

## 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)<sub>2</sub> 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 =  $1.07 \pm 0.08$  (95% CI) and  $0.81 \pm 0.08$ , 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)<sub>2</sub> 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.

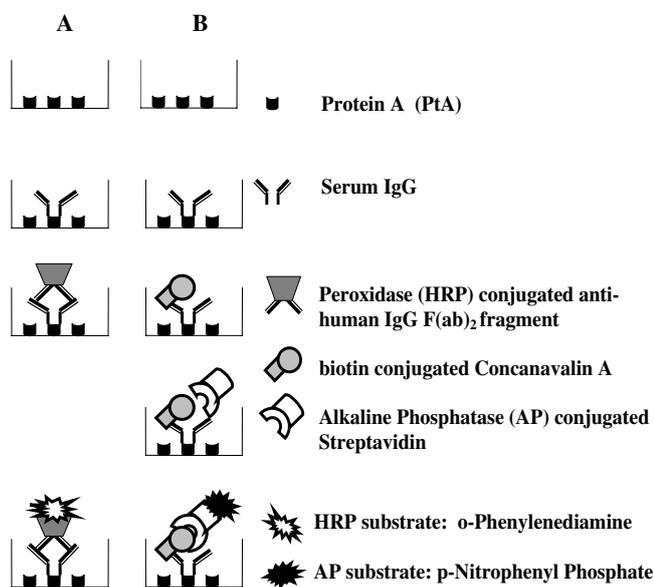
*Key words: Lectin-ELISA, Concanavalin A, IgG glycosylation; gastric cancer.*

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

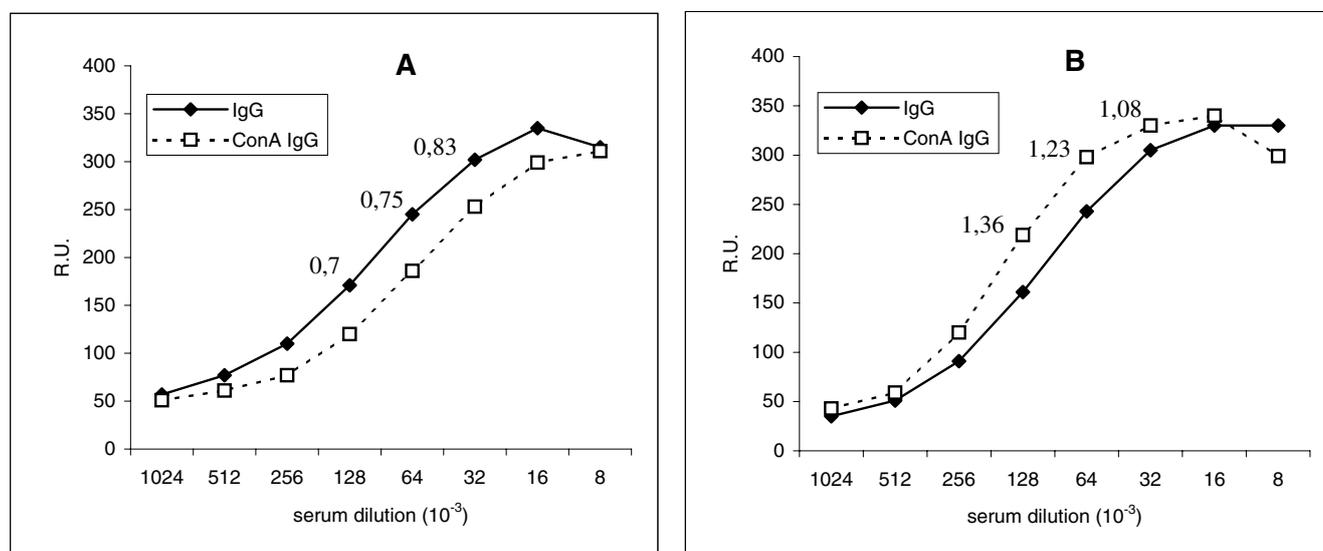
carcinoma (n=53, mean age 60.1±9.4, male/female ratio 1.78) and from healthy blood transfusion donors (n=24; mean age 52.8±7.0, male/female ratio 0.71). Tumor staging was based on the histopathological (pTNM) classification of malignant tumors. Tumor morphology was evaluated according to the classification 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<sup>-6</sup> to 0.01x10<sup>-6</sup> 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<sub>2</sub>, MgCl<sub>2</sub> and MnCl<sub>2</sub> 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 peroxidase (HRP) conjugated rabbit anti-human IgG F(ab)<sub>2</sub> 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<sub>2</sub>SO<sub>4</sub>. 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  $2.5 \times 10^{-4}$  to  $2.0 \times 10^{-6}$  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 (0.45 $\mu$ m) 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 (100 $\mu$ l 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<sub>2</sub>, 3mM MgCl<sub>2</sub> and 3mM MnCl<sub>2</sub> (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<sub>2</sub>, MgCl<sub>2</sub>, MnCl<sub>2</sub>

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 (5 $\mu$ g/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-

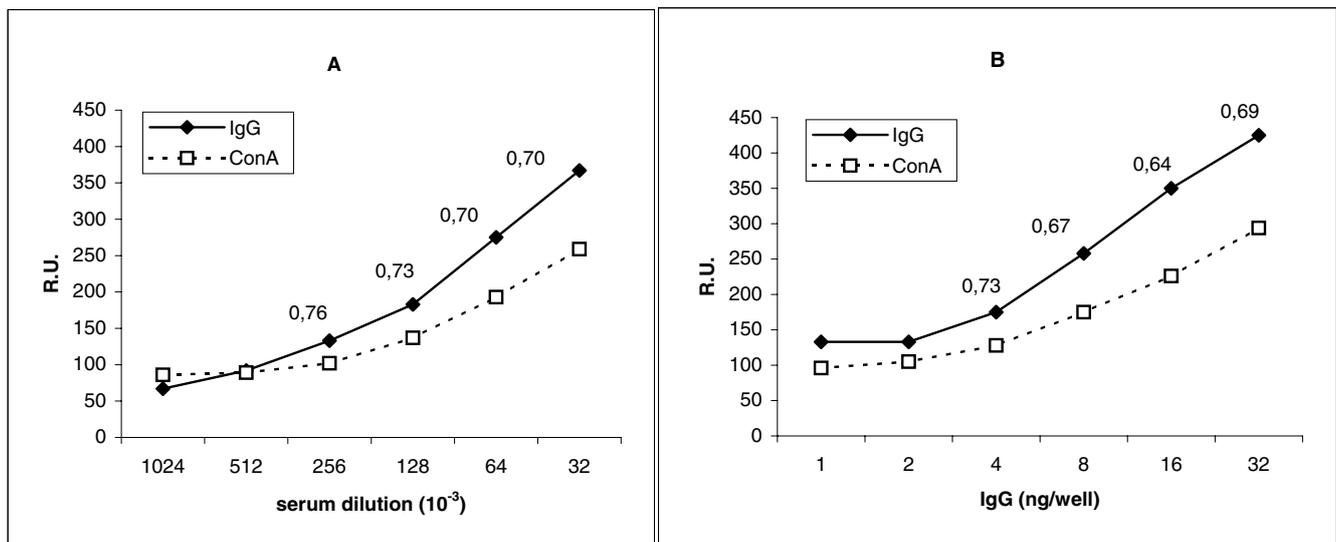


Figure 3. The LELISA titration curves for serum (A) and the IgG (B) purified from the same serum. The ConA index values are shown for a dose-dependent part of the curves.

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.

### 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  $2.5 \times 10^{-4}$  and  $5 \times 10^{-4}$ . 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  $5 \times 10^{-4}$  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

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

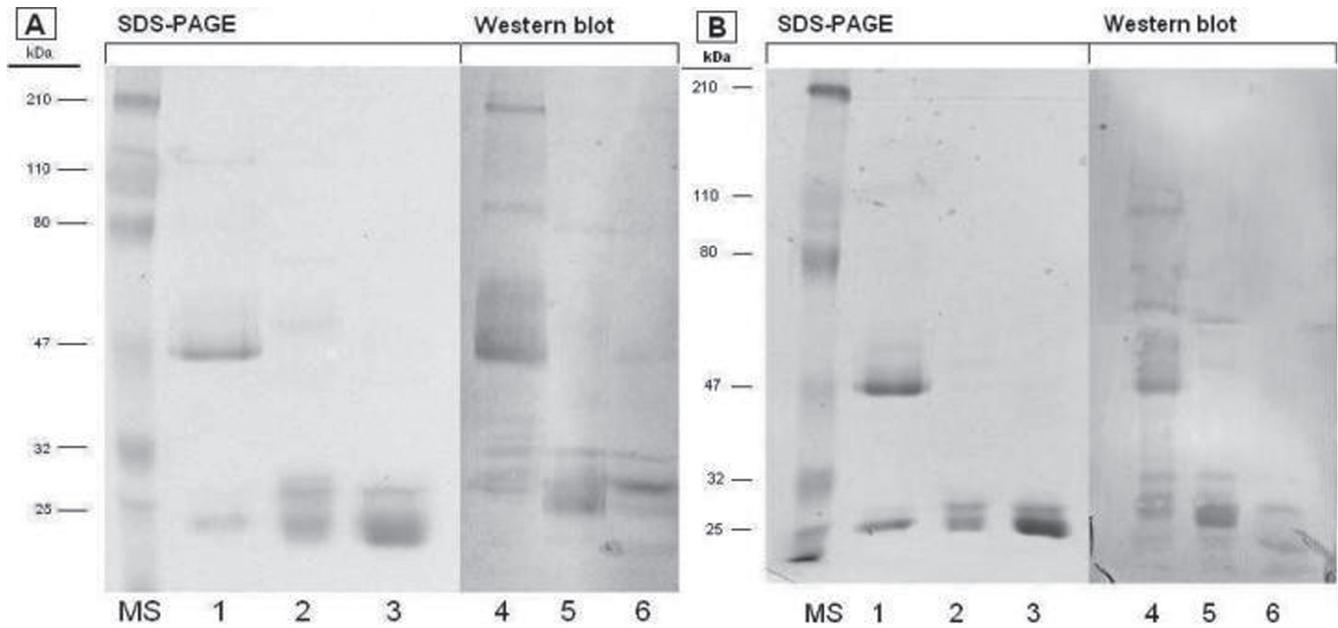


Figure 4. SDS-PAGE (lanes 1-3) and an immunoblot (lanes 4-6) analysis of purified IgG (lanes 1, 4), ConA-positive (lanes 2, 5) and ConA-negative (lanes 3, 6) Fab fragments from the serum of a blood donor (A) and a patient with gastric cancer (B).

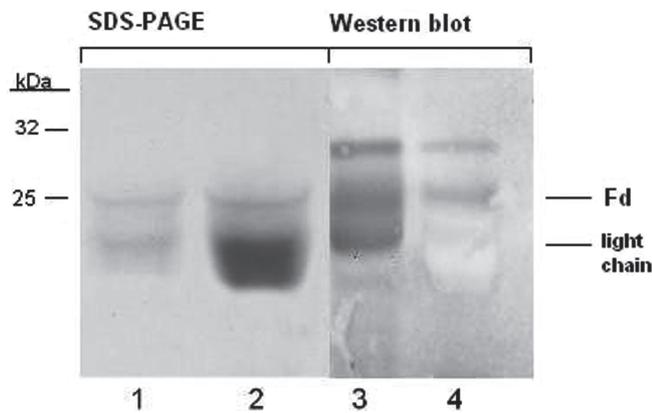


Figure 5. SDS-PAGE (lanes 1, 2) and an immunoblot (lanes 3-4) analysis of ConA-positive (lanes 1, 3) and ConA-negative (lanes 2, 4) Fab fragments purified from a ConA-positive IgG of a donor.

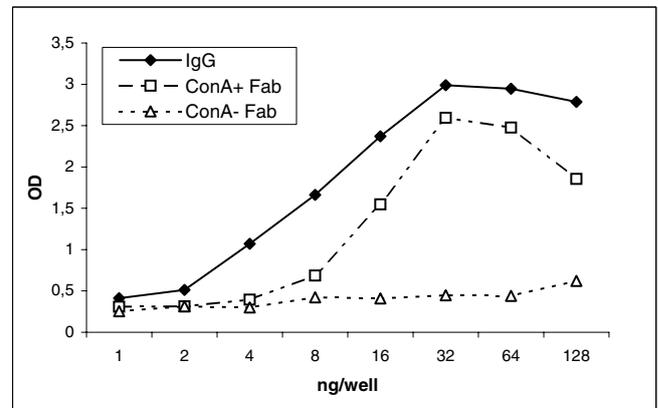


Figure 6. The ConA reactivity of purified IgG and Fab fragments as evaluated by LELISA

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-PAGE 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

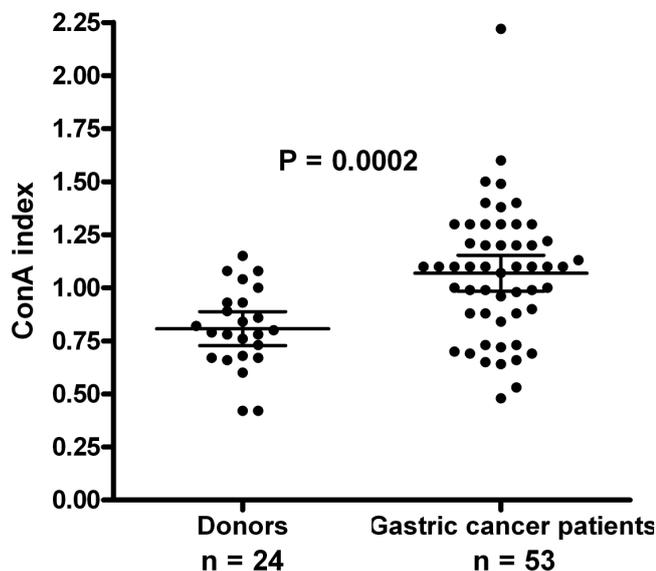


Figure 7. ConA binding patterns as evaluated by LELISA in healthy blood donors and patients with gastric cancer. Based on preliminary experiments, the dilution  $5 \times 10^{-4}$  was selected for the calculation of the ConA index. The mean values and the 95% confidence intervals are shown.

cancer contained very little amount of ConA-negative Fab fragments: only  $\sim 16 \mu\text{g}$  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 =  $1.07 \pm 0.08$  and  $0.81 \pm 0.08$ , 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) ( $P=0.19$ ).

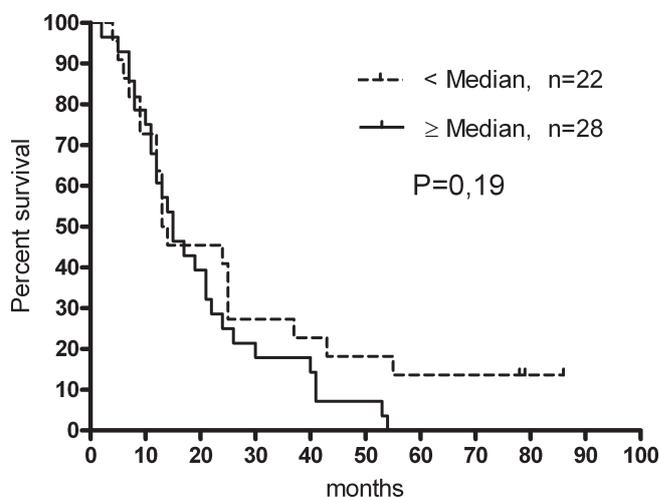


Figure 8. The survival of patients with gastric cancer in relation to the level of the ConA-positive IgG in their serum. The median of the ConA index value was taken as cut-off for groups with a low and high level of the ConA-positive IgG.

## 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)<sub>2</sub> fragment glycosylation in healthy individuals and patients with disease. An asymmetry in F(ab)<sub>2</sub> glycosylation was proposed for about 20% of the normal serum IgG which revealed the reactivity to the  $\alpha$ -D-mannose/  $\alpha$ -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)<sub>2</sub> 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 immunoglobulins 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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