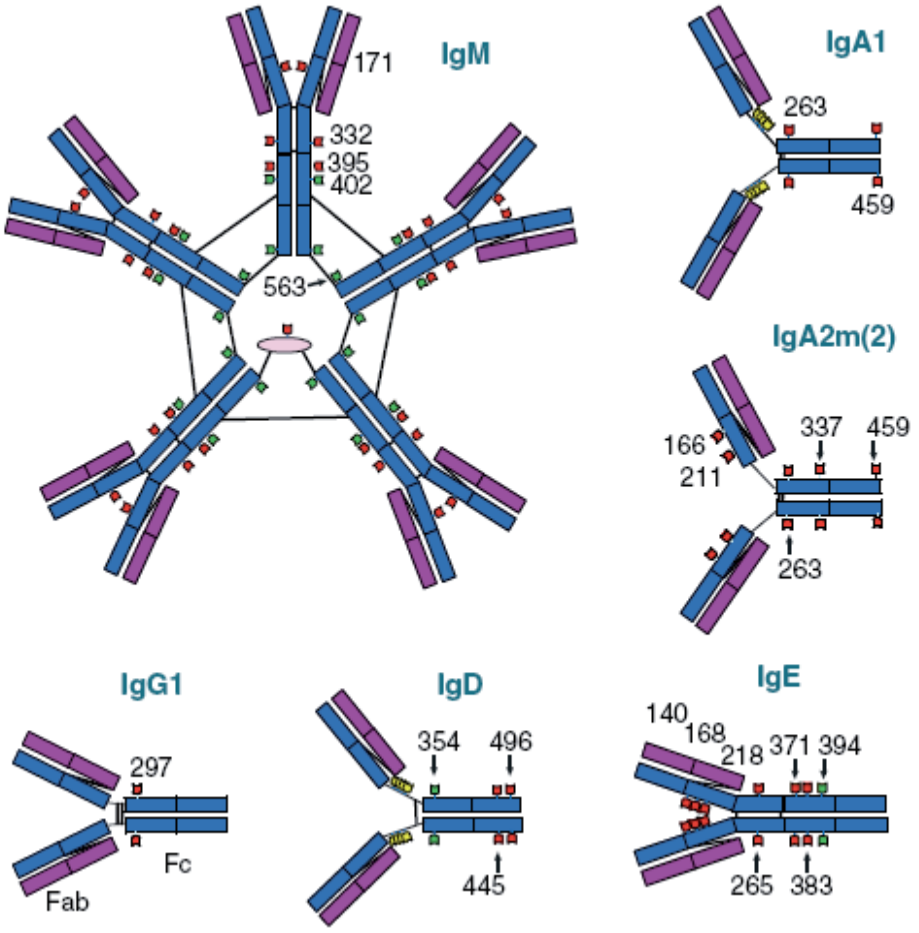

Figure 2. A schematic representation of immunoglobulins: IgM, IgA_1, $IgA_{2m(2)}$, IgE, IgD and IgG

Sites of N-glycosylation are shown on both heavy (blue) and light chain (purple) of IgG, with complex (red) and oligomannose (green) glycans (Arnold *et al*., 2007).

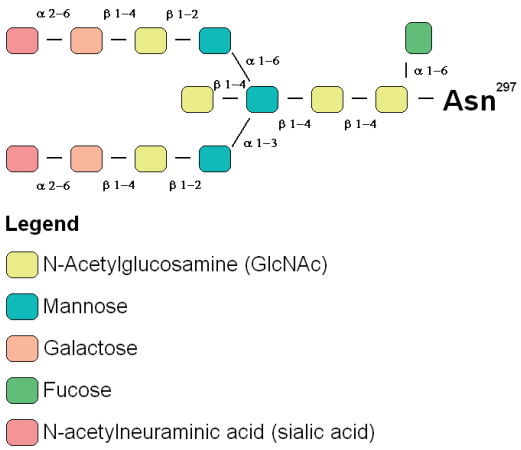

Figure 3. The structure of the complex biantennary N-glycan
The core structure of N-glycan typically consists of two N-acetylglucosamines (GlcNAc) and three mannoses (Man) with two branching GlcNAcs. In addition, the core structure can be further fucosylated, galactosylated (G1, G2) and sialylated (1Na, 2Na) and contain the bisecting GlcNAc. Thus, complex oligosaccharides can be very heterogeneous.

1.2.2 Modulation of IgG activity by glycosylation

An IgG molecule can have two completely opposite functions: it can mediate pro- and anti-inflammatory activities through the engagement of Fc with distinct FcγR (Dimotrov *et al*., 2007). The binding of Fc to FcγRs, and the consequent Ab effector functions, are influenced by several factors, including the nature of the FcγR itself, IgG subclass, the level of inhibitory FcγRIIb expression and the pattern of Fc glycosylation. It has been shown that the presence or absence of even a single sugar residue on a specific N-glycan structure has profound implications on Fc effector functions, mainly due to an alteration in the ability of Fc to bind to FcγRs (Kaneko *et al*., 2006; Nimmerjahn *et al*., 2007; Bruhns *et al*., 2009; Jefferis, 2009).
One of the most thoroughly studied modulations of antibody activity by glycosylation is the effect of the presence of core-fucose on the N-glycans of the IgG Fc fragment: the lack of core-fucose results in a major (>50-fold) enhancement of the ADCC (Mimura *et al*., 2001; Shinkawa *et al*., 2003; Shoji-Hosaka *et al*., 2006). Removal of fucose from the N-glycan selectively enhances binding to the activating FcγRIIIa, whereas binding to all other activating FcγRs remains unchanged (Lux and Nimmerjahn, 2011). Over times, this effect has

most widely been studied using the IgG_1 subclass. However, it was recently shown that all IgG subclasses show enhanced ADCC activity upon removal of fucose (Niwa *et al*., 2004). Similarly to the removal of core fucose, the effect of the presence of the bisecting GlcNAc on oligosaccharide increases IgG ADCC activity, though to much lesser extent. A possible explanation for this phenomenon may be that N-glycan containing bisecting GlcNAc is a much weaker substrate for other glycotransferases such as galactosidase and fucosyl transferase, thus less core fucose is added to the complex oligosaccharide during glycan processing (Lux and Nimmerjahn, 2011).

The data on the role of IgG N-glycan galactosylation in modulating IgG activity are conflicting. The first researches reported minor (2–3 fold) ADCC activity enhancement by highly galactosylated IgG (Kumpel *et al*., 1994, 1995), but further studies could not confirm these results (Rademacher *et al*., 1994; Boyd *et al*., 1995; Shinkawa *et al*., 2003; Forthal *et al*., 2010). Initially it was also suggested that IgG glycoforms lacking galactose in the Fc region (G0 glycoform) might be involved in increasing the pathogenicity of autoantibodies via the mannan-binding lectin (MBL) pathway of complement activation (Malhotra *et al*., 1995). However, recent studies performed in MBL knock-out mice showed that despite the capacity to bind MBL and activate the complement cascade *in vitro*, there was no significant contribution of MBL to the activity of IgG G0 antibodies *in vivo*, suggesting rather that IgG G0 antibodies work normally via the classical FcγR pathway (Nimmerjahn *et al*., 2007).

The IgG Fc N-glycan sialylation on the other hand plays central role in mediating Ab anti-inflammatory activity. The higher level of Fc sialylation in IgG is associated with reduced activity in the ADCC assay and has a profound inhibitory effect on complement and cytotoxic functions. However, the glycan structure itself is not sufficient to provide this anti-inflammatory activity indicating that both the IgG amino acid backbone and the sialic acid residue in the sugar moiety are essential (Lux and Nimmerjahn, 2011). The higher level of sialic acids can reduce the flexibility of the Ab molecule, presumably around the otherwise highly flexible hinge region, and that reduced hinge region flexibility may result in either a reduced binding to FcγRIIIa, a reduce bivalent binding to antigen or both (Jassal *et al*., 2001; Kaneko *et al*., 2006; Scallon *et al*., 2007). Similarly to the highly sialylated IgG, the IgG exposed to reactive oxygen species has weaker binding to Fcγ receptors (Uesugi *et al*., 2000).

Also, the induction of an antigen-specific immune response seems to be associated with a change in the glycosylation of IgGs: the steady-state serum IgGs show a high degree of sialylation (anti-inflammatory form) and upon an antigenic challenge, the antigen-specific IgGs shift toward glycoforms lacking sialic acid (proinflammatory form) (Kaneko *et al*., 2006).

1.2.3 Changes in IgG glycosylation in health and disease

Changes in IgG glycosylation are associated with various physiological and pathological conditions. As the presence of N-linked sugar chains was found to play a crucial role in IgG effector functions (Nose and Wigzell, 1983; Arnold *et al*., 2007; Raju, 2008), 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. Ohkura *et al*. (1985) proposed that each glycoform of IgG might have different function in the defense of human body from infectious diseases.

There are strong associations between IgG glycosylation and age. While the core fucosylation of IgG oligosaccharides stays fairly stable over time, there is an increase of glycan structures with the bisecting GlcNAc and a significant decrease of sialylation and galactosylation of IgG with age (Parekh *et al*., 1988; Yamada *et al*., 1997; Shikata *et al*., 1998; Knežević *et al*., 2010; Ruhaak *et al*., 2010; Pučić *et al*., 2011). Therefore, age-matched study populations are essential.

Along with age, gender has been shown to influence IgG glycosylation (Yamada *et al*., 1997; Chen *et al*., 2012), but a definite conclusion cannot be drawn due to the lack of studied, not to mention the sex specificity disguised beneath disease-associated changes in IgG Fc N-linked glycosylation (Chen *et al*., 2012).

Since the discovery of the decreased galactosylation of IgG in RA more than 25 years ago (Parekh *et a*l., 1985), it has become one of the most studied glycosylation features of IgG and over 50 different studies have analyzed the role of IgG galactosylation in different inflammatory diseases (Arnold *et al*., 2007; Gornik and Lauc, 2008). In addition to RA, alterations in the glycan moieties of IgG have also been described in chronic diseases such as inflammatory bowel disease, periodontal disease, Crohn's disease, tuberculosis and infection with HIV (Parekh *et al*., 1985, 1988; Dubé *et al*., 1990; Rademacher *et al*., 1994; Bond *et al*., 1997; Holland *et al*., 2002; Moore *et al*., 2005; Stefanovic *et al*., 2006; Mehta *et al*., 2008). An increased proportion of the agalactosylated IgG was found in the serum of patients with ovarian cancer (Gercel-Taylor *et al*., 2001; Alley *et al*., 2012). In contrast, Chen *et al*. (2012) observed higher incidences of galactosylated IgG glycoforms in female thyroid cancer patients than in female controls.

A higher level of the ConA-positive IgG was also observed in pregnancy (Zenclussen *et al*., 2001; Canellada *et al*., 2002). Furthermore, in RA patients, the dynamic levels of IgG G0 glycoforms correlate with disease progression, disease activity, and spontaneous remission of the disease in pregnancy and

postpartum flares of disease (Rook *et al*., 1991; Alavi *et al*., 2000; van de Geijn *et al*., 2009).
Thus, many immunological phenomena, such as aging, autoimmunity, inefficiency of antibody-dependent immune reactions to tumor cells or infections, the immunologic protection of pregnancy, might be related to the variations in antibody glycosylation. Yet, less is known about the potential role of IgG glycosylation, namely the glycosylation of antibodies specific to tumor associated antigens, in malignancy and tumor immunity.

1.3 Tumor associated antigens

The aberrant glycosylation often observed in cancer cells leads to the expression of TA carbohydrate antigens which may be autoimmunogenic and recognized by autoantibodies (Hakomori, 1989 and 2002; Springer, 1984; Springer *et al*., 1995(1); Vollmers and Brändlein, 2007; Schwartz-Albiez *et al*., 2008; Wandall *et al*., 2010; Li *et al*., 2010; Kobold *et al*., 2010). This makes TA carbohydrate antigen a promising target for cancer immunotherapy. In cancer patients, an abnormal glycosylation pattern has also been observed for many circulating glycoconjugates, such as alpha 1-antitrypsin, haptoglobin, transferrin, MUC1 mucin, alpha1-acid-glycoprotein, and Igs (Kanoh *et al*., 2004; Ang *et al*., 2006; Saldova *et al*., 2007; Abd Hamid *et al*., 2008; Bones *et al*., 2010; Comunale *et al*., 2010). This suggests a systemic impact of malignancy on glycosylation machinery or possibly represents a specific feature of the host metabolism. In both cases, such changes might be considered as a biomarker of cancer, a premalignant state, or the disposition of the host to cancer (risk factors).

1.3.1 TF antigen

The Thomsen-Friedenreich antigen (TF, CD176, core-1) (Galβ1,3GalNAcα/β-O-Ser/Thr) is expressed due to the incomplete synthesis of O-linked glycans on glycoproteins and glycolipids (Springer, 1984; Hakomori, 1989). The TF glycotope is known as a pancarcinoma antigen which is expressed in approximately 90% of all human cancers and in premalignant conditions (Springer, 1984; Springer *et al*., 1993). The TF expression is associated with more aggressive tumors and is related to the induction of invasion, metastasis and cancer surveillance mechanisms (Springer, 1997; Baldus *et al*., 2000; Desai, 2000; Schindlbeck *et al*., 2007; Yu, 2007). The TF antigen seems to play a crucial role in the adhesion of cancer cells to the endothelium through the

interaction with galectin-3, thereby promoting metastases (Glinsky *et a*l., 2001; Heimburg *et al*., 2006).
Naturally-occurring TF antigen-specific IgG (anti-TF IgG) autoantibodies are present in human serum in health and disease (Springer *et al*., 1995 (1, 2); Kurtenkov *et al*., 1999). In cancer patients, their level is related to tumor progression and prognosis, being higher in patients with the early stages of the disease, in those with more differentiated tumors (G1-2), and in better survivors (Springer, 1997; Kurtenkov *et al*., 2007; Smorodin *et al*. 2008). This suggests an immediate impact of the humoral immune response on malignancy via direct or antibody-dependent cell-mediated effector pathways. However, the mechanisms behind these associations remain to be further elucidated.
In part of the study the αGal glycotope antigen which is expressed mostly on glycolipids or N-linked carbohydrate chains (Galili *et al*.,1999) was used as non-mucin type glycotope for comparison with TF antigen.

1.4 Lectins

Lectins are non-immunological proteins (glycoproteins) that specifically bind oligosaccharides. First lectin, then referred to as hemagglutinin – ricin (*Ricinus communis*), was describe by Peter Hermann Stillmark in his doctoral thesis presented in 1888 to the University of Dorpat, now University of Tartu, Estonia. While the majority of lectins are described from plants, especially legumes seeds, they also occur naturally in bacteria, viruses, fungi and mammalians (Sharon and Lis, 2004).
Due to their specificity to particular oligosaccharides lectins are widely used to study the glycosylation of glycoconjugates (Turner, 1992; Cook *et al*., 1999; Fotinopoulou *et al*., 2003), including Igs (Margni and Malan Borel, 1998; Moore *et al*., 2005; Pasek *et al*., 2006) by various assays such as enzyme-linked immunosorbent assay (ELISA) and blotting which are more clinically adaptive tests compared to mass spectrometry-based technologies (Sumar *et al*., 1990; Routier *et al*., 1998; Miyamoto, 2006). In this work three lectins were used: 1) the Concanavalin A lectin (ConA) reactive to the terminal α-D oligomannose/α-D glucosamine structures; 2) the *Aleuria aurantia* lectin (AAL) that binds preferentially to fucose linked (α1,6) to N-acetylglucosamine or to fucose linked (α1,3) to N-acetyllactosamine related structures and 3) the *Sambucus nigra* lectin (SNA) with specificity to sialic acid attached to the terminal galactose in (α2,6), and to a lesser degree (α2,3) linkage.

In an attempt to discover and evaluate potential biomarkers for stomach cancer diagnosis and prognosis, the total and TF antigen specific IgG glycosylation profiles were investigated using lectins of various sugar specificities. In this work, we demonstrate the aberrant glycosylation of anti-TF IgG in patients with stomach cancer, and the association of these changes with the overall survival, indicating lectins potential clinical applicability.

2. AIM OF THE STUDY

A general objective of this study was to gain further knowledge about the glycosylation of human IgG in gastric cancer.

This was approached through the specific aims:

(i) to investigate the glycosylation profile of the total and antigen specific IgG in patients with gastric cancer and controls (healthy blood donors and patients with benign gastric disorders) using lectins of various sugar specificities;

(ii) to profile the Fc N glycans of IgG by the liquid chromatography electospray ionization mass spectrometry (LC-ESI-MS) method;

(iii) to evaluate whether the putative changes in the IgG glycosylation profile could be used to diagnose and prognosticate of stomach cancer.

3. MATERIALS AND METHODS

Detailed descriptions of materials and methods are provided in the publications of this thesis.

In the course of this study the following methods were used:

- Lectin enzyme-linked immunosorbent assay (lectin ELISA) (**I**, **II** and **IV**)
- Different affinity purifications of IgG (**I**, **II**, **III** and **IV**)
- IgG fragmentation with papain (**I**)
- Polyacrylamide gel electrophoresis (**I** and **III**)
- Western blot analysis (**I**)
- Glycopeptide preparation (**III**)
- LC-ESI-MS of glycopeptides (**III**)

4. RESULTS AND DISCUSSION

4.1 The ConA binding to human IgG

4.1.1 The ConA binding of human polyclonal IgG (I)

Their unique saccharide-binding properties have made lectins useful for analysis of glycoconjugates and glycans mediated processes. An increased proportion of the ConA-positive IgGs was found in the serum of patients with ovarian cancer (Gercel-Taylor *et al*., 2001). A higher level of the so-called asymmetrically (only one Fab fragment) glycosylated ConA-positive IgG was observed in pregnancy (Zenclussen *et al*., 2001; Canellada *et al*., 2002). These authors suggested that such asymmetric Abs are mostly directed to self antigens and may protect organism from autoimmunity.

In the present study, to specify the ConA binding to human IgG, a preliminary sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and the immunoblotting of the purified IgG were performed. Data showed that only the heavy chains of IgG strongly bound the ConA, whereas the light chains did not bind it at all (Figure 4 A and B lane 4 of **I**). The Fab fragments of the normal human polyclonal IgG obtained by papain proteolysis contain the ConA positive oligosaccharides which are located in the N-terminal half of the heavy chain (the Fd fragment) (Figure 5 of **I**). The Fab fragments obtained from the ConA-positive IgG fraction contain ConA-positive Fabs, while no appreciable amount of ConA-negative Fabs was found. This suggests that there is no obvious asymmetry in the glycosylation of ConA-positive IgG-$F(ab)_2$ fragments. The ConA binding to the Fab fragments derived from the ConA-negative IgG was absent or was very weak, possibly due to the presence of a certain amount of the admixture of the ConA-positive material eluted from the ConA-Sepharose together with the ConA-negative fraction (Figure 5 of **I**). The IgG and Fab preparations from healthy individuals and patients with cancer revealed a similar SDS-PAGE and ConA binding pattern (Figure 4 of **I**). This implies that differences found in the IgG-ConA interaction between cancer patients and controls (described in the sections below) reflect glycosylation changes in IgG Fc fragment, since a majority of IgG glycans (about 80 to 85%) are located in the Fc fragment (Arnold *et al*., 2007).

4.1.2 The purification of human IgG with Protein A and G (I and II)

The treatment of IgG with an acidic buffer, pH 2.0, i.e. a procedure known to unfold the structure of proteins, for 1 h has been shown to increase its ability to bind the MBL (Terai *et al*., 2006). To control whether the purification of IgG may alter its glycosylation and the interaction of IgG glycans with the ConA two titration curves were obtained in a parallel ConA-ELISA testing of the serum of a healthy individual and of the IgG purified from the same serum using Protein A (PtA) as a catcher (Figure 3 of **I**). The ConA index values in the dose-dependent part of the curves were practically the same and differed by less than 10%. This indicates that the binding of IgG to PtA in the first step of the assay does not change the ConA binding, suggesting that the data obtained with the serum reflect the real IgG glycosylation pattern. Besides, this indicates that the acid milieu (pH=2.5) used for the elution of IgG from PtA-agarose does not alter the IgG glycosylation pattern or change the proportion of ConA-positive- and -negative IgGs. In addition, the other ConA-positive glycoconjugates (IgM, IgA, other mannose-rich glycoconjugates) that are abundant in the serum or some interfering components (the MBL) are excluded from the reaction. This may be important in different pathological conditions when the amount of the mannose positive material in the serum may be appreciably increased.
The lectin reactivity of the anti-TF or anti-αGal glycotope specific IgG could not be tested by the serum-based lectin-ELISA without a preliminary purification of IgG due to the presence of the lectin positive IgM and other isotype antibodies to these glycotopes. Therefore, a group of healthy blood donors (n=15) was tested for the ConA reactivity of the total serum IgG and IgG samples purified previously on the Protein G (PtG)-Sepharose from the same sera. The ConA ELISA using PtG as catcher molecule in both tests was performed. No difference in the mean ConA index values between the total serum IgG and purified IgGs was observed (P=0.84; Figure 1A of **II**). Also, a significant correlation was found between two assays: r=0.76, P=0.001 (Figure 1B of **II**). These data show that the purification of IgG on the PtG-Sepharose using the elution at an acid pH of 2.7 does not alter its ability to bind the ConA either.

4.1.3 Lectin enzyme-linked immunosorbent assay of human serum and purified IgGs (I)

The lectin ELISA based approach used in this study (**I**) showed a good reproducibility in the evaluation of the ConA-positive IgG level in the serum. Typical titration curves obtained are represented in Figure 2 of **I**. To minimize the interassay variations, the internal standard serum and relative units (RU) were used for the calculation of the ConA/IgG ratio (ConA index) for each serum dilution. The ConA index values did not change appreciably in the dose-dependent part of the curves, for instance, for serum dilutions of $2.5x10^{-4}$ and $5x10^{-4}$. At these two serum dilutions there was no significant change in the ConA index values and a highly significant correlation between the index values calculated at both serum dilutions was observed ($r=0.90$; $n=19$; $P<0.001$). For practical convenience, the serum dilution equal to $5x10^{-4}$ was used. Among the sera tested ($n=77$) the O.D. values obtained at this serum dilution were always in the middle part of the IgG titration curves where the dose-dependent linearity is observed and they did not reach the plateau.

4.2 The ConA lectin reactivities of serum and purified total IgGs in cancer patients and controls (I)

A comparison of the lectin ELISA data for patients with gastric cancer and healthy blood donors is shown in Figure 4. In patients with gastric cancer, the ConA index was found to be significantly higher than in healthy individuals (ConA index=1.07±0.08 and 0.81±0.08 (group mean±SE), respectively; $P=0.0002$). In donors, a highly significant correlation was observed between the level of IgG bound to PtA and the ConA binding ($r=0.85$; $p<0.001$). In contrast, patients with gastric cancer showed a much less pronounced, though significant correlation ($r=0.33$, $P=0.02$) probably because in many patients the relative ConA binding to IgG (ConA index) was appreciably higher.

No difference in the ConA index values of serum IgG was observed between the males and females ($P>0.1$ for both blood donors and cancer patients). Very similar ConA index values were obtained for patients at the age below or above 60 years ($P=0.62$). Tumor morphology (intestinal and diffuse types of tumor growth according to P. Lauren's classification (1965)) did not influence the ConA index either.

The appreciable changes in the ConA index values observed during the follow-up (2–6 months) of patients with cancer (up to 29%), but not in blood donors

(-1.8% to +8.9%) or two patients with benign gastric diseases (-5.8 to +0.5%, respectively) (Table 1 of **I**) suggest that the IgG glycosylation pattern may be related to the progression of cancer. However, in repeated tests a majority of patients with low ConA index values still had lower ConA index values than those who revealed higher ConA indexes. It appears that the IgG glycosylation pattern is strongly controlled at an individual level and is rather stable in a given individual.

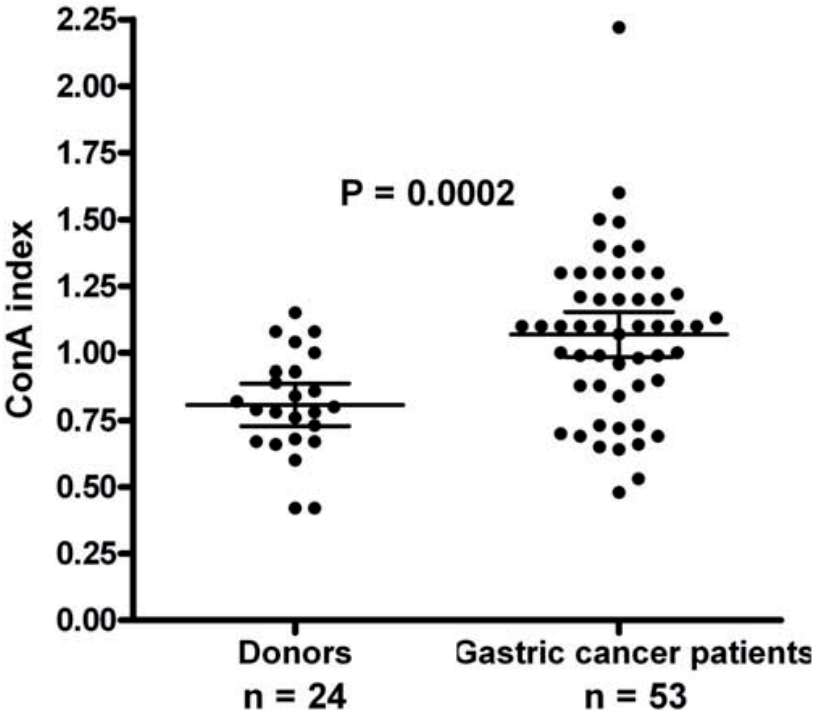

Figure 4. ConA binding patterns as evaluated by lectin ELISA in healthy blood donors and patients with gastric cancer
Based on preliminary experiments, the serum dilution $5x10^{-4}$ was selected for the calculation of the ConA index. Each dot represents an individual and the horizontal lines group mean±95% confidence interval (CI).

4.3 The characterization of TF and αGal antigen specific IgG from total IgG preparations

The role of autoantibodies to tumor-associated glycans in cancer surveillance has been mostly considered for IgM (Springer, 1984; Vollmers and Brändlein, 2007). These antibodies are not affinity-matured which speaks in favour of their inherent natural origin. In contrast, the presence of IgG anti-glycan antibodies suggests an adaptive immune response. The origin of anti-glycan autoantibodies to tumor-related glycotopes is still unclear though evidence available suggests that at least anti-TF and anti-αGal antigen specific antibodies may be induced by bacterial glycans or, possibly, by the cross-reactivity with these antigens (Springer, 1984; Galili *et al*.,1988). In any case, great and yet unexplained interindividual variations of the antibody level to these epitopes in health and disease exist (Kurtenkov *et al*., 2007; Smorodin *et al*., 2008), possibly reflecting distinct immunological histories of each individual. Moreover, the anti-TF IgG level is rather stable over time at an individual level in both patients and controls (Kurtenkov *et al*., 1995; Smorodin *et al*., 2008).

4.3.1 The level of anti-TF antigen IgG in total IgG preparations (IV)

In this study, a significantly higher level of the TF specific IgG in purified total IgG preparations from the serum of patients with stomach cancer than in both control groups was observed: P=0.002, 0.0003 and 0.00004 for donors, the patients with benign stomach disease and combined non-cancer group, respectively (Figure 5). This increase was mostly pronounced in stage I of the disease (P=0.02, P=0.0006, P=0.01 compared to stages II, III and IV, respectively), suggesting that an adaptive immune response cannot be excluded in the early stages of tumor with the following decrease of the anti-TF IgG level in advanced cancer as a result of the tumor-induced immunodepression. If this is the case, the population of the anti-TF IgG should be expected heterogeneous and includes both naturally-occurring TF glycotope specific antibodies, whose presence precedes tumor development, and those triggered by the disease, i.e. induced by the tumor-derived TF glycotope. Due to the great interindividual variations (up to tenfold) of its anti-TF IgG level, the human population may be divided into low and strong responders to the TF glycotope. Notably, donors and the benign group showed a more compact distribution. A further characterization of anti-TF IgG subpopulations is needed to specify their structural and functional diversities, and clinical significance.

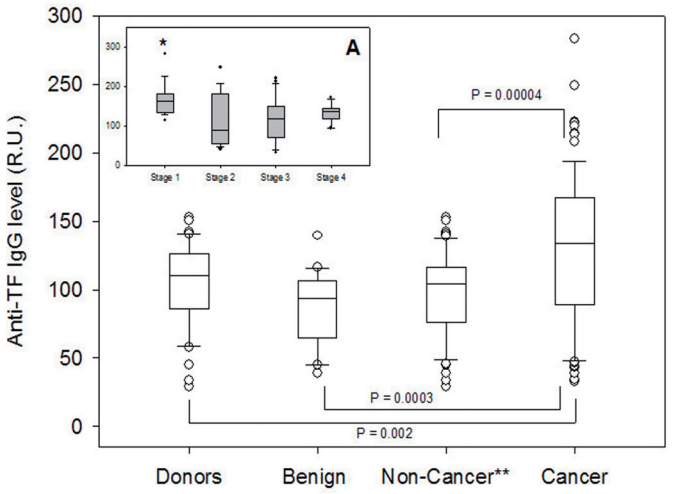

Figure 5. The TF antigen specific IgG level in patients with stomach cancer and controls

The box plots of anti-TF IgG levels (medians, ranges and quartiles) in controls and cancer patients by stage of the disease. A – anti-TF IgG levels in cancer patients by stage. *significantly higher compared to stages II, III and IV (P=0.02; 0.001 and 0.01, respectively); **combined group of donors and patients with benign stomach disease.

4.3.2 The ConA reactivity of anti-TF and anti-αGal glycotope specific IgG (II)

The appreciable inter-individual variations in the ConA reactivity of IgG specific to both tumor-related epitopes (anti-TF and anti-αGal) were observed (Figure 6). However, the mean ConA index for the anti-TF specific IgG in patients with gastric cancer was significantly higher than that in controls (1.09±0.057 (group mean±SE) and 0.82±0.054, respectively; P=0.006). In contrast, the ConA index values for anti-αGal specific IgG were lower (P<0.0001) and similar in both study groups (0.56±0.056 and 0.65±0.052 for cancer patients and donors, respectively, P=0.26), though a slight trend towards a higher ConA index in cancer patients may be observed (Figure 6). The increase of the ConA index value in patients with cancer was more pronounced in the early stages of the disease for both anti-TF and anti-αGal specific IgG (Table 1). No increase of anti-αGal IgG ConA index values at all was observed in patients with advanced stages of cancer. The ConA index values were found to be similar in males and females.

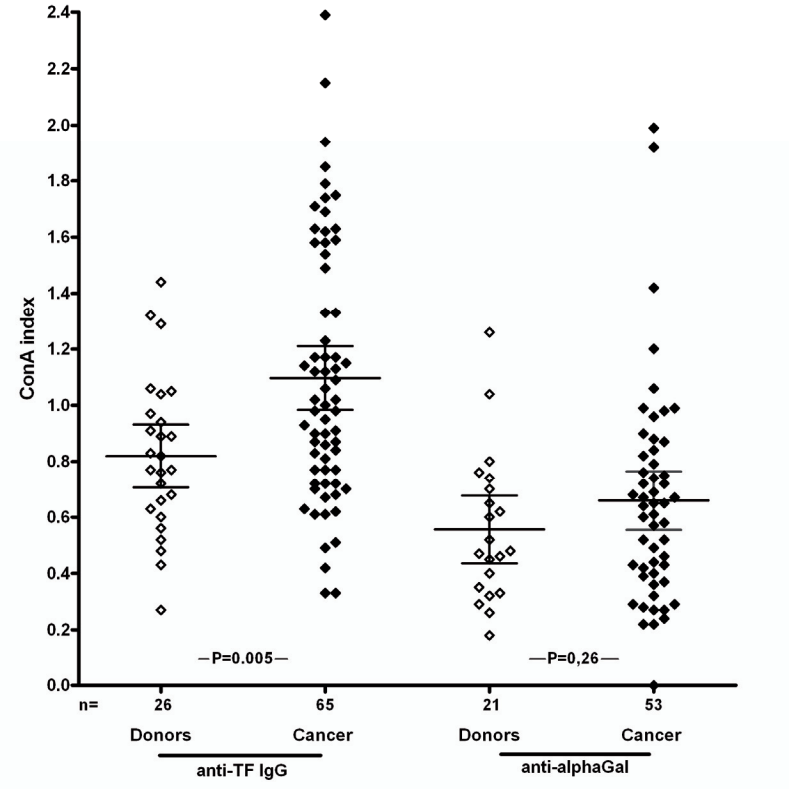

Figure 6. The ConA index values for human anti-TF and anti-αGal specific IgG
Each dot represents an individual and horizontal lines group mean±95% CI.

Table 1. The ConA index values (RU) for anti-TF and anti-αGal IgG in controls and cancer patients by stage

IgG antibody specificity	Blood donors n	ConA index mean±SE	Gastric cancer patients stage	n	ConA index Mean±SE	P value Donors vs cancer
TF	26	0.819±0.054	I–II	21	1.323±0.11	<0.0001
			III–IV	43	0.975±0.059	0.078
			All	64	1.09±0.057	0.006
αGal	21	0.556±0.057	I–II	18	0.88±0.11	0.010
			III–IV	35	0.54±0.043	0.86
			All	53	0.65±0.052	0.26

A correlation between the ConA reactivities of anti-TF and anti-αGal specific IgGs was observed in patients with cancer (r=0.591; n=54; P<0.0001), but no correlation was found in controls (r=0.21, n=20, P=0.37) (Figure 3 of **II**). The ConA reactivity of the anti-TF specific IgG was further correlated with that of the total purified IgG. No correlation was found between these parameters: the "r" value was equal to -0.15 (P=0.50) for a combined group of donors and cancer patients (n=23). Similarly, there was no correlation between the ConA reactivities of total purified IgG and anti-αGal specific IgG (r=0.06, P=0.79). This suggests that variations in the glycosylation of total and antigen specific IgGs (ConA reactivity) do not coincide and occur independently for at least two glycol-epitopes tested. This also implies that the glycosylation pattern of Ab against the target antigens involved in the pathogenesis of a specific disease may be more informative than the level of IgG antibodies to a specific antigen. Interestingly, changes in the ConA reactivity of anti-TF and anti-αGal IgGs were more pronounced in the early stages of cancer suggesting that they are not induced by tumor growth *per se* but rather precede tumor development.

4.3.3 The lectin binding profile of the TF specific IgG (IV)

To further characterize the TF antigen specific IgG glycosylation, further lectin binding profiles were acquired. In addition to ConA, fucose- and sialic acid-specific lectins (*Aleuria aurantia* lectin (AAL) and *Sambucus nigra* agglutinin (SNA), respectively) were used.
The anti-TF IgG of patients with cancer had a significantly higher level of the ConA positive IgG glycoform than that of both controls (which accords with the results of **II**): P=0.013, 0.05 and 0.005 for donors, the benign and non-cancer groups, respectively (Figures 7 and 8). In contrast, the binding of SNA was significantly lower in cancer patients compared to that of blood donors and patients with benign gastric diseases (P<0.0001). In cancer patients, the binding of AAL did not differ from that of the donors group (P=0.64), being, however, significantly lower than that of the benign (P=0.000008) or non-cancer group (P=0.005). The group of patients with chronic gastritis (n=7, one of them with atrophic gastritis), who showed a very high AAL index values, were responsible for this difference. This was the only exception in this part of the study when the benign group differed significantly from donors (P=0.01). Two of these patients also showed a high level of SNA binding. All patients with the peptic ulcer disease (n=6) demonstrated the level of binding similar to that of blood donors, for all three lectins.

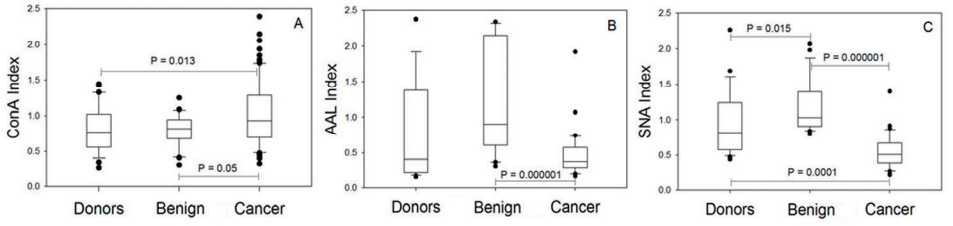

Figure 7. The binding of lectins to the TF specific IgG in gastric cancer patients and controls

The box plots of lectin index values (medians, ranges and quartiles) in patients with stomach cancer, healthy blood donors and patients with the benign stomach disease. A: ConA – Concanavalin A; B: AAL – *Aleuria aurantia* lectin; C: SNA – *Sambucus nigra* agglutinin.

No appreciable stage-dependency of the binding of any lectin to the anti-TF IgG was found (Figure 8), though a slight trend towards higher ConA index values in stage I cancer patients was observed (P=0.19 compared to stage III patients).
A strong positive correlation between the reactivities of AAL and SNA was demonstrated in all groups: cancer patients (r=0.72; P<0.0001); the non-cancer group (r=0.71; P<0.0001) as well as the combined group of all the tested subjects (r=0.72, P<0.0001). No significant correlation between the reactivities of ConA and two other lectins was observed. Thus, changes in the ConA reactivity were not related to the modification of anti-TF IgG binding sites for the fucose- or sialic acid-specific lectins (AAL and SNA, respectively).

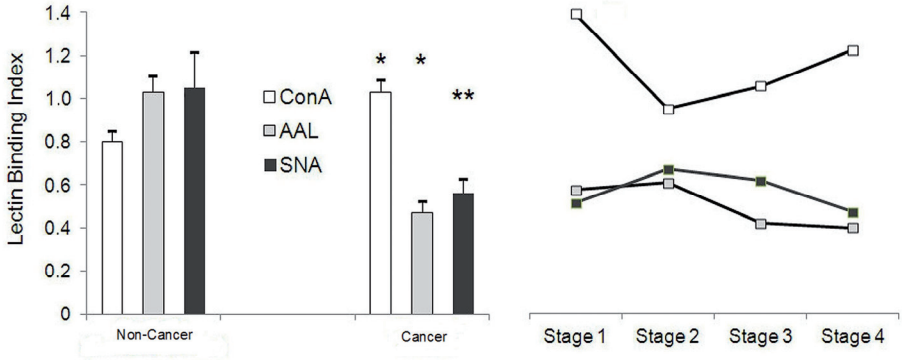

Figure 8. The anti-TF IgG glycosylation profile in cancer and non-cancer groups: relation to the stage of cancer

The results are depicted as a mean with error bars representing a standard error of the mean (SEM). *P=0.005; ** P<0.0001, compared to controls.

Given that an active immunization reduces the sialylation of IgG, especially the antigen-driven IgG (Kaneko *et al*., 2006), we hypothesize that the decreased sialylation of the anti-TF IgG observed in cancer patients may be an indicator of an adaptive immune response to the tumor-derived TF antigen, in addition to naturally-occurring anti-TF IgG antibodies, which are present in every individual in different amounts. It remains yet unclear whether changes in the sialylation and core fucosylation of anti-TF IgG glycans in both IgG fragments may be independent or concordant events, despite the positive correlation observed between the bindings of SNA and AAL lectins to the whole molecule of the TF specific IgG.

4.3.4 The anti-TF IgG level and lectin binding profile: sensitivity and specificity in stomach cancer (IV)

The receiver operator characteristic (ROC) curves analysis was used to evaluate the changes of the level and glycosylation profile of anti-TF-IgG as possible biomarkers. The diagnostic accuracy and ROC curves statistics are presented in Figure 9 and Table 2 of **IV**. Coming of the absence of the correlation between the data on the binding of ConA and two other lectins we investigated a possible interactive effect of lectin combinations using the ratios of ConA/SNA, ConA/AAL and AAL/SNA in the ROC analysis.

Despite the significant difference in anti-TF IgG levels between cancer patients and controls these changes showed a low sensitivity and specificity for cancer, possibly due to great variations within each group. The same was true for the ConA and AAL lectin index values. In contrast, the SNA binding index and, especially, the ConA/SNA ratio demonstrated rather a good sensitivity and specificity reaching 78.8–88.6% (Table 2 of **IV**), and can therefore be considered as diagnostic markers to differentiate stomach cancer from non-cancer, including benign gastric diseases. Since no notable cancer stage dependency of lectin binding was observed, the sensitivity and specificity values are presented for the combined cancer group and non-cancer controls (Figure 9). Using the combination of ConA/AAL and AAL/SNA lectin ratios did not improve the accuracy of the assay showing lower sensitivity and specificity values, usually below 70% (Table 2 of **IV**).

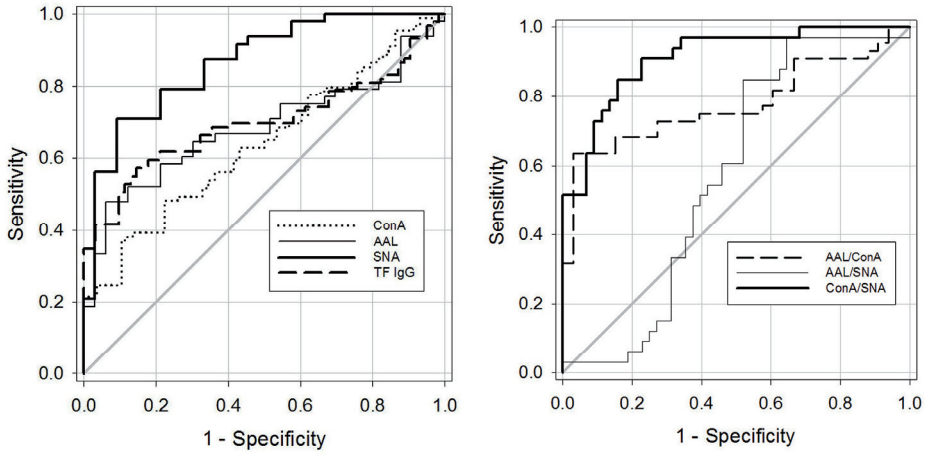

Figure 9. The sensitivity and specificity of anti-TF specific IgG glycosylation changes in stomach cancer
Reciever operator characteristic (ROC) curve analysis.

4.4 LC-ESI-MS of the total IgG Fc fragment (III)

Altogether 32 Fc glycan structures (Figure 10) from the individual serum IgG samples of healthy controls, patients with benign stomach diseases and patients with gastric cancer were analyzed. The heavy chains of the 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 liquid chromatography electrospray ionisation mass spectrometry (LC-ESI-MS) method (Stadlmann *et al*., 2008, 2009). This approach allowed the glycoprotein- and subclass-specific relative quantification of both neutral and sialylated glycan structures, including those occurring in small quantities (Stadlmann *et al*., 2010). Due to their extremely low abundance, however, we were not able to quantitatively analyze the N-glycosylation profiles of IgG_3 and IgG_4.

4.4.1 The N-glycan profile of the total IgG Fc fragment

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) the presence of the bisecting GlcNAc. The N-glycosylation profiles of the IgG Fc fragment are shown in Table 3 of **III** and Figure 11. Among the 32 IgG-typical glycan structures analyzed, the glycoforms GnGnF, GnGn(bi)F showed a significant increase ($P \leq 0.05$) in patients with cancer when compared to healthy donors. In contrast, the levels of 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 a 22% increase in the G0 ($P<0.0001$) and 4% in the non-sialylated (0Na) glycoforms ($P<0.0001$), a 25% ($P<0.0001$) and 22% ($P<0.0001$) decrease in the G2 and the monosialylated glycoforms (1Na), respectively (Figure 11, Supplementary Material Tables 2 and 3 of **III**). There is a strong negative correlation ($r>-0.9$, $P<0.001$) between the levels of 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 the levels of G2 and 1Na glycoforms indicates IgG N-glycans to be at least monosialylated if prior doubly galactosylated. 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 the result of tumor growth (i.e. a 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.

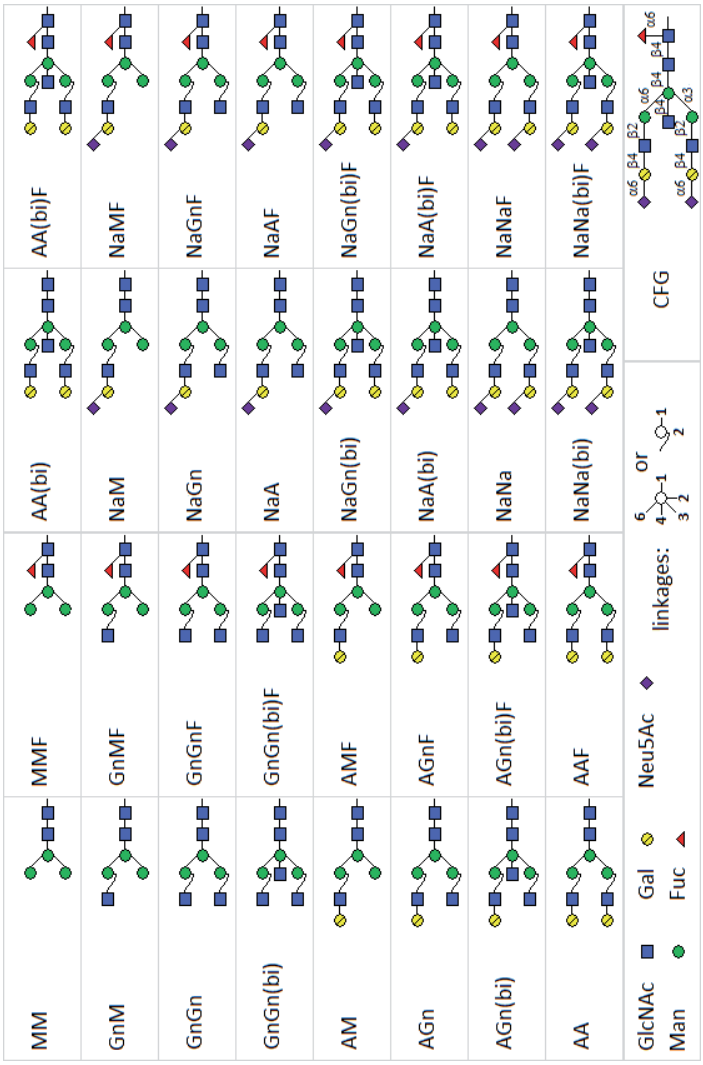

Figure 10. The structures of the human IgG Fc N-glycans dealt with in this study
Glycoforms are named according to the proglycan system (Altmann, 2010) and monosaccharide symbols are used as suggested by the Consortium of Functional Glycomics (CFG, www.functionalglycomics.com). In short, as each N-glycan of the IgG Fc fragment has a pentasaccharide core structure (except truncated glycoforms) only terminal residues are represented in the 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, the IgG N-glycan is shown in a true CFG style. The structures of potential structural isomers (for example AGnF, GnAF or A(bi)MF) are not presented.

Similar, but less pronounced changes were observed in patients with chronic gastric diseases (Figure 11). Agalactosylation was observed in both, the cancer and the benign gastric disease groups, asialylation seems 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 the benign stomach disease.

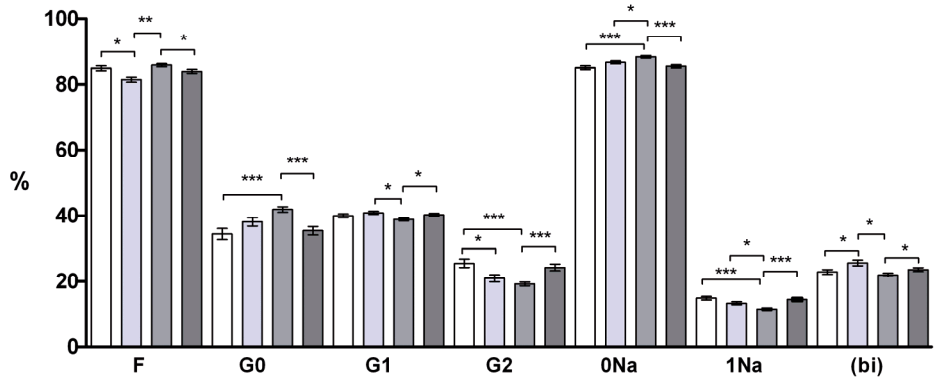

Figure 11. Glycosylation differences in total IgG Fc fragment between gastric cancer patients and controls
Shown are glycosylation differences between healthy donors (white), patients with benign stomach diseases (light grey), gastric cancer patients (medium grey) and the non-cancer group (dark grey) (group mean %±standard deviation (SD)). F – fucosylation, G – galactosylation (G0, G1 and G2 glycovariants), Na – sialylation (0Na, 1Na glycovariants), (bi) – the presence of the bisecting GlcNAc. * P≤0.05, ** P≤0.001, *** P≤0.0001.

Similar changes in the glycosylation pattern of IgG_1 and IgG_2 of all three study groups were observed, 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 and Supplementary Material Table 2 of **III**).
An additional control group was created by combining donors and patients with benign stomach diseases, i.e. the 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 analysis of variance (ANOVA) the highly

significant differences in the levels of 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 (Figure 12, Supplementary Material Table 3 of **III**).

4.4.2 IgG Fc fragment glycosylation changes with age

Age and gender have been factors that influence IgG glycosylation with decreasing galactosylation and sialylation with higher lifetime while the incidence of the bisecting GlcNAc was found to increase with age for both sexes. However, the differences between genders are minor compared to the influence of age (Pucic *et al*., 2011, Yamada *et al*., 1997). In this part of the study a significant correlation between the levels of IgG G0, G2 glycoforms and age was found in donor and cancer groups (G0: r=0.55, r=0.39; G2: r=-0.55, r=-0.43, respectively; P<0.02). Since the changes in Fc galactosylation/sialylation have been shown to reach a plateau at the age of 60 years (Ruhaak *et al*., 2010), we stratified patients and controls into subgroups by age – below and above 60 years. In both subgroups the significant differences between healthy donors and cancer patients (Figure 11) remained significant (Supplementary Material Table 4 of **III**), 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.

In cancer versus non-cancer group comparisons, differences found in G0, G2, 0Na and 1Na glycoforms between both age subgroups remained significant. 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 the 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). This may be because these are hidden by the cancer-associated changes observed and it is to be noted that the reported data on the association of IgG glycosylation with age and sex exclusively apply to healthy individuals (Pucic *et al*., 2011). Thus, the differences found in the cancer versus non-cancer group were not closely related to age and gender.

4.4.3 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 (Stadlmann *et al*., 2009). The IgG subclass analysis showed a significant increase of $IgG_{1(+3)}$ subclass (68.98% and 61.41%, P=0.002) and a decrease of IgG_2 in the cancer group (27.94% and 35.39%, P=0.001, respectively) compared to donors. $IgG_{1(+3)}$ is less galactosylated than IgG_2, however, there is no correlation between the increase of $IgG_{1(+3)}$ and the agalactosylion of IgG in gastric cancer patients. The results of the benign group were intermediate showing no significant difference from the donor or cancer group. At present, we cannot give any explanation for these changes.

4.4.4 The glycosylation of IgG from gastric cancer patients with different disease stage

No differences in the levels of total fucosylation and of the bisecting GlcNAc between cancer patients and donors were observed (Figure 11, Supplementary Material Tables 2 and 3 of **III**). However, the respective moderate differences were revealed between cancer and non-cancer groups (P=0.02). The distribution of cancer patients by stage of the disease on the other hand revealed significant stage-dependent changes, namely a higher degree of IgG fucosylation and a lower level of bisecting GlcNAc in stage II and III of cancer (Figure 12). Patients with chronic gastric diseases showed values similar to stage I cancer patients, and great variations for both parameters were found in advanced cancer (stage IV). This implies that these changes are related to tumor progression. Further, a significant negative correlation between both parameters was observed ($r=-0.81$, $P<0.001$, $n=132$). This was not unexpected, given that the IgG oligosaccharide bisecting GlcNAc modification results in the suppression of further processing and elongation of N-glycans, including core fucosylation (Takahashi *et al*., 2009)

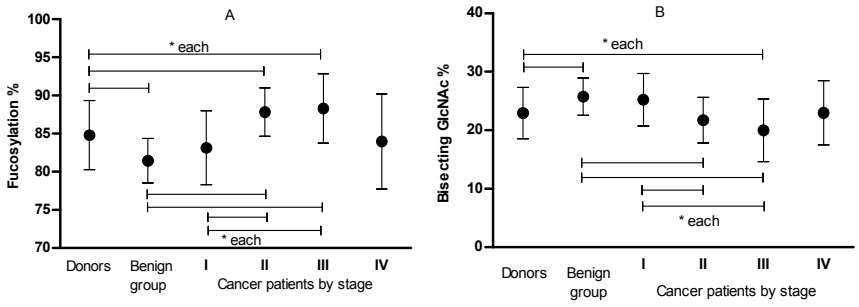

Figure 12. Differences in IgG N-glycan fucosylation (a) and presence of the bisecting GlcNAc (b)
Differences between donors, patients with benign stomach diseases and gastric cancer patients in different stages of the disease (group mean %±SD) are shown. * $P \leq 0.05$.

4.4.5 Correlations between the ConA reactivity of serum IgG (lectin ELISA) and the IgG Fc fragment glycosylation (LC-ESI-MS) (unpublished data)

LC-ESI-MS results demonstrated that the level of the agalactosylated IgG (G0 glycoform) was significantly higher and that the galactosylated IgG (especially G2 glycoforms) significantly lower in the total IgG of gastric cancer patients than in controls (**III**). Interestingly enough, this shift to the agalactosylation of IgG in cancer moderately correlated with the binding of ConA to the total serum IgG (a combined group of gastric cancer patients and donors, n=50; unpublished data) (Table 2). This indicates that the IgG asialylation/agalactosylation is associated with an increased ConA binding possibly due to a better accessibility of the D-mannose residue to the ConA because of conformational changes in the Fc G0 glycoform. The absence of the correlation between the bindings of ConA and SNA may be a reason for a positive interactive effect of using the ConA/SNA binding ratios for cancer *vs* non-cancer group discrimination.

Table 2. Correlations between the ConA reactivity (RU) of IgG (lectin ELISA) and the IgG Fc fragment glycosylation (LC-ESI-MS)

A combined group of donors and gastric cancer patients was correlated (n=50). *P<0.05

Glycoform	Pearson r	P
F	-0.09	0.535
G0*	0.38	0.006
G1*	-0.32	0.022
G2*	-0.35	0.012
0Na	0.25	0.078
1Na	-0.26	0.074
2Na	0.01	0.974
(bi)	0.028	0.845

4.5 Survival of patients with gastric cancer

4.5.1 The ConA reactivity of anti-TF and anti-αGal specific IgGs and the survival of patients with gastric cancer (II)

In a previous work, our group found that the significantly better survival of patients with gastric cancer (Kurtenkov *et al*., 2007) is associated with the higher level of total serum anti-TF IgG, yet no association was found for anti-αGal specific IgG antibodies. The mechanism behind these associations remains unclear. It is tempting to assume that in this respect the ConA-positive and ConA-negative anti-TF IgG glycoforms are functionally different. Which anti-TF IgG glycoform may inhibit or potentiate this protection remains to be clarified.

In the current study, the ConA reactivity of anti-TF antigen specific IgG was significantly associated with the survival of cancer patients (Figure 13A): better survival rate was observed in patients with low ConA index values (Hazard Ratio (HR)=0.43, 95% CI (0.19–0.95), P=0.037, n=62). A more pronounced association with the survival was observed in surgically treated patients in stage III of the disease (HR=0.40, 95% CI (0.12–1.35), n=30; P=0.005; Figure 13B). In contrast, no significant association (P>0.3) was observed in patients in the early stages (I–II) and stage IV of the disease. Thus the significant association

of the better survival with the low ConA index value observed in the combined group of patients (n=62) is mostly related to patients in stage III of the disease.
Even a more pronounced difference in the median survival time was observed if patients whose serum anti-TF IgG ConA index values falling in the zone of the group mean±95% CI (n=8) were excluded from analysis, i.e. only patients whose serum ConA index values were low and high were involved. The median survival time of patients, whose serum ConA index was low (n=29), was twice higher than that of the alternative group (34.0 versus 16.0 months; HR=0.49; 95% CI (0.14–1.15); n=17, P=0.089).
Despite the absence of the difference in anti-αGal IgG ConA reactivity between donors and cancer patients, the trend to a better survival of patients with cancer (HR=0.46, 95% CI (0.21–1.00), P=0.051; n=54) was also associated with the low anti-αGal IgG ConA index (Figure 13C). However, there was no significant difference in survival rate between patients in stage III of the disease with low and high ConA index values (HR=0.40, 95% CI (0.12–1.35), P=0.13) (Figure 13D). Excluding patients whose serum anti-αGal IgG ConA index values fall in the zone of the group mean±95% CI showed no difference in the median survival time: 34 and 32 months for patients with a low and high ConA index value, respectively (n=37, P=0.19). Thus, a more pronounced association of survival with the ConA index value was observed for anti-TF specific IgG antibodies. This indicates that changes in the glycosylation of tumor-specific IgG may be more informative than the determination of just the total IgG level. The negative impact of the increased level of ConA-positive anti-TF and anti-αGal specific IgG on survival established in this study shows that the detection of IgG glycoforms may be of clinical importance in the evaluation of anti-tumor immunity potential and in the prognostics of patients with cancer.
Since the glycosylation pattern of therapeutic Abs may strongly influence the interaction of the latter with Fcγ receptors and Ab effector functions, the optimization of IgG Ab glycosylation is considered to be a promising way to improve cancer immunotherapy (Dimitrov *et al*., 2007; Nimmerjahn and Ravetch, 2008; Raju, 2008). Our data show that the ConA reactivity of IgG antibodies specific to tumor-associated TF and αGal glycotopes may significantly differ and does not correlate with the binding of ConA to the total serum IgG. However, in patients with cancer, the content of ConA positive glycans of both total IgG (**I**) and IgG specific to the two tested tumor-related glycotopes tends to be higher. Another regularity is that the better survival of patients with cancer is associated with the lower content of ConA positive glycans in anti-TF and anti-αGal specific IgGs suggesting the important role of glycosylation of IgG in the immunosurveillance of cancer.

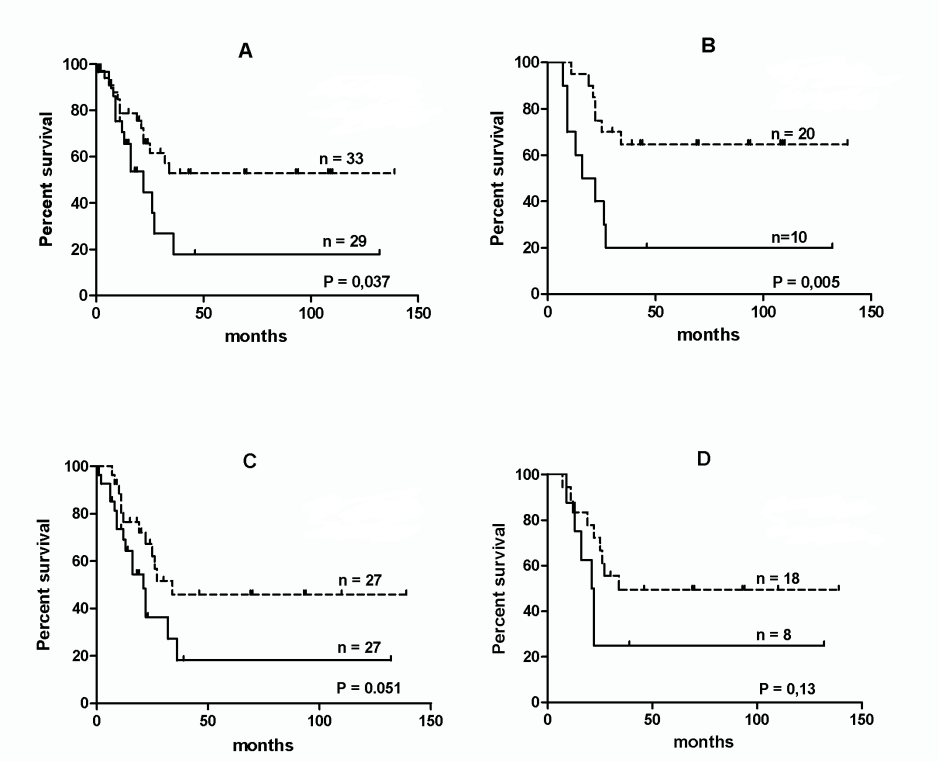

Figure 13. The dependence of the survival of gastric cancer patients on the ConA reactivity of anti-TF (A, B) and anti-αGal (C, D) specific IgGs
Patients with ConA index values, which were either lower, equal to (a dashed line) or higher (a solid line) than the median are compared.
* A, C – the combined group of patients (stages I–IV); B, D – patients in stage III of the disease.

4.5.2 The lectin binding of theTF specific IgG and the survival of cancer patients (IV)

The subgroups of cancer patients with high and low levels of lectin binding to the anti-TF IgG were compared. The cut-off levels were calculated using the time-dependent ROC curves analysis for each lectin. Despite the opposite changes in the binding of ConA and AAL or SNA lectin in cancer patients (increase *vs* decrease, Figure 7) a better survival rate was related to the lower reactivity of anti-TF IgG to ConA, especially in those with stages III-IV of the disease (HR=2.17 (95% CI 0.98–4.79), P=0.048) and AAL (HR=2.39, 95% CI

(1.0–5.7); P=0.038). The SNA reactivity revealed no significant association with survival, though a similar slight trend was observed (Figure 14). It seems that the IgG asialylation alone is not sufficient for the impact on survival and further agalactosylation is needed to attain this effect. In this part of the study, patients were subjected to follow-up for more than 10 years. The association of the binding of ConA and AAL with survival became evident after 2.5–3 years of observation, reaching maximum after 5 years.

Considering that no correlation between the bindings of ConA and two other lectins was found, a possible interactive effect of the combination of two lectins was investigated using the ratios of ConA/SNA, ConA/AAL and AAL/SNA. However, no additional information regarding association with survival was obtained (data not shown).

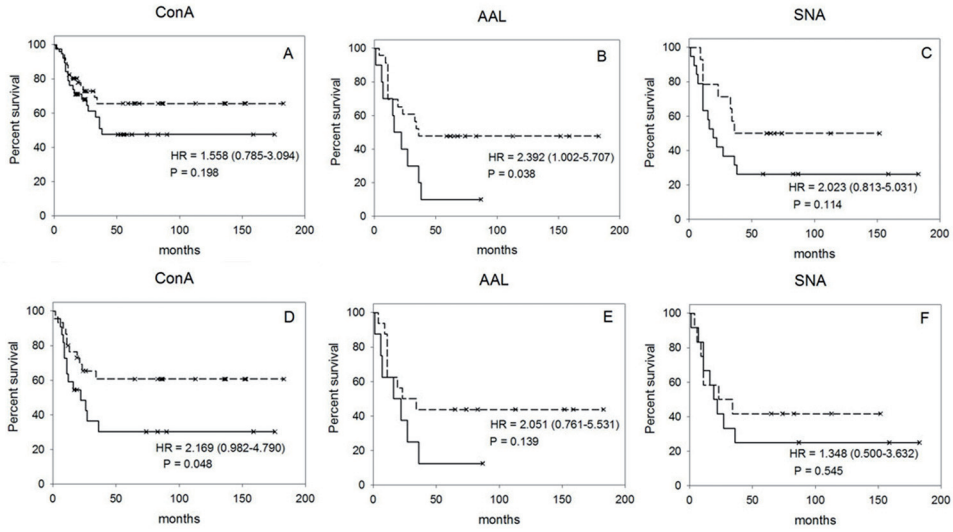

Figure 14. The binding of lectins to the TF specific IgG and the survival of patients with stomach cancer

The probability of survival (the Kaplan-Meier method) of stomach cancer patients in relation to lectin binding to the TF specific IgG. The patients' lectin index values, which were either lower, equal to (a dashed line) or higher than cut-off (a solid line), are compared. The cut-off levels were calculated using the time-dependent ROC curves analysis. ConA – Concanavalin A; AAL – *Aleuria aurantia* lectin; SNA – *Sambucus nigra* agglutinin. A, B, C – all cancer patients; D, E, F – patients in stages III–IV of the disease.

4.5.3 Fc glycoforms and survival of gastric cancer patients (III)

The subgroups of cancer patients with higher and lower than median levels of IgG Fc glycoforms were compared. We found that the high level of IgG G_2 (HR=2.06; 95% CI (0.90–4.71), P=0.08) was associated with a benefit in survival of cancer patients (Figure 15A). Notably this effect was mostly accounted for IgG_2 (HR=2.05; 95% CI (0.90–4.68), P=0.09) though a similar slight trend was also observed for IgG_1 (HR=1.51; 95% CI (0.66–3.44), P=0.33). In contrast, the higher level of IgG G0 glycoforms was associated with a lower survival rate (HR=0.52; 95% CI (0.23–1.19), P=0.12) (Figure 15B). The level of Fc N-glycans with a single galactose showed an intermediate position between the results for G0 and G2.

Cancer patients with a higher level of disialylated IgG glycoforms showed a better survival rate (HR=2.24; 95% CI (0.98–5.16), P=0.06) (Figure 15C), irrespective of IgG subclass. The higher expression of the bisecting GlcNAc was also associated with a better outcome of patients with cancer (HR=2.14; 95% CI (0.93–4.91, P=0.07), mostly the higher level of IgG_2 glycoforms with the bisecting GlcNAc (HR=2.12; 95% CI 0.92–4.88, P = 0.08) (Figure 15D and E).

No relation of IgG fucosylation to the survival of cancer patients was found, except a significantly better survival rate of patients with a higher level of IgG_1 NaGnF glycoform (HR=2.38; 95 % CI (1.04–5.46, P=0.04) (Figure 15F). However, possibly it is rather the result of the N-glycan sialylation than the presence of the core fucose. Thus, the degree of IgG galactosylation/sialylation and the presence of IgG glycoforms with the bisecting GlcNAc may predict the outcome of patients with gastric cancer.

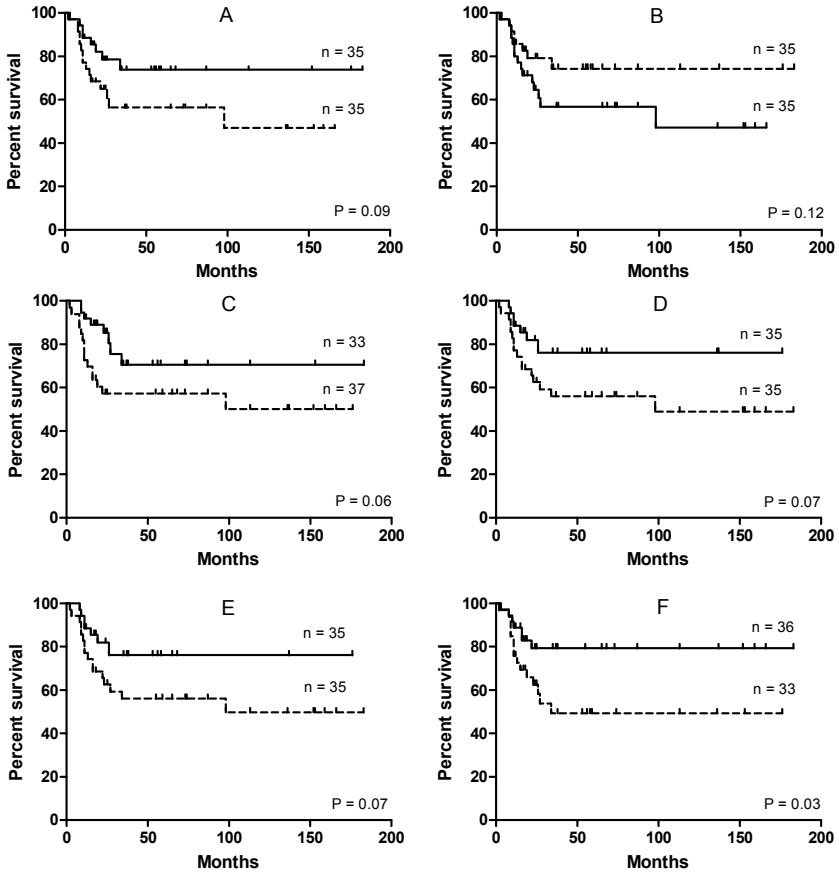

Figure 15. Probability of survival (Kaplan-Meier) of gastric cancer patients in relation to IgG glycoform levels
Patients with different glycoform levels, which were lower, equal to or higher than the median, are compared. A – G2 level, B – G0 level, C – 2Na level, D – (bi) level, E – the level of G2 glycoforms with (bi), F – NaGnF IgG1 level. A dark line – values above the median, a dotted line – values below or equal to the median.

Given that recently a similar glycosylation pattern of IgG was found for purified IgG specific to the tumor-associated TF antigen (unpublished data) we speculate that alterations observed in the total IgG of patients with gastric cancer might modulate the antibody-mediated immune response to tumor. It has been shown that the level of anti-TF IgG antibodies is significantly associated with the survival of cancer patients being higher in long-term survivors (Kurtenkov *et*

al., 1999, 2007). However, the glycoforms of the anti-TF IgG responsible for this effect remain to be determined. Substantial inter-individual variations in the glycosylation profile of IgG are present in three study groups, including glycoforms related to 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 Fcγ receptor IIB (Kaneko *et al*., 2006). 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 (Raman *et al*., 2007; Goldberg and Schwertfeder, 2010), it is possible that the selective removal of the pro-inflammatory IgG G0 or transfusion of the anti-inflammatory sialylated G2 glycoform (Kaneko *et al*., 2006, Kazatchkine and Kaveri, 2001) could be effective in optimizing of anti-tumor humoral immunity. The beneficial effect of the intravenous immune globulin transfusion in some cancer patients (Kazatchkine and Kaveri, 2001) may also be related to changes in IgG glycosylation profile, thus influencing antibody-based tumor immunity.

Taken together, the results clearly demonstrate that the IgG glycosylation pattern of gastric cancer patients differs significantly from that of healthy donors either on total serum IgG or tumor-associated Thomsen-Friedenreich antigen-specific IgG level. The appearance of these alterations already in the early stages of cancer and their association with survival suggest that they play a significant role in cancer development and progression. The lectin-based glycoprofiling ELISA assay is an informative and clinically applicable tool for the analysis of IgG glycans. The results imply that changes in the TF specific IgG glycosylation have a diagnostic and prognostic potential for stomach cancer. It appears that there is a general regularity in IgG glycosylation changes in cancer patients or in individuals prone to cancer development. This might provide useful information for predicting a disposition to malignancy (a risk factor) based upon a change in IgG glycosylation. In any case, the aberrantly glycosylated IgG 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 tumor-associated antigens on a larger scale and functional analysis of purified IgG glycoforms may provide clearer understanding of the possible impact of IgG glycosylation on tumour progression and evaluation of Fc glycans as a potentially useful (predictive) bio-marker for monitoring of patients with cancer.

CONCLUSIONS

1. The lectin-ELISA used in this study is a simple and clinically informative method to evaluate the IgG glycosylation alterations in health and disease. Protein A and G or synthetic glycan-polyacrylamide conjugates as catchers, and lectins of various sugar specificities (ConA, AAL, SNA) were used for the analysis of total and antigen-specific IgG, respectively. The IgG purification procedure did not influence the IgG interaction with lectins. The combination of different lectins provided additional information which is useful for cancer diagnostics and prognostics. **I, II, IV**

2. Despite the large interindividual variations in IgG-lectin interactions, cancer patients showed a significantly higher level of ConA positive total IgG, anti-TF glycotope specific IgG and a very low level of sialic acid specific SNA lectin binding to anti-TF IgG, irrespective of the stage of the disease. No correlation between the ConA reactivities of the total IgG and anti-TF IgG (or anti-αGal IgG) was observed, which indicates that variations in the total and antigen specific IgG glycosylation do not coincide and occur independently. **I, II, IV**

3. Changes in the SNA binding to anti-TF IgG and especially in the ConA/SNA binding ratio demonstrated a good sensitivity (72.7%) and a high specificity (88.64%) for stomach cancer. **IV**

4. The low level of the ConA and AAL reactivity to anti-TF IgG was associated with a significantly higher survival rate of patients with gastric cancer, particularly of surgically treated stage III cancer patients. No correlation between the reactivities of these two lectins was observed suggesting that despite different sugar specificities these lectins detect some interrelated IgG glycosylation changes that both have prognostic significance. **IV**

5. The LC-ESI-MS results showed that there was a significant increase of the level of agalactosylated IgG glycoforms and a decrease of the level of galactosylated and monosialylated IgG glycoforms in cancer patients. A statistically significant increase of the Fc fucosylation was observed in tumor stages II and III whereas reverse changes were found for the glycoforms with the bisecting GlcNAc with a strong negative correlation between two variables. The age-dependent variations in Fc glycosylation were not responsible for the differences found between cancer patients and controls. No significant correlation between gender and IgG glycosylation changes was observed either. **III**

6. The higher level of fully sialylated and galactosylated glycans and an elevated expression of glycans with the bisecting GlcNAc were associated with a benefit in the survival of cancer patients. **III**

Concluding remarks

The findings of this thesis evidence that changes in the glycosylation profile of total IgG and specifically anti-TF IgG have a potential for stomach cancer diagnosis and prognosis. The lectin-based glycoprofiling of antibodies is an informative and clinically applicable tool to specify sets of total and tumor-associated IgG autoantibody glycoforms which can be used as a biomarker for stomach cancer detection and/or follow-up. It appears that the evaluation of Ab glycoforms levels is more informative than just the whole level of antibodies to the antigen under study, but this approach is not yet wildely used in practical medicine. Since the glycosylation of IgG specific to various antigens may considerably differ from that of total IgG, the focus of further studies should be shifted to IgG antibodies specific to antigens directly involved in the pathogenesis of the disease under study. The higher proportion of the aberrantly glycosylated IgG in patients with gastric cancer observed in this study may be related to the alteration of immunological mechanisms against cancer. The purification and characterization of IgG glycoforms and the study of their functional activity (interaction with Fcγ receptors, complement- and antibody-dependent cellular cytotoxicity to tumor cells, impact on proliferation, apoptosis, *etc*), as well as of Ab glycosylation changes during the therapy are needed to further clarify the role of the structural and functional diversity of IgG glycans in cancer immunosurveillance, tumor progression and the survival of cancer patients.

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ABSTRACT

All human immunoglobulins are glycosylated, each with inherited set of glycoforms, which differ by number, type and oligosaccharide location. The glycan structure of the immunoglobulin G (IgG) Fc fragment strongly influences the affinity for Fcγ receptors and changes in the IgG glycosylation affect many of its functions and are related to several physiological and pathological states, such as infections and autoimmunity. However, little attention has yet been paid to the glycosylation of IgG in cancer, especially of those specific to tumor-associated autoimmunogenic antigens such as the Thomsen-Friedenreich glycotope (TF), which is expressed in a majority of carcinomas. It has been reported that naturally-occurring IgG antibodies to TF may predict a long-term survival of patients with gastric cancer which until now has no reliable diagnostic and prognostic biomarkers. We hypothesize that alterations in the glycosylation profile of the total and antigen-specific IgG in patients with cancer may influence tumor immunity, the disease outcome, and can be of clinical importance for cancer diagnostics and prognosis.

The objective of this thesis was (i) to investigate the glycosylation profile of the total and antigen specific IgG in patients with gastric cancer and controls (healthy blood donors and patients with benign gastric disorders) using lectins of various sugar specificity, (ii) to profile the Fc N glycans of IgG by the liquid chromatography electospray ionization mass spectrometry (LC-ESI-MS) method and (iii) to evaluate whether the putative changes in the IgG glycosylation profile could be used to diagnose and prognosticate of stomach cancer.

The level of TF specific IgG in purified IgG samples was significantly increased in cancer patients compared with that of non-cancer controls ($P<0.001$). Cancer patients showed a higher level of ConA binding ($P<0.05$) and a very low level of sialic acid specific SNA lectin binding ($P<0.001$) to anti-TF IgG, irrespective of the stage of the disease. Changes in the SNA binding index and especially in the ConA/SNA binding ratio demonstrated rather a good sensitivity and specificity for stomach cancer, ranging up to 80%. No correlation between the ConA reactivities of total IgG and anti-TF IgG (or anti-αGal IgG) was observed, which indicates that variations in the glycosylation of total and antigen specific IgG do not coincide and occur independently at least for the two glycotopes tested. The low level of the total IgG ConA reactivity and anti-TF IgG low ConA and AAL reactivity were associated with a significantly higher survival rate of patients with gastric cancer, particularly of surgically treated stage III cancer patients.

Analysis of IgG Fc N-glycans with LC-ESI-MS revealed a significant increase of agalactosylated, and a decrease of galactosylated and monosialylated IgG glycoforms in cancer patients. A statistically significant increase of the Fc fucosylation was observed in tumor stages II and III whereas reverse changes were found for the glycoforms with bisecting GlcNAc. The higher level of sialylated glycans and an elevated expression of glycans with bisecting GlcNAc were associated with a benefit in the survival of cancer patients.

In conclusion, the findings of this thesis evidence that the changes in the glycosylation profile of total IgG and specifically anti-TF IgG have a potential for stomach cancer diagnosis and prognosis. The lectin-based glycoprofiling of antibodies is an informative and clinically applicable tool to specify sets of total and tumor-associated IgG autoantibody glycoforms which can be used as a biomarker for stomach cancer detection and/or follow-up. The higher proportion of an aberrantly glycosylated IgG in patients with cancer observed in this study may be related to the alteration of immunological mechanisms against cancer. Therefore, a further study is needed to evaluate the possible impact of IgG glycosylation pattern on cancer immunosurveillance, as well as tumor progression and the survival of cancer patients.

KOKKUVÕTE

Kõik inimese antikehad on glükosüleeritud neile omase oligosahhariidide komplektiga, mis erinevad hulga, tüübi ja seondumise asukoha poolest. Immunoglobuliini G (IgG) Fc-fragmendis oleva glükaani struktuur mõjutab tugevalt antikeha afiinsust Fcγ-retseptorite suhtes, millest tulenevalt muutub IgG funktsioon. Muutused IgG glükosüleerituses on seotud mitmete füsioloogiliste ja patoloogiliste seisunditega, nagu näiteks infektsioonid ja autoimmuunsus. IgG glükosüleeritusele vähkkasvaja korral ei ole aga kuigipalju tähelepanu pööratud, eriti kasvajaantigeen-spetsiifilisele IgG-le, nagu näiteks Thomsen-Friedenreichi (TF) IgG-le, mis on ekspresseeritud enamiku kasvajate puhul. On andmeid, et loomulikud anti-TF antikehad võivad ennustada haigete elulemust maovähi korral, mille jaoks puuduvad siiani usaldusväärsed diagnostilised ja prognostilised markerid. Arvame, et üldise ja antigeen-spetsiifilise IgG glükosüleerituse muutused võivad mõjutada kasvajavastast immuunsust, haiguse tulemust ja olla kliinilise tähtsusega vähi diagnostikas ja ravitulemuse prognoosimisel.

Käesoleva töö eesmärgiks oli: (i) uurida maovähihaigete ja kontrollgrupi (eeldatavalt terved veredoonorid ja healoomuliste maohaiguste haiged) üldise ja antigeen-spetsiifilise IgG glükosüleeritust, kasutades erineva oligosahhariid-spetsiifilisusega lektiine; (ii) koostada IgG Fc-fragmendi N-glükaanide profiil, kasutades vedelikkromatograafia elektropihustus-ionisatsiooni mass-spektromeetria (LC-ESI-MS) meetodit; (iii) hinnata, kas eeldatavaid IgG glükosüleerituse muutusi oleks võimalik kasutada maovähi diagnostikas ja ravitulemuse prognoosimisel.

Tulemused näitasid, et maovähihaigetel on TF antigeen-spetsiifilise IgG tase puhastatud IgG-s oluliselt kõrgem võrreldes kontrollgrupiga ($P<0,001$). Vähihaigeid iseloomustas kõrgem ConA siduvus ($P<0,05$) ja väga madal siaalhappe-spetsiifilise SNA lektiini siduvus ($P<0,001$) anti-TF IgG-ga, sõltumata haiguse staadiumist. Muutused SNA siduvusindeksis ja eriti ConA/SNA siduvuse suhtes näitasid maovähi puhul üsna head tundlikkust ja spetsiifilisust, ulatudes kuni 80%-ni. Korrelatsiooni üldise IgG ja anti-TF IgG (või anti-αGal IgG) ConA reaktiivsuste vahel ei täheldatud, mis näitab, et üldise ja antigeen-spetsiifilise IgG glükosüleerituse variatsioonid korraga ei esine ning tekivad sõltumatult vähemalt kahe testitud glükotoobi puhul. Üldise IgG ConA seondumise madal tase ning anti-TF IgG ConA ja AAL-i madalam seondumine olid seotud maovähihaigete oluliselt parema elulemusega, eriti kirurgiliselt ravitud III staadiumi vähki põdevatel haigetel.

IgG Fc-fragmendi N-glükaanide analüüs LC-ESI-MS meetodil näitas agalaktosüleeritud glükovormide (G0 glükovormide) olulist tõusu vähipatsientidel ning 1Na ja G2 glükovormide vähenemist võrreldes kontrollgrupiga. Kasvaja II ja III staadiumis täheldati statistiliselt märkimisväärset Fc fukosüleerituse kasvu ning vastupidine trend ilmnes glükovormide osas, mis sisaldasid *bisecting* GlcNAc-i. Lisaks oli kõrgem sialüleeritud ning *bisecting* GlcNAc-ga glükovormide tase seotud haigete parema elulemusega.

Kokkuvõtteks võib antud doktoritöö tulemuste põhjal öelda, et muutused totaalse IgG ja konkreetsemalt TF antigeen-spetsiifilise IgG glükosüleerituse profiilis võivad omada diagnostilist ja prognostilist potentsiaali maovähihaigete ravis. Lektiinil põhinev antikehade glükosüleerituse hindamise meetod on informatiivne ja kliiniliselt kohaldatav täpsustamaks totaalse ja antigeen-spetsiifilise IgG kasvajaga seotud glükovorme, mida oleks võimalik kasutada maovähi biomarkerina kasvaja avastamisel ja/või järelkontrollil. Aberrantselt glükosüleeritud IgG tõus võib olla seotud vähi vastu suunatud immuunmehhanismide muutustega, mistõttu on vaja täiendavaid uuringuid, et hinnata IgG glükosüleerituse võimalikku mõju kasvaja progresseerumisele ja vähihaigete elulemusele.

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PUBLICATION I

An increased level of the Concanavalin A - positive IgG in the serum of patients with gastric cancer as evaluated by a lectin enzyme - linked immunosorbent assay

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An increased level of the Concanavalin A – positive IgG in the serum of patients with gastric cancer as evaluated by a lectin enzyme-linked immunosorbent assay (LELISA)

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All human immunoglobulins are glycosylated. The changes in IgG glycosylation are associated with autoimmune disorders and pregnancy. Little is known about IgG glycosylation in patients with cancer. A lectin enzyme-linked immunosorbent assay (LELISA) based method was developed for measuring the Concanavalin A – positive IgG in the serum. Its rationale is as follows: PtA was used as a capture agent for binding IgG via the Fc fragment. Then IgG and the ConA-positive glycans on the IgG were detected using an anti-human IgG-F(ab)2 alkaline phosphatase conjugate or biotinylated ConA, respectively. The index ConA binding/total IgG was calculated. Serum samples from patients with gastric carcinoma (n=53) and healthy blood transfusion donors (n=24) were analysed. The protein A-agarose and ConA-sepharose affinity chromatography was applied to the purification of IgG, ConA-positive IgG, and Fab fragments. The LELISA, SDS-PAGE and Western blot methods were used to analyse the purified IgG and Fab fragments. A significantly higher ConA binding to IgG was found in patients with cancer compared to that of blood donors (ConA index = 1.07±0.08 (95% CI) and 0.81±0.08, respectively; P=0.0002). In donors, a significant correlation between the level of IgG bound to PtA and the ConA binding (r=0.85; p<0.001) was observed. Patients with gastric cancer showed a less pronounced, though significant correlation (r=0.33; P=0.02). Only the Fd fragment of the Fabs derived from both total serum IgG and ConA-positive fraction of IgG contained the ConA-positive glycans. The comparison of the purified IgG and Fab fragments derived from healthy blood donors and patient with gastric cancer showed no difference in either SDS-PAGE, immunoblotting or LELISA pattern. The LELISA is simple, reproducible and suitable for the evaluation of IgG glycosylation changes. The level of ConA positive serum IgG was found to be increased in patients with cancer. No convincing evidence of the presence of asymmetrically glycosylated $F(ab)_2$ fragments was found. A trend towards a better survival of patients with a lower level of the ConA-positive IgG was observed suggesting a possible blocking effect of the latter on tumor immunity.

Key words: Lectin-ELISA, Concanavalin A, IgG glycosylation; gastric cancer.

Over the last decade, there has been an increasing interest in determining the glycosylation of glycoproteins because of the importance of glycosylation in affecting a wide range of biologically important parameters, such as activity, stability, solubility and biological half-life. The oligosaccharides attached to glycoproteins help orient binding faces, provide protease protection and restrict nonspecific interactions [1,2,3]. In humoral immune system, all immunoglobulins (Igs) are glycosylated [4,5,6,7]. However, the biological role of many glycoconjugates and the diversity of their glycans have no obvious functional relevance yet [1].

An abnormal glycosylation (agalactosylation) of the IgG Fc fragment was found to be characteristic in patients with rheumatoid arthritis that can be used as a diagnostic and prognostic criterion [8,9,10,11]. Alterations in the glycan moieties of IgG have been described in chronic diseases such as inflammatory bowel disease, periodontal disease and infection with HIV [8,11,12,13,14]. An increased proportion of the ConA-positive IgG was found in the serum of patients with ovarian cancer [15]. A higher level of the so-called asymmetrically glycosylated ConA-positive IgG was observed in pregnancy [16,17]. These authors suggested that such asymmetric antibodies (Abs) are

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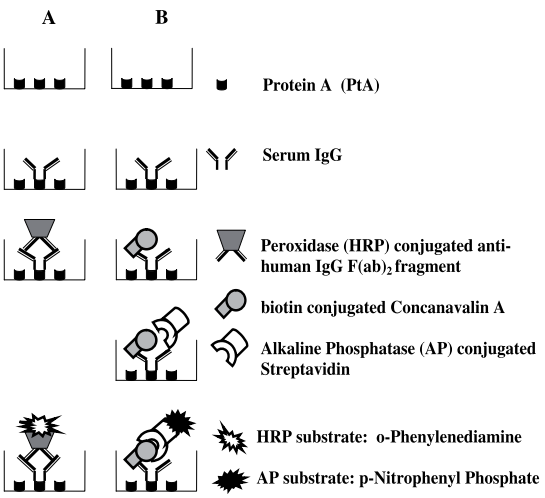

Figure 1. The design of the lectin enzyme-linked immunosorbent assay for the detection of Pt A binded IgG (A) and the ConA binding to the captured IgG (B).

mostly directed to self antigens and may protect from autoimmunity. Changes in the glycosylation of Abs alter their functional and effector functions, including affinity, complement fixation, formation of immune complexes, activation of macrophages, elimination of antigens, or antibody-dependent cellular cytotoxic activity [18,19,20,21].

Thus, many immunological phenomena, such as autoimmunity, inefficiency of antibody-dependent immune reactions to tumor cells or infections, the immunologic protection of pregnancy, might be related to the variations in antibody glycosylation. Little is known about IgG glycosylation in patients with cancer [15,22].

Due to their specificity to particular oligosaccharides lectins are widely used to study the glycosylation of glycoconjugates [23,24,25], including immunoglobulins [10,13,20]. The aim of this study was to develop a simple lectin enzyme-linked immunosorbent assay (LELISA)-based method for monitoring changes in the human IgG glycosylation in healthy individuals and patients with gastric cancer. In the present study, the Concanavalin A (ConA) was used to measure the proportion of the mannose/glucosamine-positive IgG in the serum. A comparative study of healthy blood donors and patients with gastric cancer showed the latter to have a significantly higher proportion of ConA-positive IgG, and patients with a low level of the ConA-positive IgG, a trend towards a better survival.

Material and Methods

Study population and samples. Serum samples were obtained from patients with histologically verified gastric carcinoma (n=53, mean age 60.1±9.4, male/female ratio 1.78) and from healthy blood transfusion donors (n=24; mean age 52.8±7.0, male/female ratio 0.71). Tumor staging was based on the histopathological (pTNM) classification of malignant tumors. Tumor morphology was evaluated according to the classification of P.Lauren [26] as a diffuse or intestinal type of tumor growth. The serum samples were stored in aliquots at -20° C until use.

Sandwich lectin enzyme-linked immunosorbent assay (LELISA). The experimental approach of the assay is outlined in Fig. 1. Protein A (PtA) (Sigma, USA), 100ng/per well) in phosphate buffered saline (PBS) was used to coat two parallel 96-well ELISA plates (Maxisorp, Nunc, Roskilde, Denmark). PtA was used as a specific catcher for binding the serum IgG via the Fc fragment. After incubation overnight at 4° C, a triple washing under agitation (a Tecan Washer, Austria) with PBS-0.05% Tween 20 (PBS-Tw) and blocking with 0.1% BSA in PBS (60 min, 25°C), the serum dilutions from $2.5x10^{-6}$ to $0.01x10^{-6}$ in the PBS-Tw or ConA-binding buffer (0.05M Tris-HCl buffer, pH 7.2, containing 0.2 M NaCl and 3 mM $CaCl_2$, $MgCl_2$ and $MnCl_2$ each) for the determination of the IgG level and ConA binding, respectively, were applied for 1.5 hr at 25° C. After the subsequent washing with PBS-Tw, the bound IgG was detected with horse raddish peroxydase (HRP) conjugated rabbit anti-human IgG $F(ab)_2$ antibody (Dako) and developed with p-nitro-phenyl-phosphate (Sigma) at 405 nm (Tecan Reader, Austria). Alternatively (ConA binding), the wells were incubated with biotinylated ConA (Sigma) (1mg/ml in ConA binding buffer) for 1.5 hr at 25° C. After a triple washing, a streptavidin-alkaline phosphatase conjugate (Dako) was added for 1.5 hr at 25° C and after additional washings the reaction was developed with para-nitrophenyl phosphate (Sigma, 1.0 mg/ml in 0.1M glycine buffer, pH 10.3) for 30 min and stopped with 0.5M H_2SO_4. The absorbance values were registered at 492 nm using a Tecan Reader. An optical density (O.D.) of control wells (0.1% BSA instead of PtA) was subtracted from the values of the serum-coated wells for both IgG and ConA coated wells. Each serum was analysed in duplicate.

To standardise the assay a standard serum was included in each plate for IgG determination and ConA binding measurement. The interassay variations were minimized by using the correction factor (CF= 1:(standard serum O.D. values – blank).The results were expressed in relative units (R.U.): corrected O.D. values x 100. The ConA binding was calculated as a ConA index: ConA (OD-blank) x CF / IgG (OD – blank) x CF.

To evaluate the ConA binding to purified Fab fragments the plates were directly covered with different doses of Fab fragments in PBS and after incubation overnight at 4°C the LELISA steps were performed as described above.

IgG purification on PtA agarose. The purification of polyclonal IgG was performed by affinity chromatography on Protein A-agarose (Sigma) from the serum of a healthy blood donor (male, 65 years old, ConA index -1.0) and from

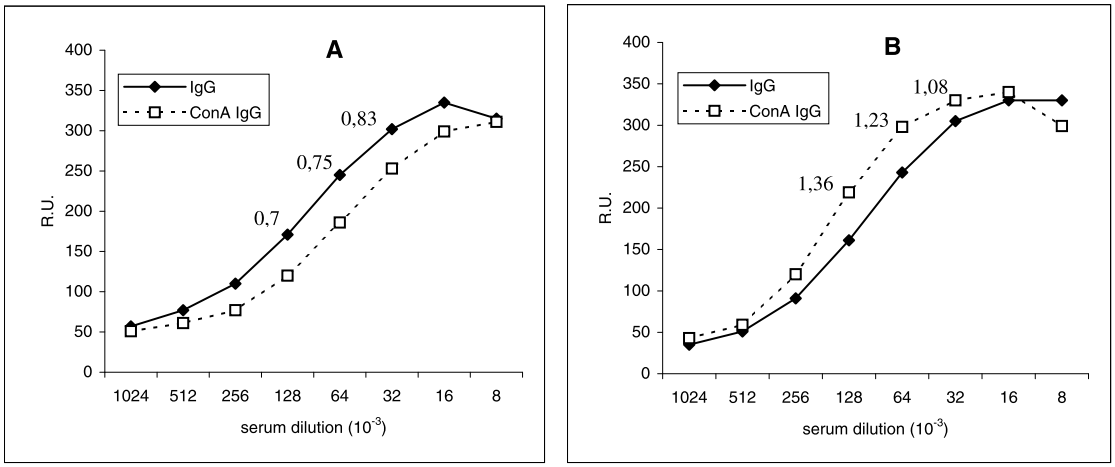

Figure 2. The LELISA of two sera with a low (A) and high (B) ConA/IgG ratio. The serum dilutions from $2.5x10^{-4}$ to $2.0x10^{-6}$ are used. The ConA index values are shown for the serum dilutions corresponding to the dose-dependent part of the curves.

a patient with gastric cancer (a male with gastric cancer of stage 3, 66 yrs old, ConA index -1.2).

After centrifugation (20,000g,10 min) and filtration (0.45µm) the serum samples were dialysed against the loading buffer overnight (50mM TBS, 0.05% Tween 20, pH 7.4) and applied on the column (2.5 ml, at a flow rate of 15ml/hr). The column was washed with 10 vol of the binding buffer. IgG was eluted (0.5 ml fractions) with 5 vol. of the eluting buffer (0.2M glycine, pH 2.5) and immediately neutralized with 1M Tris/HCl, pH 9.0 (100µl per mL of the eluate). The IgG-containing fractions were pooled, dialysed against 0.025M Tris-HCl, pH 7.2 and concentrated using an Amicon Centrifugal Concentrator with a 10-kDa cutoff. The protein concentration was measured by the method of Bradford. The IgG preparations were analysed by SDS-PAGE. The resulting bands were visualized by the Coomassie R-250 staining and molecular weights were determined by the comparison with prestained standards (Pierce) run simultaneously.

Concanavalin A affinity chromatography. The separation of ConA positive and ConA negative purified IgG or Fab fragment fractions (see below) was performed by ConA-Sepharose affinity batch-wise chromatography. The samples were dialysed overnight against 25 mM Tris-HCl (pH 7.2) containing 0.15M NaCl, 3mM $CaCl_2$, 3mM $MgCl_2$ and 3mM $MnCl_2$ (loading buffer), and ~10mg of protein was mixed with an equal volume of 50% Concanavalin A-Sepharose (Sigma,10mg lectin/mL gel) and incubated overnight at 4°C under stirring.

The unbound, normally glycosylated IgG fraction was obtained by centrifugation (1500 rpm 10 min). The gel was washed with a binding buffer twice and the fractions were pooled. The bound IgG was eluted by incubation with 0.1M Tris, 0.2M NaCl, 1M glucose and 3mM $CaCl_2$, $MgCl_2$, $MnCl_2$ each, pH 8.0, for 2 hrs at 4°C under stirring. After centrifugation the samples were dialysed against PBS and in case of need, concentrated as described above. The separation of Fab fragments into ConA positive- and -negative fractions on ConA-Sepharose was performed as described for IgG. The IgG or Fab populations were assayed in LELISA, SDS-PAGE and immunoblotting.

IgG fragmentation and purification of Fab fragments. The two IgG populations were fragmented using the ImmunoPure Fab Preparation Kit (Pierce) according to the instruction of the manufacturer. Briefly: the IgG samples were dialysed against the loading buffer and fragmented by mixing with an immobilized papain and incubation overnight at 37° C under rotation. The Fc fragments and undigested IgG were removed by chromatography on a PtA agarose column as described above.

Polyacrylamide gel electrophoresis (PAGE) and Western blot analysis. The isolated IgG and Fab fragments were analyzed by a standard SDS-PAGE procedure of Laemmli [27]. The samples were dissolved in a sample buffer (2% SDS, 10% glycerol, 5% mercaptoethanol, 0.06 M Tris and 0.025% bromphenol blue), heated at 100°C for 5 min, and subjected to SDS-PAGE using 10% acrylamide gel under reducing conditions using a Hoefer mini VE System. Then the proteins were electrophoretically transferred onto an Immobilon P membrane (Millipore) using a semi-dry blotting system (Hoefer, Amersham). The membranes were incubated with ConA-biotin (Sigma) in a ConA-binding buffer (5µg/ml) for 1.5 hr at room temperature (RT) under shaking. The membranes were washed thrice with TBS-Tw. Then the streptavidin-alkaline phosphatase conjugate (Dako) was added and incubated for 1 hr at RT. After additional washings the bound lectin was devel-

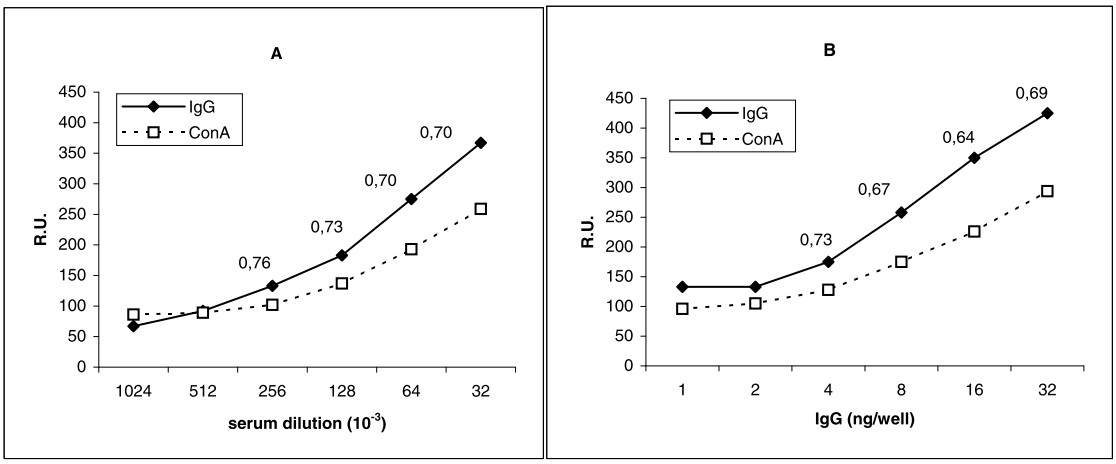

Figure 3. The LELISA titration curves for serum (A) and the IgG (B) purified from the same serum. The ConA index values are shown for a dose-dependent part of the curves.

oped by the addition of a BCIP/NBT reagent (Pierce). The blots were scanned and analysed using the ImageMaster software (Amersham).

Statistical methods

The results were analysed for normality of distribution and comparisons between the groups were performed using the Student's t test and Pearson two-tailed correlation. The survival of cancer patients with a low and high level of the ConA-positive IgG was analysed by the Kaplan-Meier method. Patients with a ConA index value below or equal to the median were classified as low responders. A difference between the groups was considered to be significant when $P \leq 0.05$. All calculations were performed using the GraphPad Prisma 4 software.

Table 1. Changes of ConA index values over time (repeated testing after 2-6 months).

Nr	ConA index 1	ConA index 2	changes (%)	person
1	0.89	0.94	+8.3	donor
2	1.08	1.09	+1.0	donor
3	0.78	0.82	+5.1	donor
4	0.89	0.97	+8.9	donor
5	1.18	1.15	-1.8	donor
6	1.04	0.98	-5.8	chronic gastritis
7	1.0	1.05	+0.5	peptic ulcer
8	1.38	1.03	- 25.4	gastric cancer patient
9	0.64	0.68	+6.2	gastric cancer patient
10	1.2	0.92	- 23.3	gastric cancer patient
11	0.82	0.86	+4.8	gastric cancer patient
12	0.48	0.68	+29.5	gastric cancer patient
13	0.53	0.64	+20.7	gastric cancer patient
14	1.1	0.94	- 6.8	gastric cancer patient
15	0.96	1.21	+26.0	gastric cancer patient

Results

Typical titration curves obtained in LELISA with two sera are presented in Fig 2. The ratio of ConA/IgG for each serum dilution was calculated. The ConA index values did not change appreciably in a dose-dependent part of the curves, for instance, for serum dilutions of 2.5 x 10^{-4} and 5 x 10^{-4}. At these two serum dilutions no significant impact on the ConA index value and a highly significant correlation between the index values calculated at both serum dilutions was observed ($r=0.90$; $n=19$; $P<0.001$). For practical use, the serum dilution equal to 5 x10^{-4} is recommended. Among the sera tested ($n=77$) the O.D. values obtained at this serum dilution were always in the middle part of the IgG titration curves where the dose-dependent linearity is observed and they did not reach the plateau. To minimize the interassay variation, the internal standard serum and arbitrary units (R.U.) were used for the calculation of the ConA index.

The intra-assay variations did not exceed 3.1%. Similarly, variations in the interassay of the donors tested at the interval of 2-6 months were also low in the range of -1.8%/+8.9% (Table 1). More pronounced differences (up to 29%) were observed in patients with cancer tested during the follow-up at the interval of 2-6 months, whereas two patients with chronic gastric diseases (chronic gastritis, peptic ulcer disease) showed fairly stable results (-5.8% and +0.5%, respectively).

No difference in the ConA index values was observed between the males and females ($P>0.1$ for both blood donors and

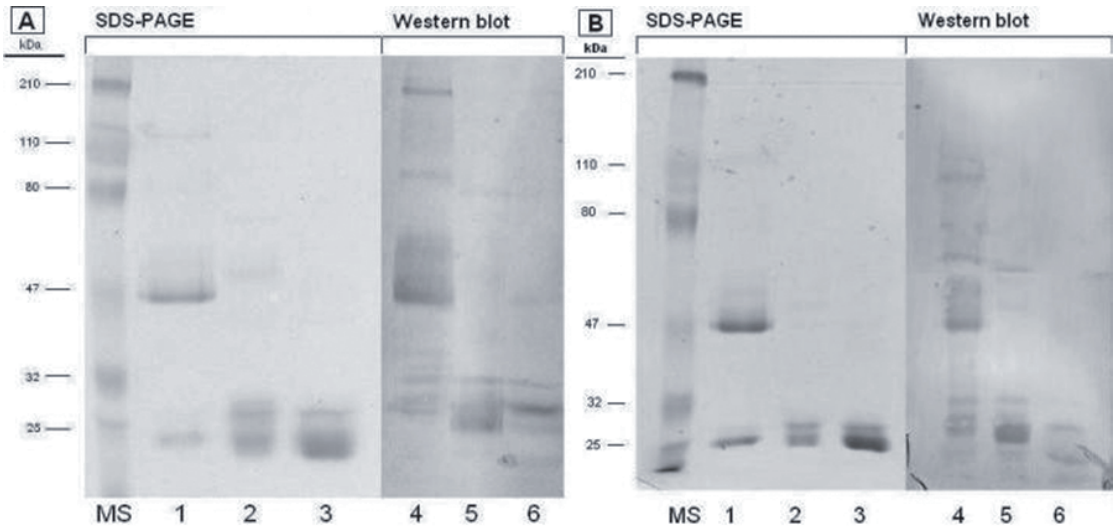

Figure 4. SDS-PAGE (lanes 1-3) and an immunoblot (lanes 4-6) analysis of purified IgG (lanes 1, 4), ConA-positive (lanes 2, 5) and ConA-negative (lanes 3, 6) Fab fragments from the serum of a blood donor (A) and a patient with gastric cancer (B).

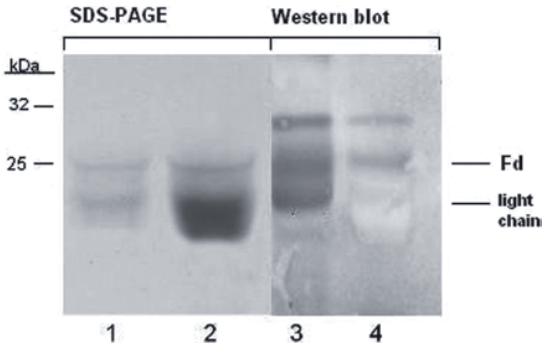

Figure 5. SDS-PAGE (lanes 1, 2) and an immunoblot (lanes 3-4) analysis of ConA-positive (lanes 1, 3) and ConA-negative (lanes 2, 4) Fab fragments purified from a ConA-positive IgG of a donor.

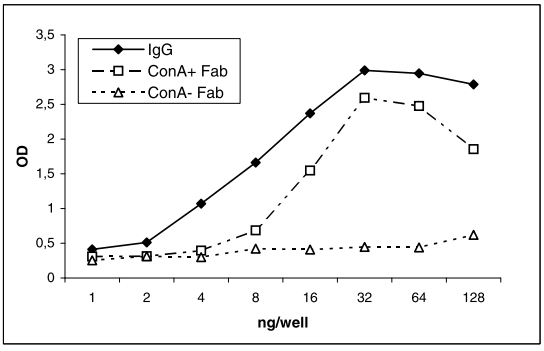

Figure 6. The ConA reactivity of purified IgG and Fab fragments as evaluated by LELISA

cancer patients). Very similar ConA index values were obtained for patients at the age below or above 60 years (P=0.62). Tumor morphology (intestinal and diffuse type of tumor growth by P.Lauren classification) did not influence the ConA index either.

Very similar titration curves were obtained in a parallel ConA-ELISA testing of the serum of a healthy individual and the IgG purified from this serum (Fig.3). The ConA index values in the dose-dependent part of the curves were practically the same and differ by less than 10%.

The SDS-PAG electrophoresis of IgG fractions and immunoblotting data showed that only IgG heavy chains strongly binded the ConA, whereas the light chains did not bind it at all. (Fig. 4 A,B lane 4).The Fab fragments of normal human polyclonal IgG contain the ConA-positive oligosaccharides which are located in the N-terminal half of the heavy chain (Fd fragment, m.w. ~25kDa) for both ConA-positive and ConA-negative Fab fragments, whereas no ConA binding was found on the light chain band (MW ~22kDa) (Fig. 4A, lane 5,6). Similar data were found for the IgG purified from the serum of a patient with gastric cancer (Fig. 4B, lane 5,6). The ConA-positive fraction of the IgG purified from the serum of both a healthy blood donor and patient with gastric

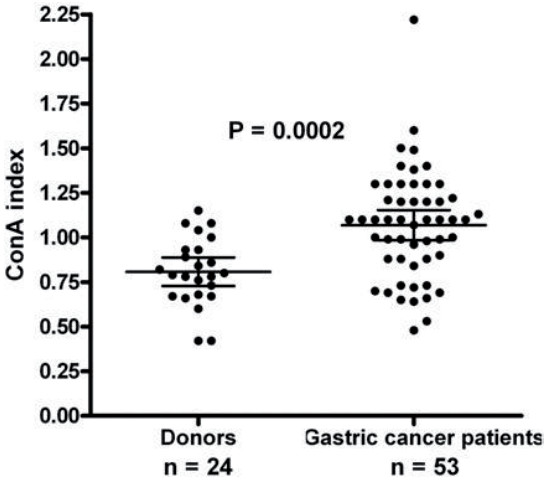

Figure 7. ConA binding patterns as evaluated by LELISA in healthy blood donors and patients with gastric cancer. Based on preliminary experiments, the dilution $5x10^{-4}$ was selected for the calculation of the ConA index. The mean values and the 95% confidence intervals are shown.

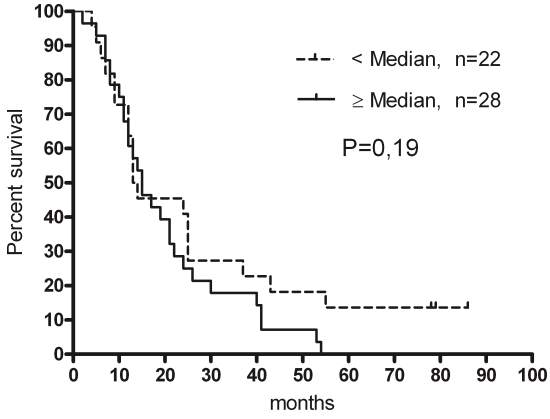

Figure 8. The survival of patients with gastric cancer in relation to the level of the ConA-positive IgG in their serum. The median of the ConA index value was taken as cut-off for groups with a low and high level of the ConA-positive IgG.

cancer contained very little amount of ConA-negative Fab fragments: only ~16 μg of protein was obtained from about 10 mg of purified IgG. The ConA binding to ConA-negative Fab fragments derived from ConA-positive IgG was absent or very weak (Fig. 5, lane 4), possibly due to the presence of a certain amount of the admixture of the ConA-positive material eluted from the ConA-Sepharose together with the ConA-negative fraction

The analysis of the ConA reactivity of Fab fragments preparations by using LELISA showed the results to be similar to those obtained in immunoblotting: the ConA-positive Fabs purified from the ConA-positive IgG of a donor or a patient with cancer revealed a dose-dependent reactivity to the ConA (Fig 6). In contrast, a minimal reactivity was detected for ConA-negative Fab preparations.

The comparison of the LELISA data for both tested groups is shown in Figure 7. In patients with gastric cancer, the ConA index was found to be significantly higher than in healthy individuals (ConA index = 1.07±0.08 and 0.81±0.08, respectively; P=0.0002). In donors, a highly significant correlation was observed between the level of IgG bound to PtA and the ConA binding ($r=0.85$; $p<0.001$). In contrast, patients with gastric cancer showed a much less pronounced, though significant correlation ($r=0.33$; $P=0.02$) because in many patients the relative ConA binding to IgG (ConA index) was appreciably higher.

A trend for a better survival (Fig.8) was found in cancer patients with low ConA index values (lower than the median) compared to those with a higher level of ConA positive IgG (ConA index higher than the median) ($P=0.19$).

Discussion

Many studies have shown the glycosylation of the IgG Fc fragment in autoimmunity to undergo changes [10,11,14,28]. Much less is known about the IgG $F(ab)_2$ fragment glycosylation in healthy individuals and patients with disease. An asymmetry in $F(ab)_2$ glycosylation was proposed for about 20% of the normal serum IgG which revealed the reactivity to the α-D-mannose/ α-D glucosyl-specific ConA lectin. This was considered a mechanism to protect "the self" from the autoimmunity and to escape the fetus from mother's immune attack [16,20,29]. However, the presence of such asymmetry for human IgG has not strongly been proved and has been shown only for the rabbit anti-DNP IgG1 [30] which may otherwise be glycosylated. Besides, other Fab fragments purification methods were used (gel filtration and DEAE-cellulose chromatography). In this study, the purified ConA-positive serum IgG was digested with papain and the purified Fab fragments were tested for ConA reactivity to evaluate whether the ConA-negative Fab fragments are present in the ConA positive IgG fraction.

Our data showed that the Fab fragments obtained from the ConA-positive IgG fraction contain the ConA-positive Fabs, while no appreciable amount of ConA-negative Fabs was found. This suggests that there is no obvious asymmetry in the glycosylation of ConA-positive IgG-$F(ab)_2$ fragments. Moreover, the IgG and Fab preparations from both normal individual and patient with cancer revealed a similar SDS-PAGE and ConA binding pattern (Fig.4 A,B), indicating that

the higher ConA index observed in patients with cancer reflects quantitative rather than qualitative changes in IgG ConA-positive glycans.

The LELISA based approach described in this study showed a good reproducibility in the evaluation of the ConA-positive IgG level in the serum. The comparison of LELISA data obtained with the whole serum and the IgG purified from this serum showed the results to be identical. This indicates that the binding of IgG to PtA in the first step of the assay does not change the ConA binding (Fig.3), suggesting that the data obtained with the serum reflect the real IgG glycosylation pattern. Besides, this indicates that the acid milieu (pH=2.5) used for the elution of IgG from protein A agarose does not alter the IgG glycosylation pattern or change the proportion of the ConA-positive- and -negative IgG. In addition, other ConA-positive glycoconjugates (IgM, IgA, other mannose-rich glycoconjugates) that are abundant in the serum or some interfering components (mannan binding lectin) are excluded from the reaction. This may be important in different pathological conditions when the amount of the mannose positive material in the serum may be appreciably increased.

There are very little data available on the changes of IgG glycosylation in cancer patients. Gercel-Taylor et al. [15] have demonstrated that in patients with ovarian cancer the level of ConA positive IgG in the serum and, especially, in the tumor derived IgG, is increased. This suggests that the serum ConA positive IgG may be of tumor origin. The ConA-binding sites were mostly located in the Fc fragment. In another recent study, Radcliffe et al. [22] reported that the follicular lymphoma cells-derived IgG/IgM immunoglubulins reveal mostly oligomannose structures located in the antigen binding site that may interfere with antigen binding. The present study showed patients with gastric cancer to have a significantly higher ConA reactivity than healthy blood donors. However, it remains unclear to what extent the significantly higher level of ConA-positive IgG found in patients with gastric cancer is related to the glycans of IgG-Fab, -Fc or both fragments. In autoimmune diseases, such as rheumatoid arthritis, the Fc fragment was reported to be predominantly involved (hypogalactosylated) [10]. It has been recently reported that changes in the IgG glycans profile may occur independently in Fab and Fc fragments [31]. Therefore it should be expected that the simultaneous characterization of ConA-binding sites in Fab and Fc fragments might be more informative.

The appreciable changes in the ConA index values observed during the follow-up of patients with cancer, but not in blood donors or patients with benign gastric diseases, suggest that IgG glycosylation patterns may be related to the progression of cancer. However, in repeated testings a majority of patients with low ConA index values had lower ConA index values than those who revealed higher ConA indexes. It appears that the IgG glycosylation patterns are strongly controlled at the individual level and are rather stable in a given individual. A trend to a better survival of patients with a lower level of ConA-positive IgG implies a possible blocking effect of the latter on tumor immunity.

In conclusion, a simple lectin-ELISA based method for the evaluation of the serum IgG glycosylation has been developed. The assay combines in one procedure the IgG purification and the analysis of IgG glycosylation using lectins of various specificity. Due to its sensitivity, the method can be easily performed in testing biological liquids containing a very low amount of IgG, such as saliva, cerebrospinal liquid, cell extracts, and can be used in various clinical applications (autoimmune diseases, infections, cancer). Given the important role of glycosylation in fundamental biological processes [1,3] and the existing data on the role of IgG glycosylation in physiological and pathological states [10,11,16,17,20] it should be expected that the IgG glycosylation pattern may appreciably affect the functional properties of IgG antibodies, thus altering the immunological mechanisms involved in the pathogenesis of many diseases. The higher proportion of an aberrantly glycosylated IgG in patients with cancer observed in this study may be related to the alteration of immunological mechanisms against cancer. A further study is needed to evaluate the possible impact of IgG glycosylation changes on tumor progression and the survival of cancer patients. An investigation of putative alterations in the glycosylation of the IgG antibody specific to tumor-related antigens is now under way.

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PUBLICATION II

The Thomsen-Friedenreich antigen and αGal- specific human IgG glycoforms: Concanavalin A reactivity and relation to survival of cancer patients

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The Thomsen-Friedenreich Antigen and αGal-specific Human IgG Glycoforms: Concanavalin A Reactivity and Relation to Survival of Cancer Patients

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Glycan structures of IgG strongly influence the affinity for Fcγ receptors and antibody effector functions. However, no particular attention has been paid yet to the glycosylation of tumor antigen-specific IgG. The objectives of this study were (i) to investigate the concanavalin A lectin (ConA) reactivity of human anti-Thomsen-Friedenreich (TF) and anti-αGal specific IgG in gastric cancer patients and healthy controls and (ii) to evaluate whether the ConA-reactivity of anti-TF and anti-αGal specific IgG is associated with the survival rate of patients with cancer. Total IgG was purified from the sera of patients with gastric cancer and healthy blood donors. The anti-TF and anti-αGal glycotope specific IgG were detected with ELISA using synthetic saccharide-polyacrylamide conjugates as antigen. In parallel plate, the ConA reactivity of the anti-TF or anti-αGal IgG was determined and the ConA index was calculated. Results show that serum anti-TF specific IgG antibodies of patients with cancer contain significantly higher content of ConA positive IgG glycoform compared to IgG of controls. No correlation between the ConA reactivity of anti-TF IgG and anti-αGal IgG was observed. High level of anti-TF IgG ConA reactivity was associated with a significantly lower survival rate of patients with gastric cancer.

Keywords Tumor immunity, IgG glycosylation, Thomsen-Friedenreich antigen, Alpha-Gal, Concanavalin A, Cancer patients survival, Lectin-ELISA.

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INTRODUCTION

The Thomsen-Friedenreich antigen (TF) is a mucin-type glycotope (Galβ1,3GalNAcα/β-O-Ser/Thr) expressed in many carcinomas due to aberrant glycosylation, which is a common feature of malignant transformation (Hakomori, 1991; Springer, 1997; Karsten et al., 2005). A positive correlation has been found between the expression of TF and aggressiveness of several types of cancer (Baldus et al., 2000; Desai, 2000; Yu, 2007). The presence of natural antibodies (Abs) to tumor-associated antigens, including TF, has been described in patients with various cancers (Springer, 1984; Kurtenkov et al., 1999, 2007; Coronella-Wood and Hersh, 2003; Pavoni et al., 2006; Chapman et al., 2007; Macher and Galili, 2008). An appreciable amount of the anti-TF specific IgM and IgG antibodies are present in normal human serum being significantly decreased in patients with cancer and premalignant conditions though there are large inter-individual variations (Springer, 1984, 1997; Desai, 2000; Kurtenkov et al., 1999).

We reported recently that gastric cancer patients with a higher level of serum anti-TF IgG before surgery showed significantly better survival rate compared to those with low anti-TF IgG level (Kurtenkov et al., 2007). In contrast, the anti-TF IgM antibody level showed no relation to the survival. This is indicative of the significant role of anti-TF IgG antibodies in the immunosurveillance of cancer. However, the anti-tumor potential of tumor specific Abs remains to be further elucidated because the latter may actually have various effects (Fuster and Esko, 2005; Heimburg et al., 2006; Johansson et al., 2008; Tan and Coussens, 2007; Yu et al., 1997) suggesting that these antibodies are heterogeneous functionally and structurally.

Although multiple mechanisms are involved in the anti-tumor activity of tumor specific antibodies such as the activation of apoptosis, complement dependent cytotoxicity (CDC) and antibody dependent cellular cytotoxicity (ADCC), it has been shown that the engagement of Fc gamma receptors on effector cells is a crucial component determining the activity of antibodies against tumors (Clynes et al., 2000; Nimmerjahn and Ravetch, 2007; Strome et al., 2007). There is much evidence that the glycosylation is important for the interaction of IgG with Fc receptors. (Clynes et al., 2000; Dimitrov et al., 2007; Strome et al., 2007) and for antibody effector functions (Arnold et al., 2007; Burton and Dwek, 2006; Raju, 2008). An IgG molecule contains *N*-glycan in the CH(2) domain (Asn297) of the Fc fragment. It consists of a biantennary core of *N*-acetylglucosamine and mannose with added terminal and branching carbohydrate residues such as fucose, sialic acid, and galactose (Arnold et al., 2007; Raju, 2008; Ravetch and Bolland, 2001). It has been reported that the deficiency of even a single terminal carbohydrate of the IgG molecule may drastically change Ab function (Kaneko et al., 2006).

Lectins are widely used ligands to detect different glycans on glycoproteins by various assays such as ELISA and blotting which are more clinically adaptive

tests compared to mass spectrometry-based technologies (Miyamoto, 2006; Routier et al., 1998; Sumar et al., 1990). The concanavalin A lectin (ConA) reveals reactivity to the α-D oligomannose structures, which are usually further galactosylated/sialylated. Removal of the IgG galactose results in an increase of the ConA binding affinity (Baenzieger and Fiete, 1979). Hence the ConA binding to IgG is related to the degree of IgG molecule asialylation/agalactosylation.

The objectives of this study were (i) to determine the ConA reactivity of human anti-TF and anti-αGal glycotope specific IgG antibody in cancer patients and healthy controls and (ii) to find it out whether the clinical outcome of patients with gastric cancer is associated with changes in the ConA reactivity of IgG antibodies to tumor-related glycotopes. We report that serum anti-TF specific IgG antibodies of patients with cancer contain a significantly higher amount of ConA positive IgG glycoform and that the significantly lower survival rate of cancer patients is associated with this increase.

MATERIALS AND METHODS

Subjects

Serum samples were obtained from patients with histologically verified gastric carcinoma and from healthy blood transfusion donors (Table 1). Tumor staging was based on the histopathological (pTNM) classification of malignant tumors. The serum samples were stored in aliquots at –20°C until use.

Serum IgG Purification on Protein G Sepharose

To test the ConA reactivity of the anti-TF and anti-αGal specific IgG by lectin-ELISA a preliminary purification of serum total IgG was performed on Protein G HP Spin Trap column as described by the manufacturer (GE Healthcare). The samples were immediately neutralized, dialyzed against PBS-0.1% NaN_3 and stored at +4°C until tested. About 8.5 mg of IgG was obtained from 1 ml of serum applied onto the Protein G Sepharose column.

Table 1: Characteristics of the subjects tested.

	n	Males	Females	m/f	Median age (range)
Donors	26	6	20	0,3	52.5(27 – 70)
Cancer patients Stage I - IV	64	34	30	1,133	66 (28 – 84)
Stage I	13	6	7	0,857	65 (38 – 81)
Stage II	8	5	3	1,667	65.5(46 – 78)
Stage III	33	15	18	0,833	66 (28 – 81)
Stage IV	10	8	2	4	70 (61 – 84)

Lectin Enzyme-Linked Immunosorbent Assay (LELISA) of IgG

The assay was performed as described elsewhere (Klaamas et al., 2008) with small changes. Briefly, Protein G (Sigma, USA) (100 ng per well) in phosphate buffered saline (PBS) was used to coat two parallel 96-well ELISA plates (Maxisorp, Nunc, Roskilde, Denmark). After incubation overnight at 4°C, a triple washing with PBS-0.05% Tween 20 (PBS-Tw) and blocking with SuperBlock Blocking Buffer (Pierce) for 15 min at 25°C, the purified IgG (20 ng/well) or serum diluted 1: 50 000 in the PBS-Tw were applied for 1.5 hr at 25°C. After the subsequent washing with PBS-Tw, the bound IgG was detected with horseradish peroxidase (HRP) conjugated rabbit anti-human IgG $F(ab)_2$ antibody (Dako), developed with o-phenylenediamine dihydrochloride (Sigma) and stopped with 0.5 M H_2SO_4. The absorbance values were read at 492 nm (Tecan Reader, Austria).

Alternatively (ConA binding), the second plate was incubated with biotinylated ConA (Sigma) (5 μg/ml in ConA binding buffer: 0.05M Tris-HCl buffer, pH 7.2, containing 0.2 M NaCl and 3 mM $CaCl_2$, $MgCl_2$ and $MnCl_2$ each) for 1.5 hr at 25°C. After a triple washing, a streptavidin-alkaline phosphatase conjugate (Dako) was added for 1.5 hr at 25°C and after additional washings the reaction was developed with p-nitrophenylphosphate disodium hexahydrate (Sigma) for 60 min and stopped with 0.5M NaOH. The absorbance values were registered at 405 nm using a Tecan Reader. The optical density value (O.D.) of control wells (blank: PBS-Tw instead of sample) was subtracted from that of the serum-coated wells for both IgG and ConA binding determination. Each sample was analyzed in duplicate.

To standardize the assay a standard serum was included in each plate for IgG determination and measurement of ConA binding. The inter-assay variations were minimized by using the correction factor (CF): CF = 1 / (standard serum O.D. values – blank) x 100. The results were expressed in relative units (R.U.): R.U. = sample O.D. value x CF. The ConA reactivity of IgG was calculated as a ConA index: (sample ConA binding R.U.) / (sample IgG binding R.U.).

ConA Reactivity of Anti-TF and Anti-αGal Specific IgG

The ConA reactivity of anti-TF and anti-αGal glycotope specific IgG was measured in a similar way except TF-polyacrylamide (TF-PAA) and αGal-PAA (Lectinity, Russia) were used to cover plates: both diluted 0.5 μg/well in carbonate buffer, pH 9.2. No cross-reactivity between IgG antibodies to these glycoconjugates was observed (Smorodin et al., 2004). After overnight incubation, triple washing and blocking with Superblock solution (Pierce) for 15 min at 25°C the purified IgG samples (50 μg/well to measure anti-TF IgG and 10 μg/well for anti-αGal IgG, respectively) in PBS-Tw were applied and incubated for 1.5 h at 25°C. Relatively high doses of total IgG were applied because of low concentration of anti-TF and anti-αGal IgG in serum; these IgG doses approximately

correspond to 1:25-1:50 serum dilution used in our previous studies (Kurtenkov et al. 2007). Murine monoclonal anti-TF specific antibodies JAA-F11 of IgG_3 isotype (Rittenhouse-Diakun et al., 1998) were purified on PtG sepharose and tested at concentration of 160 ng/well. After additional washings the alkaline phosphatase conjugated goat anti-human IgG or anti-mouse Ig's-alkaline phosphatase conjugate (Sigma) and ConA-biotin was added to detect bound IgG and ConA, respectively, followed by further steps as described here.

Concanavalin A Affinity Chromatography

The separation of ConA positive and ConA negative fractions of purified IgG or MAb JAA-F11 was performed by ConA-Sepharose affinity batch-wise chromatography (Klaamas et al., 2008). The samples were dialyzed overnight against ConA binding buffer and mixed with in excess 50% Concanavalin A-Sepharose (Sigma, 10 mg lectin/mL gel) and incubated overnight at 4°C under stirring.

The unbound IgG fraction was obtained by centrifugation (at $380 \times g$, 10 min). The gel was washed with a binding buffer twice and the fractions were pooled. The bound IgG was eluted by incubation with 0.1M Tris, 0.2M NaCl, 1M glucose and 3mM $CaCl_2$, $MgCl_2$, $MnCl_2$ each, pH 8.0, for 2 h at 4°C under stirring. After centrifugation the samples were dialyzed against PBS-0.05% NaN_3 and stored in aliquots at 4ºC.

Statistical Methods

The results were analyzed for normality of distribution and comparisons between the groups were performed using the Student's *t*-test and Pearson two-tailed correlation. The survival of cancer patients with a low and high ConA index values for anti-TF and anti-αGal specific IgG was analyzed by the Kaplan-Meier method. Patients with a ConA index value below or equal to the median were classified as low responders. A difference between the groups was considered to be significant when $p \leq 0.05$. All calculations were performed using the GraphPad Prism 4 software.

RESULTS

The ConA Reactivity of Serum IgG and Preliminarily Purified Total IgG

To control whether the purification of IgG may alter its glycosylation and the interaction of IgG glycans with the ConA a group of healthy blood donors (n=15) was tested for the ConA reactivity of the total serum IgG and IgG samples purified previously on PtG sepharose from the same sera. The ConA

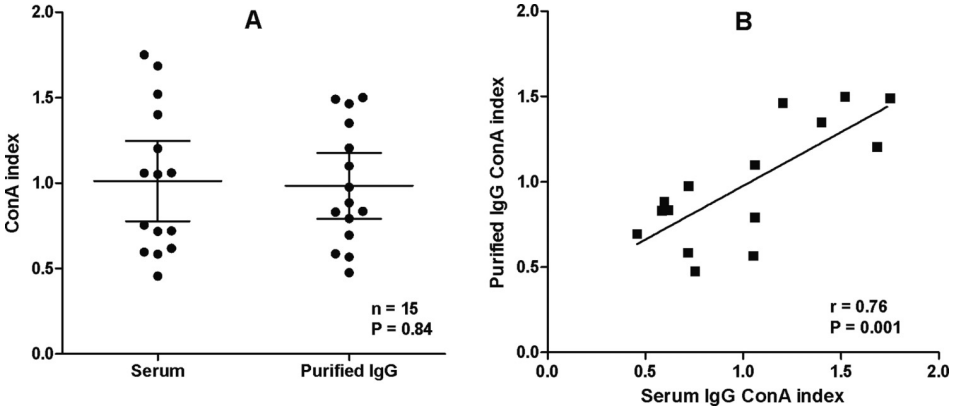

Figure 1: The ConA index values for the serum and purified total IgG.

Protein G covered plates were used in both cases. A - the ConA index values (each dot represents one individual and horizontal lines group mean ± 95% confidence interval). B - the Pearson correlation between the ConA index values for the serum and purified IgG.

ELISA using PtG as catcher molecule in both cases was performed. No difference in the mean ConA index values between the total serum IgG and purified IgG was observed (p = 0.84; Fig. 1A). Also a significant correlation was found to exist between two assays: r= 0.76, p = 0.001 (Fig. 1B). These data show that the purification of IgG on PtG sepharose using the elution at an acid pH of 2.7 does not alter the ability of IgG to bind the ConA.

The ConA Reactivity of Human Anti-TF, Anti-αGal Glycotope Specific IgG and Anti-TF Specific Monoclonal Antibody JAA-F11

The appreciable inter-individual variations in the ConA reactivity of IgG specific to both tumor-related glycotopes were observed (Fig. 2). However, the mean ConA index for anti-TF specific IgG in patients with cancer was found to be significantly higher than that in controls (1.09 ± 0.057 (SE) and 0.82 ± 0.054; p = 0.006). In contrast, the ConA index values for anti-αGal specific IgG in cancer patients and controls were similar (0.56 ± 0.056 and 0.65 ± 0.052, p = 0.26) though a slight trend towards a higher ConA index in cancer patients can be observed. The increase of the ConA index value in patients with cancer was more pronounced at the early stages of the disease for both anti-TF and anti-αGal specific IgG (Table 2). No increase of anti-αGal IgG ConA index values at all was observed in patients with advanced stages of cancer. The ConA index values were found to be similar in males and females.

Compared to anti-TF specific IgG the significantly lower ConA index values were observed for anti-αGal specific IgG in both cancer patients (P < 0.0001) and controls (p = 0.002). (Fig. 2). The anti-TF specific mouse monoclonal antibody JAA-F11 ConA index values were similar to that of blood donors (Fig. 2).

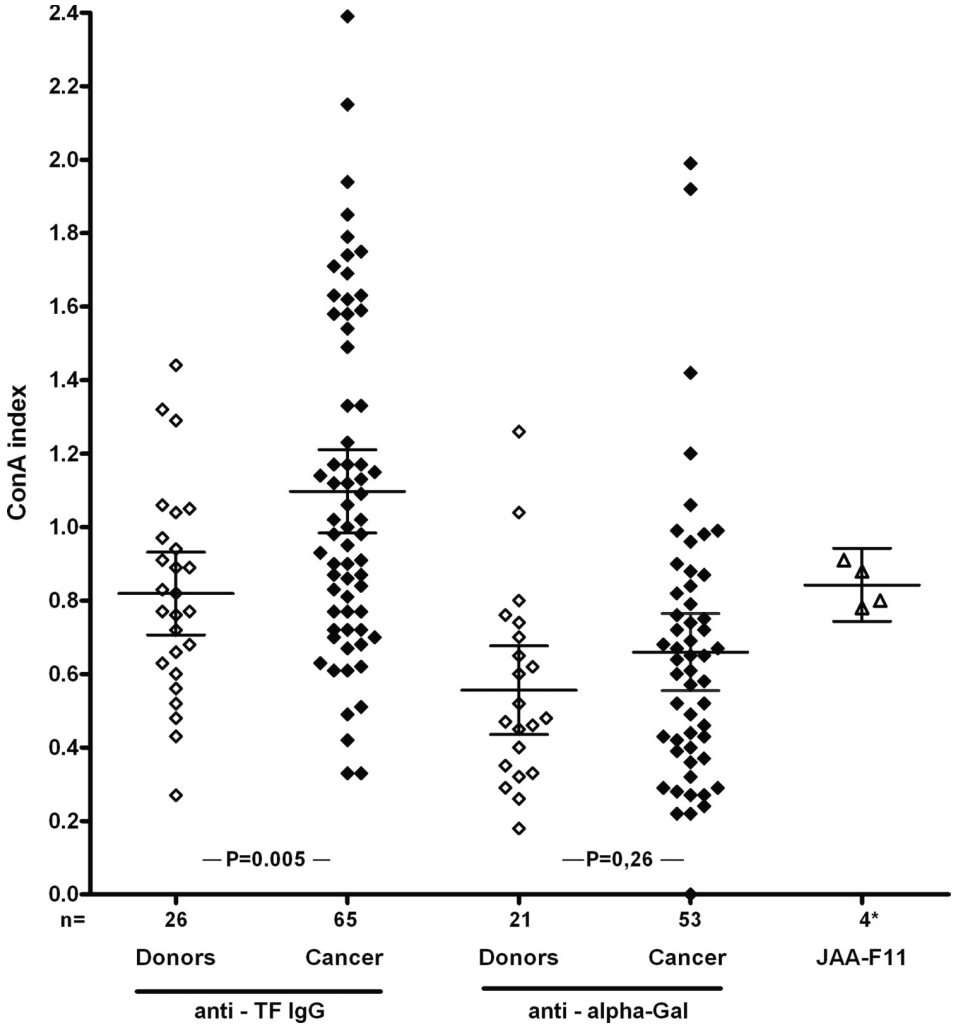

Figure 2: The ConA index values (each dot represents one individual and horizontal lines group mean ± 95% confidence interval) for human anti - TF and anti αGal specific IgG, and anti - TF specific monoclonal antibody JAA-F11. The JAA-F11 Mabs were purified using chromatography on PtG sepharose.

*number of experiments.

About 17.1% (16.2 and 18% for two separate preparations) of JAA-F11 MAb was bound to the ConA-Sepharose. A similar proportion of the ConA positive IgG was found for the total IgG preparation from two healthy individuals (19.6 and 16%). No correlation was found between the ConA reactivity of anti-TF and anti-αGal specific IgG antibodies (Fig. 3) in controls ($r = 0.21$, $n = 20$, $p = 0.37$) whereas a significant correlation between the above reactivities was observed in patients with cancer ($r = 0.591$; $n = 54$; $p < 0.0001$).

Table 2: The ConA index values for anti-TF and anti-αGal IgG in controls and cancer patients by stage.

	Blood donors		Gastric cancer patients			
IgG antibody specificity	n	ConA index mean ± SE	stage	n	ConA index Mean ± SE	P value Donors vs cancer
TF	26	0.819 ± 0.054*	I-II	21	1.323 ± 0.11	< 0.0001
			III-IV	43	0.975 ± 0.059	< 0.0001
			All	64	1.09 ± 0.057	0.006
αGal	21	0.556 ± 0.057	I-II	18	0.88 ± 0.11	0.010
			III-IV	35	0.54 ± 0.043	0.86
			All	53	0.65 ± 0.052	0.26

* Relative units (R.U.).

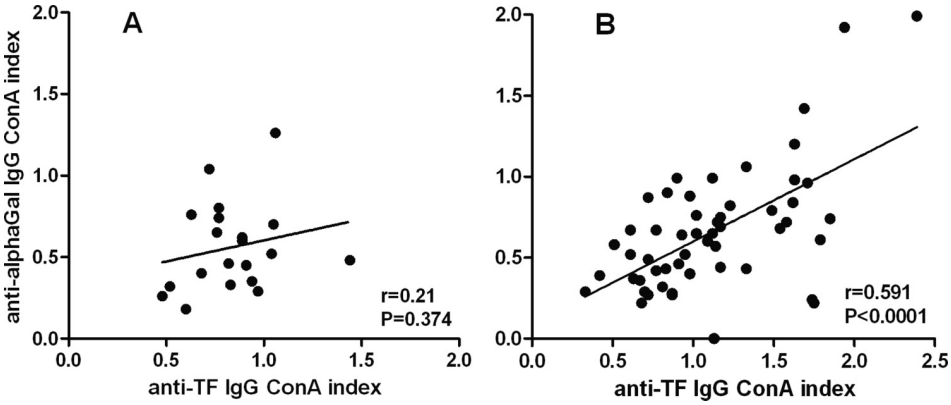

Figure 3: The correlation between the ConA index values for anti - TF and anti - αGal specific IgG in patients with gastric cancer and controls (dots represent individuals). A - healthy blood donors; B - patients with gastric cancer.

The ConA reactivity of the total purified IgG was further correlated with that of anti-TF specific IgG. No correlation was found between these parameters: the "r" value was equal to –0.15 (p = 0.50) for a combined group of donors and cancer patients (n = 23). Similarly, there was no correlation between the ConA reactivity of total serum IgG and anti-αGal specific IgG (r = 0.06, p = 0.79). This suggests that variations in the total and antigen specific IgG glycosylation (ConA reactivity) do not coincide and occurs independently.

The Anti-TF and Anti-αGal Specific IgG ConA Reactivity and the Survival of Patients with Gastric Cancer

The ConA reactivity of anti-TF antigen specific IgG was significantly associated with the survival of cancer patients (Fig. 4A): better survival rate

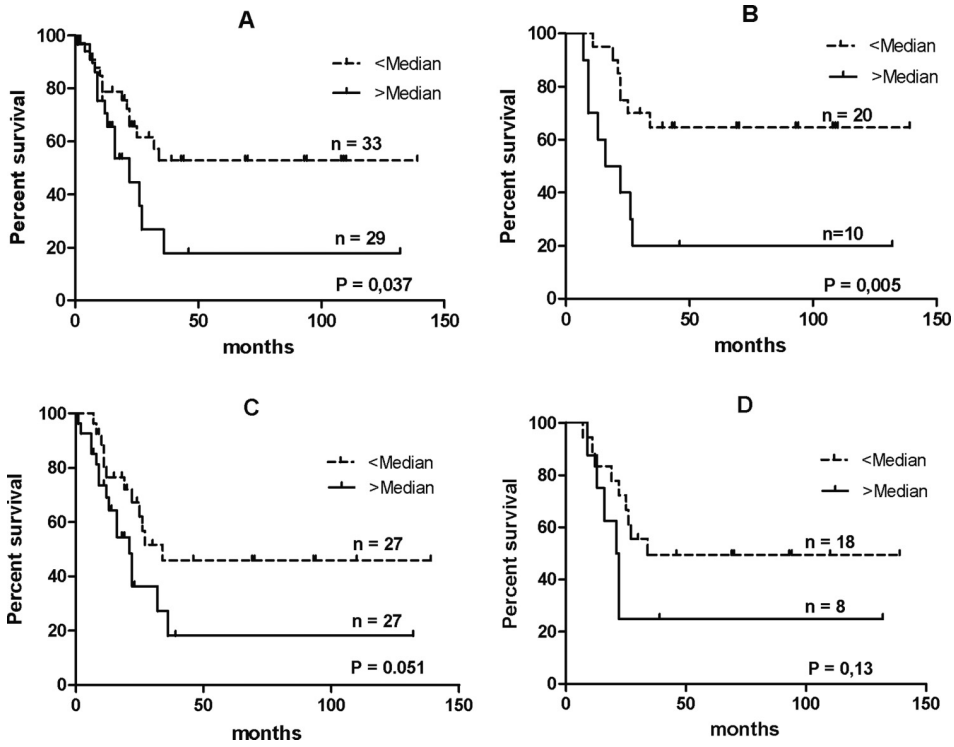

Figure 4: The dependence of the survival of gastric cancer patients on the ConA reactivity of anti- TF (A, B) and anti-αGal (C, D) specific IgG. Patients with ConA index values, which were either lower, equal to or higher than the median are compared.

*A, C - the combined group of patients (stages I-IV); B, D-patients in stage III of the disease.

was observed in patients with low ConA index values ($p = 0.037$, $n = 62$). A more pronounced association with the survival was observed in surgically treated patients in stage III of the disease ($n = 30$; $p = 0.005$; Fig. 4B). In contrast, no significant association ($p > 0.3$) was observed in patients in early stages (I-II) and stage IV of the disease. Thus the significant association of the better survival with the low ConA index value observed in the combined group of patients ($n = 62$) is mostly related to patients in stage III of the disease.

Despite the absence of the difference in anti-αGal IgG ConA reactivity between donors and cancer patients, the trend to a better survival of patients with cancer ($p = 0.051$; $n = 54$) was also associated with the low anti-αGal IgG ConA index (Fig. 4C). However, there was no significant difference in survival rate between patients in stage 3 of the disease with low and high ConA index values ($p = 0.13$) (Fig. 4D).

Even a more pronounced difference in the median survival time was observed if patients whose serum anti-TF IgG ConA index values falling in

the zone of the group mean ± 95% CI (n = 8) were excluded from analysis, i.e. only patients whose serum ConA index values were low and high were involved. The median survival time of patients, whose serum ConA index was low (n = 29), was twice higher than that of the alternative group (34.0 versus 16.0 months; hazard ratio 0.49; 95% CI of 0.14 to 1.15; n = 17, P = 0.089). A similar analysis for anti-αGal IgG showed no difference in the median survival time: 34 and 32 months for patients with low and high ConA index value, respectively (n = 37, p = 0.19). Thus, a more pronounced association of survival with the ConA index value was observed for anti-TF specific IgG antibodies.

DISCUSSION

More than 30 glycoforms have been described for Fc fragment (Asn297) related glycans of IgG purified from the serum indicating that IgG glycans are structurally highly heterogeneous (Dwek et al. 1995; Kobata, 2007; Raju, 2008). The terminal sugars of Fc glycans affect the binding of IgG to the FcRIIIa receptor and C1q, thus influencing Ab effector functions such as ADCC and CDC (Mimura et al., 2001; Raju, 2008). Fc glycans may contain 0, 1, or 2 terminal Gal residues i.e. G0, G1 (galactose on one arm) or G2 glycoforms (Raju, 2008).

The ConA reactivity of anti-TF or anti-αGal specific IgG could not be tested by the serum-based lectin-ELISA without the preliminary purification of IgG due to the presence of the ConA positive IgM antibodies to these glycotopes. The treatment of IgGs with an acidic buffer, pH 2.0, a procedure known to unfold/denaturate the structure of proteins structure, for 1 h has been shown to increase the ability of Ig's to bind to mannose-binding lectin (Terai et al., 2006). We used milder conditions and found no appreciable changes in the ConA reactivity of total serum IgG after its elution from PtG sepharose with an acidic buffer, pH 2.7 (Fig. 1).

Using the lectin-ELISA method we recently showed that the total serum IgG of gastric cancer patients contains a significantly higher amount of ConA positive glycans than the IgG of healthy persons. This shift was associated with a negative effect on the survival of cancer patients (Klaamas et al., 2008). In the present study, a significant increase of the content of ConA positive glycans for anti-TF specific IgG was observed in patients with gastric cancer, contrary to blood donors.

It is to be noted that IgG specific to another tumor-related αGal epitope did not show appreciable changes in the binding of ConA. There were appreciable inter-individual variations in the ConA index values of IgG for both glycotopes in cancer patients and controls. However, the anti-TF IgG ConA index mean value was significantly higher than that of anti-αGal IgG. In addition, no correlation between the ConA reactivity of anti-TF and anti-αGal IgG ConA reactivity was found nor between each of them and the total IgG, suggesting

an independent character of their changes. This indicates that the evaluation of the total IgG glycosylation profile does not reflect the pattern of glycosylation of antigen-specific IgGs at least for two tested glycotopes. This also implies that the glycosylation pattern of Ab against the target antigens involved in the pathogenesis of a specific disease may be more informative than the level of IgG antibodies to a specific antigen. Interestingly, changes in the anti-TF and anti-αGal IgG ConA reactivity were more pronounced at early stages of cancer suggesting that they are not induced by tumor growth *per se* but rather precede tumor development.

The inhibition of the adhesion of circulating TF positive tumor cells or micrometastasis to the endothelium *via* a galectin-3 pathway has been proposed as a mechanism for the anti-metastatic action of anti-TF specific antibodies (Glinsky et al., 2001) including JAA-F11 anti-TF specific MAb (Heimburg et al., 2006; Rittenhouse-Olson et al., 2007). The JAA-F11 MAb is unique among anti-TF specific MAbs because it is an IgG_3 isotype, is highly specific for tumor derived alpha-anomeric TF, and has no tumor growth enhancing effect (Rittenhouse-Diakun et al., 1998; Heimburg et al., 2006). In spite of their low CDC activity and the absence of a proapoptotic effect (Heimburg et al., 2006), the *in vivo* administration of JAA-F11 protects mice against metastases suggesting that the blockade of the tumor cell adhesion or other mechanisms may be involved in this protection (Glinsky et al., 2001; Rittenhouse-Olson, 2007).

In a previous work our group found that the significantly better survival of patients with gastric cancer (Kurtenkov et al., 2007) is associated with the higher level of total serum anti-TF IgG antibodies. The mechanism behind this association remains unclear. It is tempting to assume that in this respect the ConA- positive and ConA-negative anti-TF IgG Ab glycoforms are functionally different. Which anti-TF IgG glycoform may inhibit or potentiate this protection remains to be clarified.

In a previous study (Kurtenkov et al., 2007) we found no association between the level of the serum anti-αGal specific IgG antibodies and survival of gastric cancer patients. However, in this study it was demonstrated that the lower survival rate of cancer patients (P=0.051, Fig. 4B) was associated with the higher ConA reactivity of purified anti-αGal IgG. This indicates that changes in the glycosylation of tumor-specific IgG may be more informative than the determination of just total IgG level. The negative impact of the increased level of ConA-positive anti-TF and anti-αGal specific IgG on the survival established in this study shows that the detection of IgG glycoforms may be of clinical importance in the evaluation of anti-tumor immunity potential and in the prognostics of patients with cancer.

Since the glycosylation pattern of therapeutic Abs may strongly influence the interaction of the latter with Fcγ receptors and Ab effector functions, the optimization of IgG Ab glycosylation is considered to be a promising way to

improve cancer immunotherapy (Dimitrov et al., 2007; Nimmerjahn and Ravetch, 2007; Raju, 2008). Our data show that the ConA reactivity of IgG antibodies specific to tumor-associated TF and αGal glycotope may significantly differ and does not correlate with the binding of ConA to the total serum IgG. However, in patients with cancer, the content of ConA positive glycans of both total IgG (Klaamas et al., 2008) and IgG specific to two tested tumor-related glycotopes tends to be higher.

Another regularity is that the better survival of patients with cancer is associated with the lower content of ConA positive glycans in anti-TF and anti-αGal specific IgG suggesting the important role of glycosylation of IgG in the immunosurveillance of cancer.

In summary, these findings are the first evidence that the ConA reactivity of IgG antibodies to the tumor-associated Thomsen-Friedenreich carbohydrate antigen significantly differs in healthy individuals and patients with gastric cancer. This reactivity varied appreciably between individuals in both cancer patients and controls but higher level of ConA reactive anti-TF specific IgG was associated with a lower survival rate of patients with cancer. This information can be exploited for the structural-based functional study of antibodies to tumor-related glycans to further evaluate the clinical relevance of tumor-specific IgG glycovariants.

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PUBLICATION III

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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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

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

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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

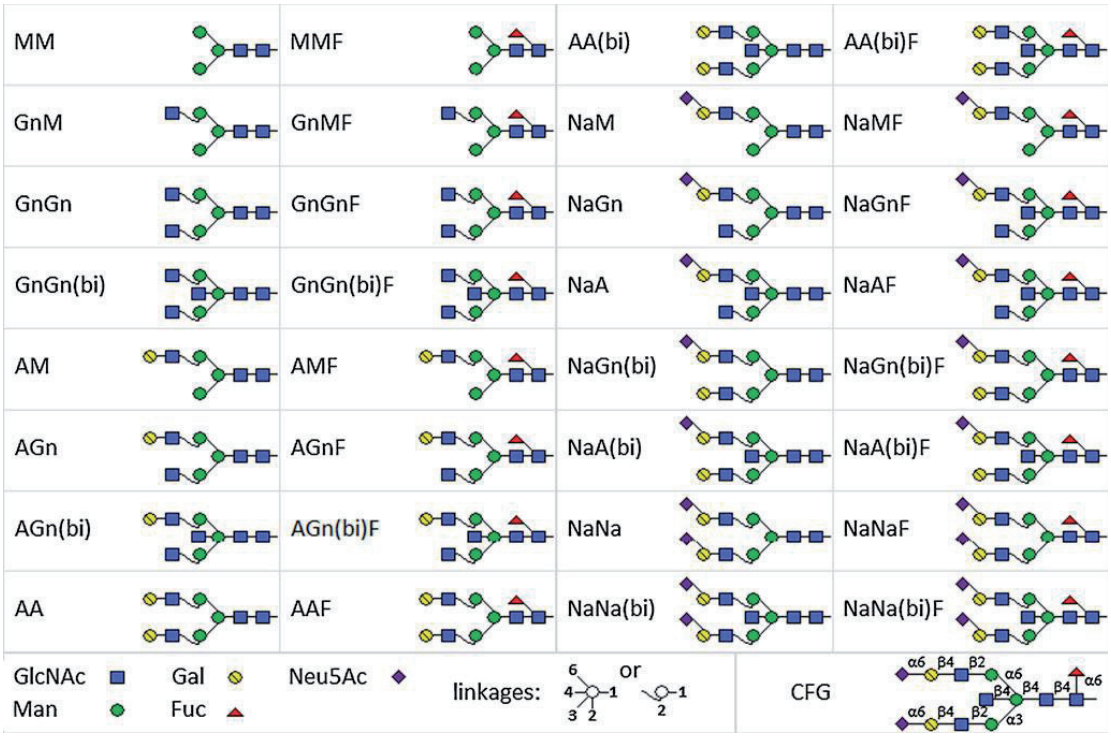

Fig. 1 Structures of human IgG Fc N-glycans dealt with in this study. Glycoforms are named according to the proglycan system [26] and monosaccharide symbols are used as suggested by the Consortium of Functional Glycomics (CFG, www.functionalglycomics.com). In short, as each N-glycan of IgG Fc fragment 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

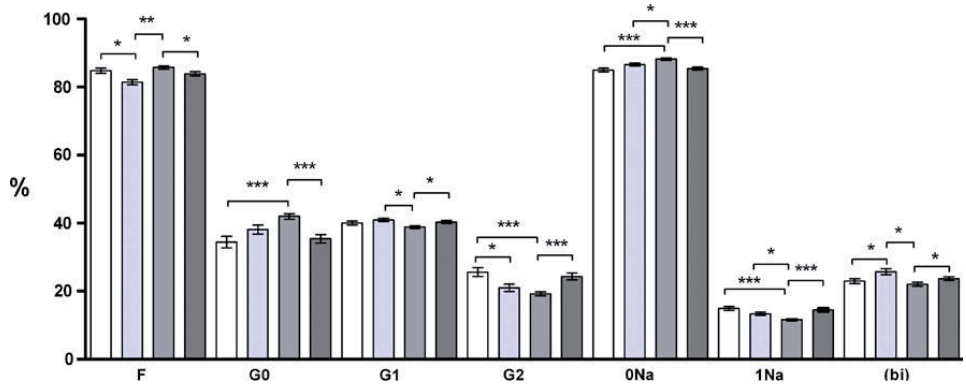

Fig. 2 Glycosylation differences in total IgG Fc fragment between gastric cancer patients and controls. Shown are glycosylation differences between healthy donors (white), patients with benign stomach diseases (light grey), gastric cancer patients (medium grey) and non-cancer group (dark grey) (group 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.

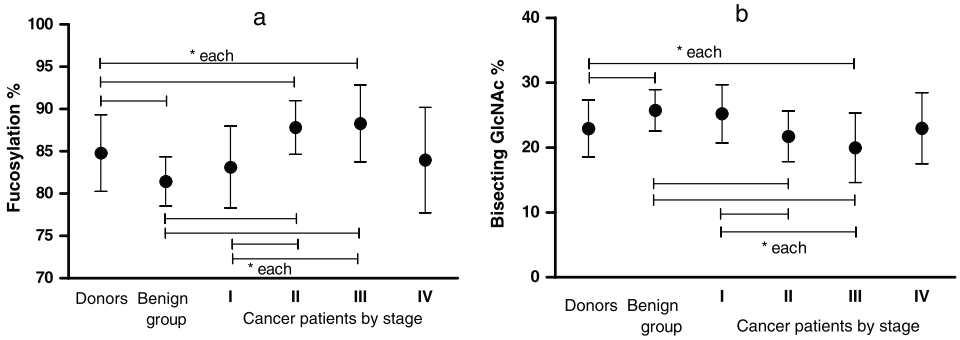

Fig. 3 Differences of IgG N-glycan fucosylation **a** and presence of bisecting GlcNAc **b**. Shown are differences between donors, patients with benign stomach diseases and gastric cancer patients in different stage of the disease (group mean %±SD). * $P \leq 0.05$

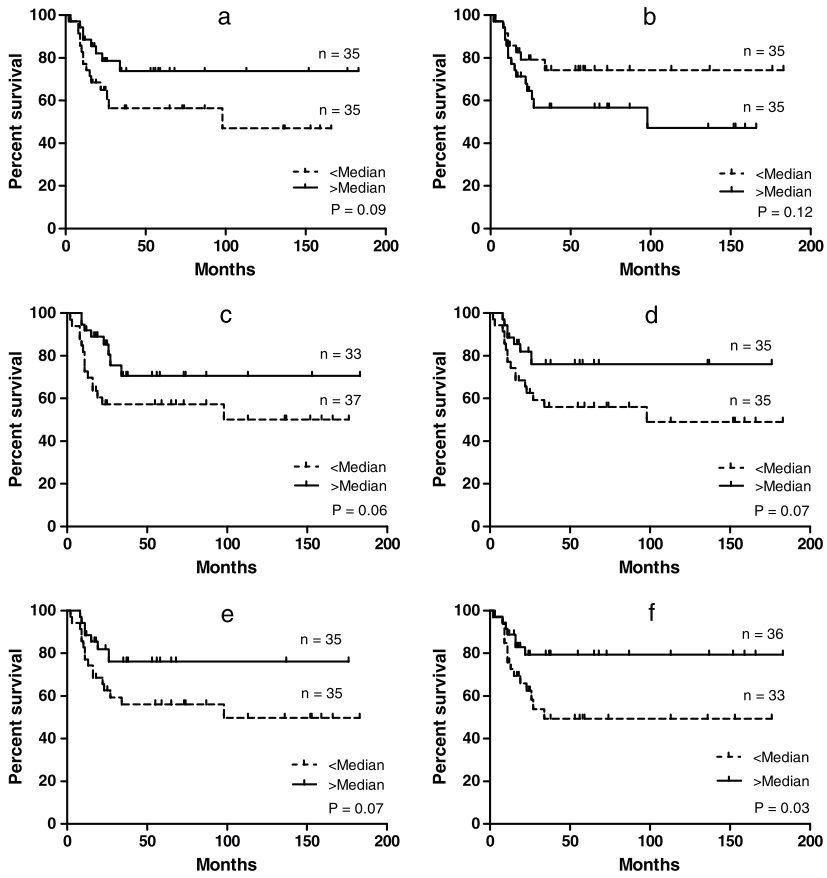

Fig. 4 Probability of survival (Kaplan-Meier) of gastric cancer patients in relation to IgG glycoform levels. Patients with different glycoform levels, which were lower, equal to or higher than the median, are compared. **a**—G2 level, **b**—G0 level, **c**—2Na level, **d**—(bi) level, **e**—level of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

An aberrant glycosylation of the Thomsen-Friedenreich glycotope specific IgG in patients with stomach cancer: the diagnostic and prognostic relevance

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An aberrant glycosylation of the Thomsen-Friedenreich glycotope specific IgG in patients with stomach cancer: the diagnostic and prognostic relevance

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ABSTRACT

The glycosylation of naturally occurring IgG antibodies specific to the Thomsen-Friedenreich antigen (TF) was studied in patients with gastric cancer and controls. Cancer patients showed a higher level of ConA lectin binding ($P<0.05$) and a very low level of SNA lectin binding ($P=0.0001$) to anti-TF IgG. These changes demonstrated a good sensitivity and specificity for stomach cancer, ranging up to 80%. The low level of ConA and AAL bindings were associated with a benefit in patients survival. The results indicate that changes in the TF specific IgG glycosylation can be used as a biomarker for stomach cancer detection and follow-up.

Keywords: TF antigen, anticarbohydrate antibodies, stomach cancer, IgG glycosylation, survival, lectins.

INTRODUCTION

The aberrant glycosylation often observed in cancer cells leads to the expression of tumor-associated carbohydrate antigens (TACA) which may be autoimmunogenic and recognized by autoantibodies[1-9]. This makes TACA a promising target for cancer immunotherapy. In cancer patients, an abnormal glycosylation pattern has also been observed for many circulating glycoconjugates, such as alpha 1-antitrypsin, haptoglobin, transferrin, MUC1 mucin, alpha1-acid-glycoprotein, and immunoglobulins[10-17]. This suggests a systemic impact of malignancy on glycosylation machinery or possibly represents a specific feature of the host metabolism. In both cases, such changes might be considered as a biomarker of cancer, a premalignant state, or the disposition of the host to cancer (risk factors).

The Thomsen-Friedenreich antigen (TF, CD176, core-1) (Galβ1,3GalNAcα/β-O-Ser/Thr) is expressed in many carcinomas due to the incomplete synthesis of O-linked glycans on glycoproteins and glycolipids[1,3]. The TF glycotope is known as a pancarcinoma antigen which is expressed in approximately 90% of all human cancers and in premalignant conditions[3,18]. The TF expression is associated with more aggressive tumors and is related to the induction of invasion, metastasis and cancer surveillance mechanisms[19-23]. The TF antigen seems to play a crucial role in the adhesion of cancer cells to the endothelium through the interaction with galectin-3, thereby promoting metastases[24,25].

Naturally-occurring TF antigen-specific immunoglobulin G (anti-TF IgG) autoantibodies are present in human serum in health and disease[4,26,27]. In cancer patients, their level is related to tumor progression and prognosis, being higher in patients with the early stages of the disease, in those with more differentiated

tumors (G1-2), and in better survivors[19,28,29]. This suggests an immediate impact of the humoral immune response on malignancy via direct or antibody-dependent cell-mediated effector pathways. However, the mechanisms behind these associations remain to be further elucidated.

The human serum IgG contains N-linked glycans attached to Asn297 on the fragment crystallizable (Fc) region. The Fc glycan structures are highly variable and differ in the level of terminal sialic acid, galactose (G0, G1, G2), core fucose and bisecting GlcNAc[30]. Changes in IgG Fc glycosylation strongly influence the Fc-receptor-mediated activities of antibody[30-32] and are associated with various pathologies, including cancer. However, little attention has been paid yet to the glycosylation of antibodies specific to tumor-associated antigens[33]. During the last decade, the diversity of IgG glycans has thoroughly been studied as defined by the interaction of IgG with lectins[33- 36], as well as by the mass spectrometry-based methodology[15,16,37]. Our recent studies demonstrated an increase of the level of the ConA lectin-positive glycoform of both total serum IgG and TF antigen specific IgG in patients with cancer[14,38]. Moreover, the low level of such IgG glycoform was associated with a benefit in an overall survival of patients with gastric cancer[14], indicating its functional relevance, as well as a potential clinical value of the approach. Similar changes in the ConA reactivity have been reported for tumor-reactive IgG in patients with ovarian cancer[33]. However, the antigenic specificity of these antibodies has yet remained unknown.

In an attempt to discover and evaluate potential biomarkers for stomach cancer diagnosis and patients prognosis, the TF antigen specific IgG glycosylation profile was investigated using lectins of various sugar specificities. In this work, we demonstrate the aberrant glycosylation of anti-TF IgG in patients with stomach cancer, and the association of these changes with the overall survival, indicating their potential clinical applicability.

MATERIALS AND METHODS

Subjects

Serum samples were obtained from healthy blood donors, patients with benign stomach diseases and those with histologically verified gastric carcinoma (Table 1). The investigation was carried out in accordance with the ICH GCP Standards and approved by the Tallinn Medical Research Ethics Committee, Estonia. A written informed consent was obtained from each subject.

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*

Groups	n	Males	Females	m/f	Median age (range)
Donors	40	12	28	0.43	52.2 (23–70)
Benign group **	22	19	3	6.3	62.0 (44–76)
Non-cancer***	62	31	31	1	59.5 (23–76)
Cancer patients Stage 1–4	89	53	36	1.47	66.0 (22–84)
Stage 1	18	8	10	0.86	65.0 (28–84)
Stage 2	19	14	5	1.67	66.5 (46–83)
Stage 3	42	22	20	0.83	65.0 (38–81)
Stage 4	10	9	1	9	65.0 (44–76)

*All subjects were tested for anti-TF IgG levels and the ConA binding. AAL and SNA binding test was performed in all donors and patients with benign gastric disease, and in 33 patients with stomach cancer (stage 1, n=4; stage 2, n=5; stage 3, n=19; stage 4, n=5).
** peptic ulcer disease, n=6; chronic gastritis, n=7; atrophic gastritis, n=9.
*** combined group of donors and patients with benign stomach disease.

Serum IgG purification on Protein G Sepharose

To remove the anti-TF IgM and IgA isotype antibodies a preliminary purification of serum total IgG was performed on a Protein G HP Spin Trap column as described by the manufacturer (GE Healthcare, USA). The samples were immediately neutralized, dialysed against PBS-0.1% NaN_3 and stored at +4˚C until tested. About 8.5 mg of IgG was obtained from 1 ml of the serum applied onto the Protein G Sepharose column.

The anti-TF IgG antibody assay

The anti-TF IgG level was detected by the enzyme-linked immunosorbent assay (ELISA) as described elsewhere[28], with minor modifications. Briefly, the plates (Maxisorp, Nunc, Roskilde, Denmark) were coated with a synthetic TF-polyacrylamide conjugate (Lectinity, Russia, 10 mol% of carbohydrate) in the carbonate buffer, pH 9.6, 5 μg per well. After the overnight incubation, triple washing and blocking with a Superblock solution (Pierce, USA) for 15 min at 25ºC, the purified IgG samples (50 μg/well) in PBS-0.05% Tween (Tw) were applied for 1.5 h at 25˚C. After the subsequent washing with PBS-Tw, the

bound anti-TF IgG was detected with the alkaline phosphatase conjugated goat anti-human IgG (Dako, USA) and p-nitrophenylphosphate disodium hexahydrate (Sigma, USA). The absorbance values were read at 492 nm (Tecan Reader, Austria). The relatively high doses of total IgG were applied because of the low concentration of anti-TF IgG in the serum. These IgG doses approximately correspond to the 1:25–1:50 serum dilution used in our previous studies[28].

Lectin reactivity of the TF specific IgG

The lectin reactivity of the TF glycotope specific IgG was measured in a similar way, except that the bindings of the mannose specific Concanavalin A (ConA), the fucose specific *Aleuria aurantia* lectin (AAL) and the neuraminic acid (sialic acid) specific *Sambucus nigra* agglutinin (SNA) to the absorbed anti-TF IgG were measured as described by Kodar *et al*., 2009[14]. The biotinylated ConA (Sigma, USA) in the ConA binding buffer (0.05M Tris-HCl buffer, pH 7.2, containing 0.2 M NaCl and 3 mM $CaCl_2$, $MgCl_2$ and $MnCl_2$ each), AAL (Vector Laboratories Inc, USA) in 10 mM Hepes, 0.15M NaCl buffer, pH 7.5 and SNA (Vector Laboratories Inc, USA) in 10 mM Hepes, 0.15M NaCl, 0.1 mM $CaCl_2$, pH 7.5, were applied at a concentration of 5 μg/ml each, for 1.5 h at 25˚C. The bound lectins were detected with a streptavidin-alkaline phosphatase conjugate (Dako, USA) and p-nitrophenylphosphate (Sigma, USA). The optical density value (O.D.) of control wells (blank: the Superblock solution instead of TF-PAA for anti-TF or no sample for lectin binding testing) was subtracted from that of the IgG-coated wells to determine the binding of both IgG and lectin. Each sample was analysed in duplicate.

To standardize the assay the standard IgG was included in each plate for IgG determination and lectin binding measurement. The interassay variations were minimized by using the correction factor (CF): CF = 1 / (standard serum O.D. values – blank) x 100. The results were expressed in relative units (R.U.): R.U. = sample O.D. value x CF. The lectin reactivity of anti-TF IgG was calculated as the lectin index: (sample lectin binding R.U.) / (sample anti-TF IgG binding R.U.).

Statistical analysis

Comparisons between the groups were performed using the nonparametric Mann-Whitney U test for unpaired data or regression analysis with the Spearman test. Survival analysis was carried out using the Kaplan-Meier method. The receiver operator characteristic (ROC) curve analysis as generated in SPSS 15.0 was used to evaluate the sensitivity and specificity of the changes found for stomach cancer. The area under the ROC curve and the P value of the ROC curve were calculated. The time-dependent ROC curve statistics were

applied to determine cut-off values for survival analysis. The difference between the groups was considered to be significant when $P \leq 0.05$. All calculations were performed using the GraphPad Prism 5 and SPSS 15.0 software.

RESULTS

TF antigen specific IgG antibody level in total IgG preparations

The significantly higher level of TF specific IgG in purified total IgG preparations from the serum of patients with stomach cancer was found compared with that in controls: P=0.002, 0.0003 and 0.00004 for donors, the benign or combined non-cancer group, respectively (Fig 1). This increase was mostly pronounced in stage 1 of the disease (P=0.02, P=0.0006, P=0.01 compared to stages 2, 3 and 4, respectively). Up to tenfold interindividual variations in anti-TF IgG antibody levels were observed in all the groups subjected to study especially in cancer patients.

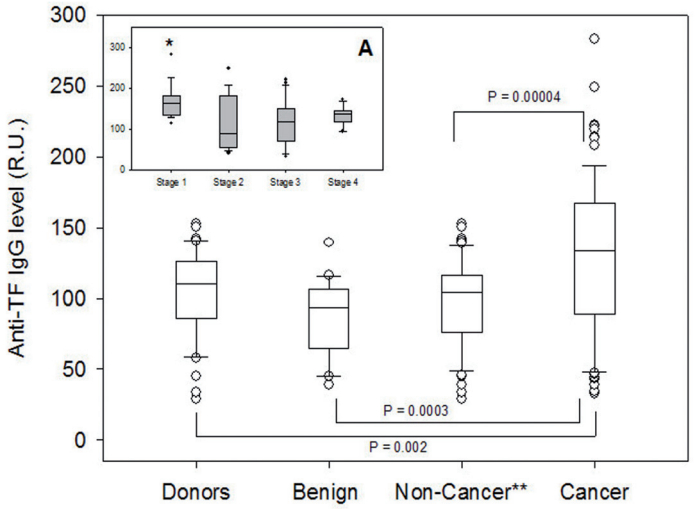

Figure 1. The TF antigen specific IgG level in patients with stomach cancer and controls

The box plots of anti-TF IgG levels (medians, ranges and quartiles) in controls and cancer patients by stage of the disease. A – anti-TF IgG levels in cancer patients by stage. *significantly higher compared to stages 2, 3 and 4 (P=0.02; 0.001 and 0.01, respectively); **combined group of donors and patients with benign stomach disease. P values were calculated by the Mann-Whitney U test.

Lectin binding profile of TF specific IgG

The anti-TF IgG of patients with cancer showed a significantly higher level of the ConA positive IgG glycoform than that of both controls: P=0.013, 0.05 and 0.005 for donors, the benign and non-cancer group, respectively (Fig 2 and 3). In contrast, the binding of SNA was significantly lower in cancer patients compared to that of blood donors and patients with benign gastric diseases (P<0.0001). In cancer patients, the binding of AAL did not differ from that of the donors group (P=0.64), being, however, significantly lower than that of the benign (P=0.000008) or non-cancer group (P=0.005). A group of patients with chronic gastritis (n=7, one of them with atrophic gastritis), who showed a very high AAL index values, were responsible for this difference. This was the only exception in this study when the benign group differed significantly from donors (P=0.01). Two of these patients also showed a high level of SNA binding. All patients with the peptic ulcer disease (n=6) demonstrated the level of binding similar to that of blood donors, for all three lectins.

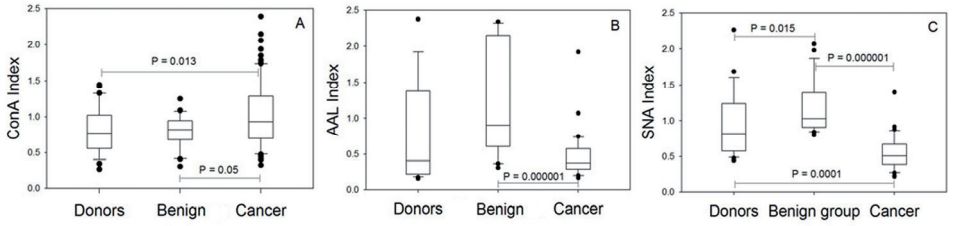

Figure 2. The binding of lectins to the TF specific IgG in gastric cancer patients and controls

The box plots of lectin index values (medians, ranges and quartiles) in patients with stomach cancer, healthy blood donors and patients with benign stomach disease. A: ConA – Concanavalin A; B: AAL – *Aleuria aurantia* lectin; C: SNA – *Sambucus nigra* agglutinin. P values were calculated by the Mann-Whitney U test and are shown for significant differences.

No appreciable stage-dependency of the binding of any lectin to the anti-TF IgG was found (Fig 3), though a slight trend towards higher ConA index values in stage 1 cancer patients was observed (P=0.19 compared to stage 3 patients).

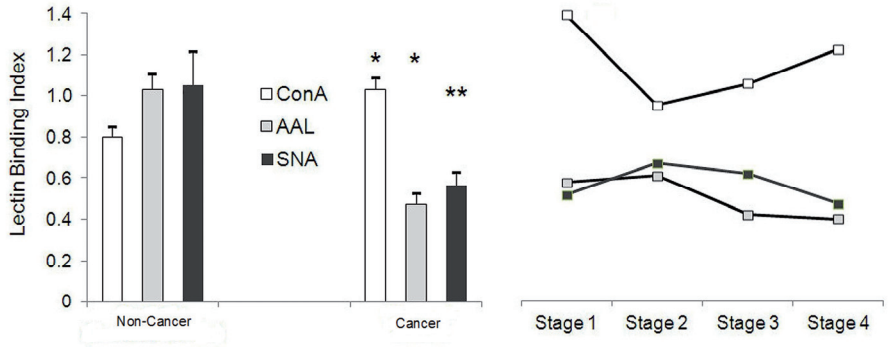

Figure 3. The anti-TF IgG glycosylation profile in cancer and non-cancer groups: relation to the stage of cancer
The results are depicted as a mean with error bars representing a standard error of the mean (SEM). P values were calculated by the Mann-Whitney U test: *P=0.005; ** P<0.0001, compared to controls.

A strong positive correlation between the reactivities of AAL and SNA was demonstrated in all groups: cancer patients (r=0.72; P<0.0001); the non-cancer group (r=0.71; P<0.0001) as well as the combined group of all the tested subjects (r=0.72, P<0.0001). No significant correlation between the reactivities of ConA and two other lectins was observed. Thus, the changes in the ConA reactivity were not related to the modification of anti-TF IgG binding sites for the fucose- or sialic acid-specific lectins (AAL and SNA).

Anti-TF IgG level and lectin binding profile: sensitivity and specificity in stomach cancer

The receiver operator characteristic (ROC) curves analysis was used to evaluate the changes of the level and glycosylation profile of anti-TF-IgG as possible biomarkers. The diagnostic accuracy and ROC curves statistics are presented in Fig 4 and Table 2. Coming of the absence of the correlation between the data on the binding of ConA and two other lectins we investigated a possible interactive effect of lectin combinations using the ratios of ConA/SNA, ConA/AAL and AAL/SNA in the ROC analysis.

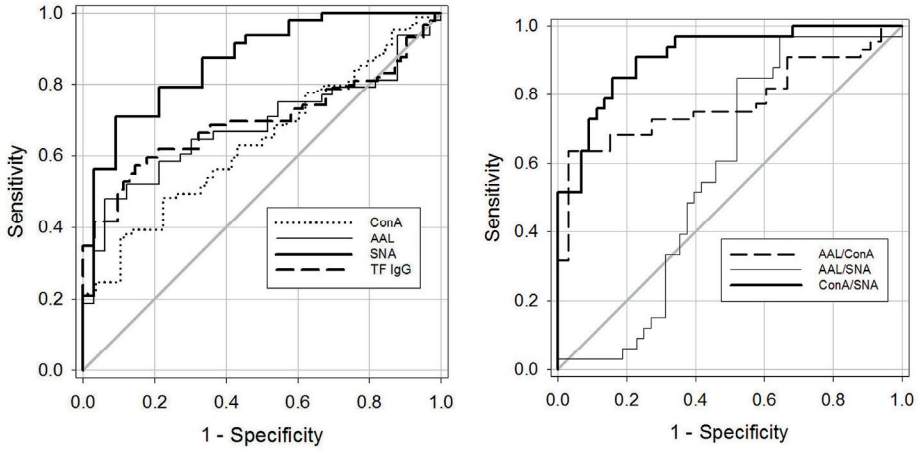

Figure 4. The sensitivity and specificity of anti-TF specific IgG glycosylation changes in stomach cancer
Reciever operator characteristic (ROC) curve analysis.

Despite the significant difference in anti-TF IgG levels between cancer patients and controls these changes showed a low sensitivity and specificity for cancer, possibly due to great variations within each group. The same was true for the ConA and AAL lectin index values. In contrast, changes in the SNA binding index and, especially, ConA/SNA ratio values demonstrated rather a good sensitivity and specificity reaching 78.8–88.6% (Table 2). Since no notable cancer stage dependency of lectin binding was observed, the sensitivity and specificity values are presented for the combined cancer group and non-cancer controls (Fig 4 and Table 2). Using the combination of ConA/AAL and AAL/SNA lectin ratios did not improve the accuracy of the assay showing lower sensitivity and specificity values, usually below 70% (Table 2).

Table 2. Anti-TF IgG level and lectin binding profile: sensitivity and specificity for gastric cancer

Parameter	Sensitivity % (95% CI)	Specificity % (95% CI)	ROC statistics		Sensitivity at Specificity 90%
			Area under curve	P value	
Anti-TF IgG	65.17 (51.34% –76.26%)	67.74 (56.66% –76.98%)	0.70	<0.0001	50.56
ConA	62.92 (48.37% –74.49%)	56.90 (46.37% –67.74%)	0.64	0.004	24.72
SNA	78.79 (61.09% –91.02%)	79.17 (65.01% –89.53%)	0.87	<0.0001	57.58
AAL	69.70 (51.29% –84.41%)	64.58 (49.46% –77.84%)	0.68	0.006	12.12
ConA/SNA	72.73 (57.21% –85.04%)	88.64 (71.80% –96.60%)	0.91	<0.0001	63.64
AAL/ConA	84.85 (68.10% –94.89%)	68.18 (52.42% –81.39%)	0.78	<0.0001	33.33
AAL/SNA	84.85 (72.24% –93.93%)	47.92 (30.80% –66.46%)	0.68	0.006	3.03

Lectin binding of TF specific IgG and the survival of cancer patients

The subgroups of cancer patients with high and low levels of lectin binding to anti-TF IgG were compared. The cut-off levels were calculated using the time-dependent ROC curves analysis for each lectin. Despite the opposite changes in the binding of ConA and AAL or SNA lectin in cancer patients (increase *vs* decrease) a similar association with survival was demonstrated for all the three lectins tested, with a common trend towards the early and advanced stages of the disease (Fig 5).

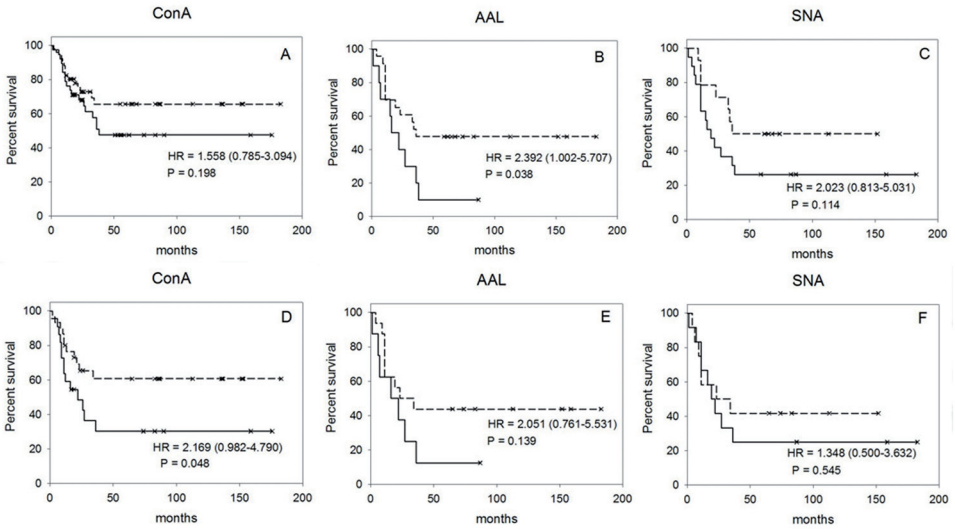

Figure 5. The binding of lectins to the TF specific IgG and the survival of patients with stomach cancer

The probability of survival (the Kaplan-Meier method) of stomach cancer patients in relation to lectin binding to TF specific IgG. Patients' lectin index values, which were either lower, equal to (a dashed line) or higher than cut-off (a solid line), are compared. The cut-off levels were calculated using time-dependent ROC curves analysis. ConA - Concanavalin A; AAL – *Aleuria aurantia* lectin; SNA – *Sambucus nigra* agglutinin. A, B, C – all cancer patients; D, E, F – patients in stages 3–4 of the disease

The low level of the ConA positive anti-TF IgG was associated with a benefit in the survival of cancer patients (Hazard Ratio (HR)=1.56 (95% CI 0.78–3.09); P=0.19), especially in those with stages 3-4 of the disease (HR=2.17 (95% CI 0.98–4.79), P=0.048). A significantly better survival rate was found in patients with a low reactivity of anti-TF IgG to the fucose specific AAL lectin: HR=2.39, 95% CI (1.0–5.7); P= 0.038, for all cancer patients. The relation of the SNA lectin reactivity to survival showed a similar trend. Considering that no correlation between the bindings of ConA and two other lectins was found, a possible interactive effect of the combination of two lectins was investigated using the ratios of ConA/SNA, ConA/AAL and AAL/SNA. However, no additional information regarding association with survival was obtained (data not shown).

DISCUSSION

The role of autoantibodies to tumor-associated glycans in cancer surveillance has been mostly considered for IgM[3,5]. These antibodies are not affinity-matured which speaks in favour of their inherent natural origin. In contrast, the presence of IgG anti-glycan antibodies suggests an adaptive immune response. The origin of anti-glycan autoantibodies of both isotypes is still unclear though evidence available suggests that at least anti-TF and anti-alpha Gal antigen specific antibodies may be induced by bacterial glycans or, possibly, by the cross-reactivity with these antigens[3,39]. In any case, great and yet unexplained interindividual variations in their level in health and disease exist[28,29], possibly reflecting distinct immunological histories of each individual. Moreover, the anti-TF IgG level is rather stable over time at an individual level in both patients and controls[29,40].

In this study, a significantly higher level of TF specific IgG in purified total IgG preparations from the serum of patients with stomach cancer than in both control groups was observed. This increase was mostly pronounced in stage 1 of the disease, suggesting that an adaptive immune response can not be excluded in the early stages of tumor with the following decrease of the anti-TF IgG level in advanced cancer as a result of the tumor-induced immunodepression. If this is the case, the population of anti-TF IgG should be expected heterogeneous and includes both naturally-occurring TF glycotope specific antibodies, whose presence precedes tumor development, and those triggered by the disease, i.e. induced by the tumor-derived TF glycotope. Due to great interindividual variations of its anti-TF IgG level, the human population may be divided into low and strong responders to the TF glycotope. Notably, donors and the benign group showed a more compact distribution. A further characterization of anti-TF IgG subpopulations is needed to specify their structural and functional diversities, and clinical significance.

A significantly higher binding of ConA (P=0.005) and a highly significant (P=0.00000003) decrease in SNA lectin binding was characteristic of the anti-TF IgG from the samples of cancer patients compared to the non-cancer group (Fig 3). The ConA binding index was higher in stage 1 patients, whereas the SNA and AAL lectin binding index values were low irrespective of the disease stage. We previously found similar changes in the binding of ConA to the total IgG from the serum of patients with gastric cancer.

The changes in anti-TF IgG glycosylation showed rather a high level of sensitivity and specificity in cancer and non-cancer group discrimination (Table 2). It appears that the SNA binding index and, especially, the ConA/SNA ratio are promising as diagnostic markers to differentiate stomach cancer from

controls, including benign gastric diseases. As there are no reliable markers for gastric cancer yet, these findings may be of clinical value.

We reported recently that the level of agalactosylated IgG (G0 glycoform) in the total IgG of gastric cancer patients was significantly higher than that of controls [15]. Interestingly enough, this shift positively correlated with the binding of ConA to the total serum IgG, and was also observed in purified TF specific IgG samples (unpublished data), indicating that the IgG asialylation /agalactosylation is associated with an increased ConA binding possibly due to a better accessibility of the d-mannose residue to the ConA because of conformational changes in Fc G0 glycoform. The absence of the correlation between the bindings of ConA and SNA may be a reason for a positive interactive effect of using the ConA/SNA binding ratios for cancer *vs* non-cancer group discrimination.

Despite the opposite changes in the binding of ConA and two other lectins to anti-TF IgG in patients with cancer (Fig 2), a better survival rate was related to the lower reactivity of anti-TF IgG to AAL and ConA. The SNA reactivity revealed no significant association with survival, though a similar slight trend was observed. It seems that IgG desialylation alone is not sufficient for the impact on survival and further degalactosylation is needed to attain this effect. In this study, the patients were subjected to follow-up for more than 10 years. The association of the binding of ConA and AAL with survival became evident after 2.5–3 years of observation, reaching maximum after 5 years.

Several studies have demonstrated that agalactosylated IgGs show an increased inflammatory activity[41-44] that may promote tumor growth[45,46]. In addition, the IgG_1 lacking the branching fucose or with an additional bisecting GlcNAc shows an enhanced ADCC activity through an increased interaction with Fc gamma receptors[43,47-49]. The association of the high level of the G0 IgG glycoform with a lower survival rate we reported recently[15] and the decreased anti-TF IgG fucosylation resulting in a better survival we established by the present findings may be related to these mechanisms.

It has been shown that sialylated glycans predominate in glycosylated antibody binding fragments (Fab) whereas the Fc fragment N-glycans are mostly monosialylated[37,50]. Our findings can not answer a question of whether the Fc or Fab fragment sialylation is responsible for the changes observed in anti-TF IgG glycosylation.

In addition, it remains yet unclear whether changes in the sialylation and core fucosylation of anti-TF IgG glycans in both IgG fragments may be independent or concordant events, despite the positive correlation observed between the bindings of SNA and AAL lectin to the whole molecule of the TF specific IgG. Given that an active immunization reduces the sialylation of IgG, especially the antigen-driven IgG[43], we hypothesize that the decreased sialylation of anti-TF

IgG observed in cancer patients may be an indicator of an adaptive immune response to tumor-derived TF antigen, in addition to naturally-occurring anti-TF IgG antibodies, which are present in every individual in different amounts.
In conclusion, the results show the glycosylation of TF specific IgG antibodies in patients with gastric cancer to undergo significant changes when compared to that of controls. The appearance of these alterations already in the early stages of cancer and their association with survival suggest that they play a significant role in cancer development and progression. The lectin-based glycoprofiling ELISA assay is an informative and clinically applicable tool for the analysis of IgG glycans. The results imply that changes in the TF specific IgG glycosylation have a diagnostic and prognostic potential for stomach cancer. However, a further study is needed to support these findings on a larger scale using different control groups. The MS-based methodology might help to further specify different subsets of anti-TF IgG of clinical importance.

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