DIPLOMARBEIT

Longitudinal evaluation of alpha-Gal antibody titers in young healthy adults

zur Erlangung des akademischen Grades

Doktor der gesamten Heilkunde
(Dr. med. univ.)

an der

Medizinischen Universität Wien

ausgeführt an der

Universitätsklinik für Chirurgie
unter der Anleitung von

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Wien, am 25.08.2010 .....

Ich bedanke mich an dieser Stelle bei Univ. Doz. Dr. Hendrik J. Ankersmit für die intensive und vor allem stets motivierende Betreuung meiner Diplomarbeit.

Ein besonderer Dank gilt meinem Kollegen Dr. Andreas Mangold, der mir jederzeit im Labor und bei der Ausarbeitung meiner Diplomarbeit mit Rat und Tat zur Seite stand.

Bedanken möchte ich mich auch bei meiner gesamten Arbeitsgruppe, bei meinen Studienkollegen und vor allem bei meinen besten Freunden, die mir meine Studienzeit zu einem unvergesslichen Lebensabschnitt gemacht haben.

Ein großes und spezielles Dankeschön gilt meinen Eltern Maria und Kurt Liepert, sowie meiner gesamten Familie, die mir das Studium überhaupt ermöglichten und mir seit jeher Rückhalt und Liebe entgegenbringen.

Julia Liepert Wien, am 25.08.2010

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ABSTRACT

Background: Alpha-Gal is an epitope on the surface of membranes of mammals and bacteria. Humans do not express alpha-Gal, they have anti-Gal antibodies. Alpha-Gal has been investigated intensively by *Galili* and colleagues in the eighties, and they demonstrated that humans display high titers of anti-Gal antibodies in every isotype class. The dominant hypothesis explains these high titers as a result of constant antigenetic stimulation provided by bacteria of the normal intestinal flora.

Materials and Methods: We wanted to examine how anti-Gal antibodies act in young and healthy adults. 19 volunteers gave blood samples once a week for eight weeks in total. We performed ELISA technique in order to measure alpha-Gal and total immunoglobulin titers. For statistical evaluation we used SPSS software and performed Kolmogoroff-Smirnoff Tests to evaluate the normal distribution of our data, Dependent T-Tests to examine alterations between each time point as well as repeated measurement and the Wilcoxon Signed Rank Test for IgE and total Ig titers which were not normally distributed. Correlations were calculated with the aid of the Pearson product-moment correlation coefficient.

Results: The specific anti-Gal immunoglobulin types IgG, IgM, IgA and IgD were mainly constant over the period of 8 weeks. Most probands were anti-Gal IgE negative and IgE was not constant. The total immunoglobulin titer levels of every isotype stayed constant as well. No correlations with the demographic data were demonstrated except from IgM with sex. Anti-Gal titers of IgG and IgE correlated with the total IgG and IgE titers.

Discussion: To the best of our knowledge, this is the first longitudinal description of all immunoglobulin isotypes. Committed statements can be made by saying that α-Gal antibodies stay mainly constant in young healthy humans.

ZUSAMMENFASSUNG

Hintergrund: Alpha-Gal ist ein Epitop, das auf Oberflächen von Glykolipiden und Glykoproteinen von Säugetieren und Bakterien vorkommt. Menschen und Affen der Alten Welt besitzen Antikörper gegen die Struktur Alpha-Gal, so genannte Alpha-Gal Antikörper. In den 80er Jahren wurde Alpha-Gal intensiv von *Galili* und seinen Kollegen untersucht und es stellte sich heraus, dass nahezu 1% aller Antikörper Alpha-Gal Antikörper sind und somit die am häufigsten natürlich vorkommenden Antikörper des Menschen darstellen. Die vorherrschende Hypothese besagt, dass Alpha-Gal Antikörper durch ständige Stimulation durch Bakterien der natürlichen Darmflora des Menschen entstehen.

Material und Methoden: In dieser Studie wollten wir herausfinden, wie sich Alpha-Gal Antikörper in jungen, gesunden Erwachsenen verhalten. 19 Probanden wurde über einen Zeitraum von 8 Wochen einmal pro Woche Blut abgenommen und mittels ELISA Technik untersucht um die Titerhöhe zu bestimmen. Unsere Daten wurden mit Hilfe des Kolmogoroff-Smirnoff Tests auf Normalverteilungen untersucht und die Veränderungen der Anti-Gal Antikörper zwischen den Messpunkten mit abhängigen T-Tests gemessen. Da IgE und die Titer der totalen Immunglobuline nicht normalverteilt waren, wurde hierfür der Wilcoxon Signed Rank Test verwendet. Korrelationen wurden berechnet mit Hilfe des Pearson Korrelationskoeffizienten.

Ergebnis: Die Titer der alpha-Gal spezifischen Antikörper der Klassen IgG, IgM, IgA und IgD blieben alle vorwiegend konstant über den Zeitraum von 8 Wochen genauso wie die Titer der Gesamtimmunglobuline. Anti-Gal IgE zeigte sich sehr variant in diesem Zeitraum. Es wurden keine Korrelation mit den demographischen Daten gefunden bis auf IgM und Geschlecht, welche eine starke Korrelation aufwiesen. Die alpha-Gal Titer von IgG und IgE korrelierten mit den Titern der Gesamtglobulinen derselben Klassen.

Diskussion: Nach unserem besten Wissen ist dies die erste longitudinale Beobachtung von α-Gal Antikörpern. Es können verbindliche Aussagen getroffen werden da die α-Gal Antikörper in jungen, gesunden Erwachsenen überwiegend konstant sind.

1. INTRODUCTION

1.1. Background

The alpha-Gal epitope (Galα1-3Galβ1-4GlcNAc-R or Galα1-3Galβ1-3GlcNAc-R) is a unique carbohydrate structure which is present on various cell surfaces of non-primate mammals, prosimians and New World monkey [1, 2]. In contrast, humans, apes and Old World monkeys do not express the alpha-Gal epitope. Instead, they have large amounts of circulating natural alpha-Gal antibodies that specifically bind the alpha-Gal epitope. The anti-Gal antibody is the only IgG antibody known to be abundantly present in all humans (~1% of circulating immunoglobulins) [2, 3]. Furthermore, anti-Gal is also present in humans as IgM, IgE, IgA and IgD isotypes. The absence of the alpha-Gal epitope in humans, apes and Old World monkeys is the result of an evolutionary event that is estimated to have occurred less than 28 million years ago. This event led to an inactivation of the glycosyltransferase (α1,3GT) in Old World primates [4].

1.2. Gycolipids and glycoproteins with α-Gal epitopes

In 1968, *Yamakawa* and coworkers first issued a glycolipid from rabbit red blood cells, ceramide pentahexosidose (CPH), which contains the non-reducing terminal sequence Galα1-3Galβ1-4GlcNAc [5]. The structure of CPH was first characterized by *Hakomori* and coworkers in 1972. Subsequently, *Dabrowski, Egge, Hanfland* and coworkers showed that rabbit red blood cells contain a range of glycolipids of varying length and size that terminate with the structure Galα1-3Galβ1-4GlcNAc-R [6-8].

The identification of a naturally occurring human antibody, anti-Gal [1, 2, 9], and development of a mouse monoclonal antibody, Gal-13, was an essential condition to evaluate the species distribution of these types of glycoconjugates [10]. These distributions were evaluated in kidney and thymus tissues from sheep, pig, rabbit, cow and rat by *Hendricks et al.* [11]. Furthermore, sialic acid-containing glycolipids (gangliosides) with the structure Galα1-3Galβ1-4GlcNAc-R were found in pig and sheep [12]. Afterwards, thymus tissue from various species (sheep, pig and rabbits) were examined and the results demonstrated that this tissue contains a range of neutral glycolipids

terminating with Galα1-3Galβ1-4GlcNAc. Brain tissue was examined too, but these glycolipids could not be detected which speaks for a tissue specific expression of these compounds [13].

Next, the research groups of *Spiro* and *Bhoyroo* [14] and *Vliegenthart* [15] pointed out that thyroglobulin, a glycoprotein, isolated from various mammals contains Galα1-3Galβ1-4GlcNAc terminal structures as well. *Thall* and *Galili* [16] figured out that glycoproteins such as fibrinogen and immunoglobulins from various species also comprise varying numbers of Galα1-3Galβ1-4GlcNAc structures. Further studies of different research groups resulted in findings of various mammalian cells containing α-Gal epitopes in large amounts on cell surface glycoconjugates [17-19].

1.3. Anti-Gal Antibodies

In 1984, *Galili et al.* showed that 1% of circulating IgG antibodies are anti-α-Gal antibodies. These anti-Gal antibodies were found in high titers in the human sera [1]. The high titers of antibodies are induced by the presence of the alpha-Gal epitopes on gastrointestinal bacteria of the normal flora. Lipopolysaccharides from various gastrointestinal bacteria were isolated and *Galili* and colleagues found that anti-Gal binds to various types of bacteria, such as *Klebsiella pneumonia*, *Escherichia coli* and *Serratia marcescens* [20]. Consequently, anti-Gal antibodies are a result of continuous antigenic stimulation and produced throughout life.

The initial studies from *Galili et al.* in 1984 aimed to characterize the specificity of anti-Gal. The method mainly used was immunostaining of glycolipids of known structures separated by thin layer chromatography (TLC). It could be demonstrated that anti-Gal binds to glycosphingolipids with non-reducing terminal Galα1-3Galβ1-4GlcNAc-R structures, but not to a range of closely related glycosphingolipids. Anti-Gal neither binds to Galα1-4Galβ1-4GlcNAc-R, which means that anti-Gal distinguishes between structures that differ in the linkage position of the terminal Gal residues [2].

The reason for the high specificity is that the α-Gal epitope lacks sialic acid and thus, is devoid of electrostatic charges. In other words, the binding of anti-Gal to α-Gal epitopes is

facilitated only by hydrogen bonds, hydrophobic interactions and van der Waals` forces so the fitting has to be very precise to enable this antigen- antibody interaction [9, 20, 21].

Differences in the fine specificity of natural anti-Gal antibodies were demonstrated in individuals of various blood types. *Galili et al.* described similarities of the alpha-Gal epitope with ABO blood antigens. In individuals of blood type AB, anti-Gal only binds to α-Gal epitopes, whereas some anti-Gal clones in individuals of blood types 0 and A can bind to the core α-Gal epitope that includes part of blood group B (i.e. Galα1-3[Fucα1-2]Galβ1-4GlcNAc-R). The analysis of *Galili* showed that approximately 85% of purified anti-B antibodies are anti-Gal antibodies [9].

1.4. Evolution

1.4.1. Evolutionary relationship between anti-Gal and the α-Gal epitope

The question of an evolutionary relationship between anti-Gal and the α-Gal epitope led to further investigations. Anti-Gal from normal human blood type AB serum was isolated and the binding to red blood cells and nucleated cells from various species was tested. The results displayed that α-Gal epitopes are present on red blood cells and nucleated cells of marsupials as well as non-primate placental mammals, but is absent on cells from non-mammalian vertebrates including fish, amphibians, reptiles and birds. The α-Gal epitope is also abundantly expressed on cells of lemurs (prosimians) and of New World monkeys (monkeys of South and Central America) such as marmoset, squirrel monkey and spider monkey. In contrast, no α-Gal epitopes were found on cells of primates including Old World monkeys (monkeys of Asia and Africa), apes and humans, they produce a large amount of anti-Gal antibodies instead. Due to the observation that α-Gal epitope is absent in fish, amphibians, reptiles and birds it is supposed to be a relatively recent event in the evolution of vertebrates. This event must have happened before placental and marsupial mammals diverged from each other, i.e. 125 million years ago, because the epitope is present in both groups of mammals [4].

Mammals	α-gal epitope expression	α1,3GT activity	Anti-Gal production
Marsupials	+	+	–
Non-primate mammals	+	+	–
Prosimians	+	+	–
New World monkeys	+	+	–
Old World monkeys	–	–	+
Apes	–	–	+
Humans	–	–	+

Figure 1: Expression of α-Gal epitope, α1,3GT activity and anti-Gal production, adapted from Macher and Galili et al.[4]

1.4.2. α1,3GT expression and evolution

The enzyme UDP-Gal: β-Galactosyl α1-3-Galactosyltransferase (α1,3GT) catalyzes the synthesis of the α-Gal epitope. α1,3GT utilizes UDP-Gal (nucleotide sugar donor) and either a glycoshingolipid or glycoprotein carrying Galβ1-4GlcNAc-R or Galβ1-3GlcNAc-R (acceptor substrate) as substrates and catalyzes the following reaction [4]:

Galβ1-4GlcNAc-R + **UDP-Gal** → **Galα1-3Galβ1-4GlcNAc-R +UDP**

Acceptor substrate | uridine phosphate-Galactose | α1,3GT | α-Gal epitope

The α1,3GT activity was first detected in rabbit bone marrow cells, in rabbit intestinal submucosa, in mouse plasmacytoma cells and in bovine thymocytes [22-25]. Fifteen years later the enzyme was cloned from mouse and bovine cells [26, 27]. With the cloned enzyme it was possible to demonstrate that the α1,3GT functions in the Golgi apparatus and competes with sialyltransferase for *N*-acetyllactosamine non-reducing terminal residues on glycolipids and glycoproteins. Generally speaking, the species distribution of α-Gal epitope expression corresponds to the distribution of α1,3GT activity – the α1,3GT is present in cells and tissues of non-primate mammals and New World monkeys, but not in Old World monkeys and humans [4].

The availability of the α1,3GT gene sequence revealed an opportunity to gain an understanding of the molecular basis for the evolutionary distribution of α-Gal epitopes in various species. For several species such as bats, minks, dogs, sheep and dolphins, coding sequences for α1,3GT were assigned and all of them displayed to contain Galα1-3Galβ1-4GlcNAc-R glycoconjugates. Although some variation in amino acid sequence is found in the N-terminal region (amino acids 1-99) of the α1,3GT sequences from this wide range of species, there is a remarkably high degree of sequence homology (90%) among the sequences from amino acid 100 to the C terminus [28].

1.4.3. Possible causes for the evolutionary inactivation of α1,3GT

The fact that α1,3GT is only present in mammals and not in other primates indicates that it must have been a relatively recent evolutionary event. Since α1,3GT was inactivated in Old World monkeys after they diverged from New World monkeys presumes that it was not essential for survival [4]. The function of the α-Gal epitope, the product of the α1,3GT, is not known. It is conjecturable that this enzyme has certain biological roles, such as cell-cell or cell-matrix interactions. This assumption is based on the fact that KO mice for the α1,3GT gene have cataracts [29, 30]. *Koike et al.* [31] compared the α1,3GT gene in primates (i.e. prosimians and New World monkeys) that express an active α1,3GT with the pseudogene in Old World monkeys and apes. They suggested that the inactivation of the α1,3GT gene was a coincidence and that the function of the α1,3GT was accidentally replaced by other glycosyltransferases.

The South American continent, the home of the New World monkeys on which the α-Gal epitope and the α1,3GT gene is still present, was separated from the African continent for over 35 million years ago. The same with Madagascar, where lemurs live and in which the α-Gal epitope and the α1,3GT have been conserved, diverged from Africa over 60 million years ago. Thus, the inactivation of the α1,3GT gene must have occurred along distinct geographical boundaries, in other words, in primates that resided on the "Old World" land mass comprised of Africa, Asia and Europe [32, 33]. It is likely that the inactivation was associated with a strong selective pressure on primates living on these continents, but not on primates living on "New World" continent like South America or Madagascar. It is conjecturable that the inactivation was exerted by an infectious agent that was endemic to

the connected continents of the Old World. It may have been a virus, bacteria or protozoa which was harmful to monkeys and apes and expressed α-Gal epitopes or an immunologically cross-reactive carbohydrate structure. This event led to an elimination of immune tolerance to this epitope and to production of antibodies against α-Gal. These assumptions are attributable to the findings that viruses, bacteria and protozoa express α-Gal epitopes when propagated in cells with active α1,3GT and anti-Gal has been shown to destroy viruses and protozoa expressing α-Gal epitopes [34-36].

An alternative reason for inactivation of the α1,3GT gene could have been the use of the α-Gal epitope as a cellular receptor by a pathogen, for example a docking receptor for a virus or a receptor for a bacterial toxin. *Clostridium difficile* is an example for such an activity, because the primary ligand for this toxin on intestinal cells is the α-Gal epitope [37]. Subsequently, an endemic infection of Old World primates with a bacteria producing an α-Gal binding toxin could have induced a selective pressure for the evolution of primates which lacked α-Gal epitopes and were not vulnerable to the toxin´s effect. Furthermore, individuals who were successful in suppressing the epitope, would have lost immune tolerance and would have produced anti-Gal antibodies in response to antigenetic stimulation by gastrointestinal bacteria expressing carbohydrate epitopes similar to α-Gal epitopes [20, 38].

1.5. Clinical Implications

1.5.1. Xenotransplantation

The production of anti-Gal antibodies in ancestral primates is the result of the evolutionary suppression of the α1,3GT gene. This has generated a quantitative important immunologic barrier between humans producing large amounts of anti-Gal and mammals with cells that express the ligand for this antibody, the α-Gal epitope [4].

Because of the shortage of human transplantable organs, nonhuman donor organs for transplantation are in the center of attention. Pigs are regarded as the most likely species to serve as donors for xenotransplantation because of the similarity to humans in size and physiology. Extensive research has been performed on the possible use of pig organs (xenografts) as grafts instead of human organs (allografts) and finally, *Galili et al.* identified the major factor of human rejection – the α-Gal/anti-Gal interaction. Pig organs (e.g. kidney or heart) transplanted into Old World monkeys are usually rejected within 30 minutes to several hours [39].

After binding of anti-Gal to α-Gal epitope on xenograft cells the Fc portion of the antibody readily interacts with the Fc receptor on antigen presenting cells (APC) such as macrophages, granulocytes, monocytes and NK cells. This leads to secretion of proteolytic enzymes and causes lysis of the xenograft cells. This mechanism causing such cell lysis is called *antibody dependent cell mediated cytotoxicity* (ADCC) and is much slower than that mediated by complement. Furthermore, these high affinity anti-Gal IgG molecules bind effectively to α-Gal epitopes on various cell surface receptors of pig endothelial cells. Activation of endothelial cells and expression of proinflammatory molecules such as E selectin are the result of this reaction and mediate the anti-xenograft inflammatory response [40-43].

1.5.1.1. Attempts to overcome xenograft rejection

There were many tries to overcome the xenograft rejection problem, starting with the study of *Cooper* and coworkers [44] , who tried to bind the α-Gal antibodies in serum through infusion of the disaccharide Galα1-3Gal in monkeys. This infusion process retarded the hyperacute rejection by several hours. *Sachs et al.* [45] showed the removal of anti-Gal from the blood of monkeys using affinity column with alpha-Gal epitopes. Anyway, due to the continuous production of anti-Gal antibodies, in both studies hyperacute rejection occurred again.

The successful knockout of α-Gal (α1,3GT) in mice was an important step in xenotransplantation research [30]. Next, transgenic pigs for *α1,2fucosyltransferase* (*α1,2FT*) were contrived [46, 47]. They induce a competition between α1,3GT and α1,2FT for capping the common N-acetyllactosamine sugar acceptor. Consequently, the α-Gal epitope expression should decrease. In vivo and in vitro studies showed that approximately 70% decrease in α-Gal epitopes could be achieved with this procedure. As pig cells like endothelial cells express 10_x10^6 to 30_x10^6 α-Gal epitopes per cells, even a decrease of 95% of these epitopes would not be sufficient for preventing rejection of the xenograft. Consequently, the anti-Gal/α-Gal epitope barrier cannot be overcome without complete elimination of α-Gal epitopes from pig cells. This goal was reached in 2002 by *Lai et al.* with the production of α1,3GT KO pigs [48]. Transplantation of kidneys or hearts from these pigs into monkeys were perfomed by *Sachs, Cooper* and coworkers and by *Zhong* and coworkers [49-52]. The studies did not result in hyperacute rejection since anti-Gal did not bind to the grafted cells and xenografts functioned for a view weeks to several months. However, the organs were rejected although the α-Gal epitope/anti-Gal barrier finally has been overcome. Hence, most pig proteins are likely to be immunogenic in humans and induce the production of antibodies, also called "anti-non Gal antibodies".

Galili et al. also studied the anti-Gal response in a primate model with monkeys transplanted with cartilage xenografts [53]. As the cartilage is avascular and thus, hyper acute rejection does not occur, anti-Gal IgG titer increased by 30- to 300-fold four weeks post transplantation and remained at the high level as long as the cartilage was present within the monkeys. The increase in anti-Gal IgM was much lower. This intensive anti-Gal IgG response is the result of activation of the many quiescent B cells capable of producing this antibody (designated anti-Gal B cells). The activation of B cells by α-Gal

epitopes of the xenograft is T-cell dependent and induces anti-Gal B-cells to proliferate, undergo isotype switch and somatic mutations for the ultimate production of large amounts of high affinity anti-Gal IgG. Such high-affinity antibodies are very effective in mediating rejection of the graft, even in the absence of complement activation, primarily by the mechanism of ADCC.

The T-cell dependency in the immune response to xenografts is due to histologic analysis of cartilage xenografts [54, 55]. Pig cartilage were transplanted into monkeys and examined two month later. These xenografts contained extensive mononuclear infiltrates, of which approximately 70% were T-cells and 30% were macrophages. Subsequently, the cartilage was treated with recombinant α-Galactosidase in order to remove α-Gal epitopes from this tissue. This cartilage showed after transplantation and removal a >95% decrease in T-cell infiltration in comparison to the untreated xenograft. Thus, the absence of α-Gal epitopes leads to reduction of T-cell infiltration [54].

However, this enzymatic treatment of xenografts is not applicable to organs such as heart, kidney or liver because elimination of α-Gal epitopes by α-Galactosidase is followed by reappearance of these epitopes within 24-48 hours due to the turn-over of the cell membrane and not like cartilage, which does not contain live cells [56].

Another alternative effort to overcome the anti-Gal/α-Gal epitope barrier has been the induction of immune tolerance to the α-Gal epitope. Studies on tolerance induction have been performed in α1,3GT KO mice that lack α-Gal epitopes and can produce anti-Gal.

Attempts are being made to induce tolerance toward the α-Gal epitopes by introducing either pig bone marrow cells into xenograft recipients or by transducing the recipient´s bone marrow cells with retrovirus vectors that contain the α1,3GT gene [57-60]. According to the first treatment the bone marrow cells expressing α-Gal epitopes are supposed to educate the immune system and regard the α-Gal epitope as a self antigen. The second approach in which tolerance was achieved by gene therapy with KO bone marrow cells transduced in vitro with a retrovirus containing the α1,3GT gene resulted in the induction of long-term tolerance and an inability to produce anti-Gal following immunization with pig lymphocytes.

1.5.2. Bioprostheses

As described above, the shortage of human donor organs drew the attention to xenografts. Porcine and bovine heart valves are used to replace diseased heart valves of humans. The valves are usually treated with glutaraldehyde to sterilize the tissue and remove the antigenicity. These bioprostheses have a superior haemodynamic profile and no requirement of lifelong anticoagulation compared to mechanical valves. Despite of all the advantages of bioprotheses, the durability is reduced to approximately 10 years and younger patients suffer from increased valve degeneration more than elderly because of an increased immunologic activity directed against the xenograft [61]. The younger the patient the worse is the outcome of biological valves [62].

The pathophysiology of continuous bioprosthetic valve degeneration is mainly composed of two major components: calcification and structural impairment of collagen. The background for this process is not clear yet. On the one hand, there are defenders of a non-immunological theory like *Schoen* and *Levy [63]*, who consider that mineralization with calcium phosphate of valve tissue is influenced through host metabolism, implant structure, chemistry and mechanical factors. They elucidate that the usual pretreatment of commercially available bioprostheses with glutaraldehyde results in calcium phosphate accumulation and subsequent to calcification of the valve. On the other hand, there are defenders of a pro-immunological theory like *Human* and *Zilla* [64], who are of the opinion that preformed antibodies, macrophage recruitment, antigen processing and presentation to the immune system and mild, specific IgG response to these antigens are responsible for valve destruction.

In 2005, *Ankersmit and colleagues* [61] investigated the role of α-Gal as an initial immunological trigger for calcification and xenograft rejection. In their study they explored a) the presence of α-Gal epitope on native and fixed porcine valves, b) the increased formation of cytotoxic anti α-Gal IgM antibodies and c) the potentially increased lysis of α-Gal-bearing PK15 porcine cells from serum obtained prior and 10 days after xenograft implantation. The results illustrate that the hypothesis came true as commonly implanted porcine valve prostheses contain α-Gal epitopes within the connective tissue and thus elicit a specific cytotoxic immune response mediated by IgM antibodies directed against α-Gal.

The following immune mechanism can be suggested: Immunoglobulines, especially IgM and IgG, infiltrate the valve matrix and cause opsonization and initiate a specific Fc-receptor-mediated macrophage recruitment with antigen processing and presentation. Macrophages deposite at the valve surface and within the valve matrix and mediate effector functions such as release of MMPs (metallo-matrix proteinase) and phagocytosis. This results in collagen breakdown and calcification.

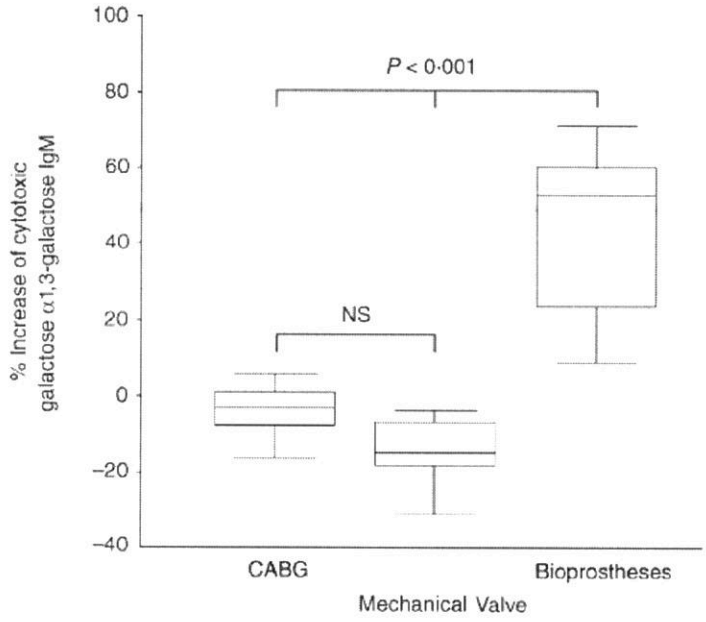

Figure 2: Recipients of bioprostheses demonstrating a significant increase of cytotoxic anti-Galactose α1,3-Galactose IgM ABs as compared with control patients. Sera of recipients of bio-prosthesis (n=12), mechanical bioprosthesis (n=12) and patients who underwent a CABG operation (n=12) were analyzed. Box plot shows the median, quartiles and extreme concentrations of percent increase of anti-α-Gal IgM ABs in serum ten days after the operation. A significantly increased mean±SEM at OD value 405 nm in the concentration of anti-α-Gal was observed in bioprosthesis valve recipients (45.1±10.5%) as compared with recipients of mechanical prostheses (-13.8±4.9%) and CABG patients (-2.2±13.6%) (both, p<0.001). Adapted from Ankersmit et al.[61].

Next, *Mangold et al.* [65] investigated whether that immune response as described in the study above continues after valve implantation. In this study plasma samples from patients who underwent bioprostheses implantation or mechanical valve replacement were collected before, 10 days and 3 month after cardiac surgery, and examined by use of ELISA technique and confocal laser scanning microscopy (CLSM). Indeed, the result showed a significant increase of α-Gal specific IgG 3 month after bioprotheses implantation compared to preoperative values and compared to the control group who underwent mechanical valve replacement. IgG subclasses were also quantified and IgG3 turned out to be the major subclass directed against α-Gal. In patients who underwent reoperation because of complications, heart valves were explanted 1 week after surgery and analyzed by CLSM what showed that bioprostheses contained IB4/DAPI positive cells within the collagen matrix. In contrast, patients who underwent reoperation 12 months later, porcine tissue showed a complete lack of IB4/DAPI what suggests a specific degradation of α-Gal bearing cells through previous exposure to the human blood circuit.

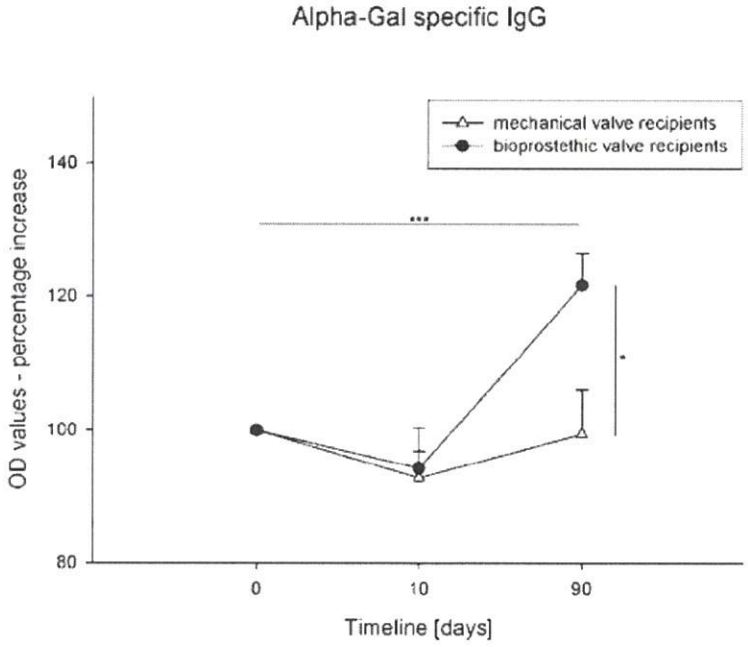

*Figure 3: Evaluation of α-Gal specific IgG ABs revealed a significant increase three months after bio valve implantation (***p<0.001) compared to preoperative values and compared to a control group (*p<0.05), evidencing a specific long-lasting humoral immune response against the α-Gal epitope. Adapted from Mangold et al.[65].*

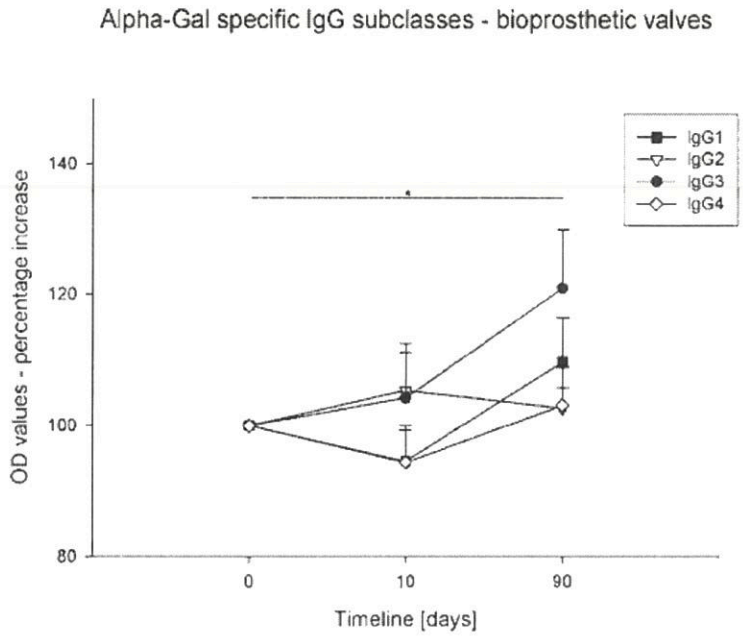

*Figure 4: The IgG subclass specification is depicted. Interestingly, IgG3 subclass levels are most affected (significant increase of IgG3, *p<0.05, significantly higher than in the control group, p<0.01), whereas the other subclasses hardly respond to α-Gal structures of the implanted valve tissue. Adapted from Mangold et al. [65]*

In 2009, *Park et al.* [66] designed a study similar to the one of *Mangold* [65], but they determined the anti-Gal immune response after bioprosthesis implantation in Korean children and not in adults. Sera from 19 patients were obtained 5 times, immediately before pulmonary valve replacement surgery (porcine bioprostheses), and at one day, one week, 3 weeks and 2 month postoperatively. The sera were analysed using ELISA technique and the results displayed an increased formation of anti-Gal antibodies. IgM antibody response was fast and transient, while the IgG antibody response was longer and slower.

Recently, *Lila et al. [67]* evaluated the calcification tendency of α-Gal-containing porcine pericardium tissue compared to pericardium from α-Gal-knockout pigs. They implanted GA-fixed tissue, GA-fixed + formaldehyde, ethanol and Tween 80 (FET)-treated tissue, with and without pre-incubation with human anti-Gal antibodies, subcutaneously into rats during one month. *Lila et al.* demonstrated a significant reduction of calcification of Gal-

KO pericardium compared to wild-type tissue. Additionally, calcification was greatly reduced through addition of FET. When the implants were pre-incubated with human anti-Gal antibodies, Gal-positive tissue revealed to have strongly increased calcification compared to Gal-negative tissue, or any tissue not pre-incubated with such antibodies.

As our data provided the basis on this work [61, 65] , *Mangold* needed to comment on it [68]. He criticized the methologic concept chosen in the work *of Lila et al.*; a) the tissue samples were implanted subcutaneously which is not an ideal environment as it is physiologically simply different, b) rats are the wrong model because humans and Old World monkeys are the only mammals lacking α-Gal epitopes and displaying high anti-Gal titers. Therefore, a specific immune response is hardly possible and c), they expected the pre-incubated tissue with human anti-Gal antibodies to accomplish effector functions in a xenogenic system such as human antibodies in a rat organism. From our point of view, this is unsustainable and the presented results of *Lila et al.* are lacking explanations.

Cryolife Inc., USA came up with a new decellularized, non-glutaraldehyde fixed porcine bioprosthesis in Europe, called Synergraft^{TM}. These newly designed valves were supposed to induce repopulation of the matrix with host cells after implantation in vivo and thus, reduction of antigenicity and creation of a living tissue. In 2003, *Kasimir et al.* [62] first reported of the rapid failure of these grafts after implantation of four porcine Synergraft^{TM} heart valves in children requiring right ventricular outflow tract reconstruction. Three of four children died, 2 due to sudden cardiac death after 6 month and 1 year, the third child due to Synergraft^{TM} rupture. The fourth graft was explanted prophylactically 2 days after implantation. All four grafts showed severe inflammatory reactions.

Anyway, a lot of research due to the role of immunological process in xenograft valve degeneration has been done and recent convincing results have been reported. Nevertheless, the research concerning the major causes remains ongoing.

1.5.3. Vaccines

In general, attenuated live virus vaccines are more effective than inactivated virus or subviral vaccines. Due to the fact that just a small number of attenuated virus vaccines are available and that there can be risks associated with using such preparations, inactivated virus vaccines or subviral vaccines are in center of interest [69-71]. These types of vaccines can be more readily developed with any type of virus. The fact that inactivated virus vaccines or subviral vaccines are generally limited in their ability to elicit an effective immune response for protection against viral infections constitutes a problem in production. It seems probable that the low immunogenicity of inactivated virus vaccines is a result of insufficient processing and presentation of viral antigens to T-cells by antigen presenting cells (APC), such as macrophages or Langerhans-cells. A potential method for increasing the uptake of antigens by APC is the formation of antigen-antibody complexes which would adhere to the Fc receptors on these cells and would be actively internalized by the APC [72] .

In the year of 2007, α-Gal epitopes were successfully produced on carbohydrate chains on virus envelope hemagglutinin with the aid of enzymatic engineering [73]. The hemagglutinin molecule is a glycoprotein with seven carbohydrate chains capped with N-acetyllactosamines (Galβ1-4GlcNAc-R) on which α-Gal epitopes can be synthesized by using recombinant α1,3GT. They hypothesize that the synthesis of α-Gal epitopes on inactivated influenza virus envelope leads to a significant higher level of immune response than immunization with conventional influenza virus vaccine without α-Gal epitopes.

Such a difference in immune response is to reduce to the fact that influenza virus vaccine expressing α-Gal is opsonized by anti-Gal immunoglobulin IgG. Thus, APC internalize the vaccine and transport it to draining lymph nodes. There they present the immunogenic viral peptides on cell surface MHC class I and class II molecules for activation of virus-specific CD8+ and CD4+ T-cells. Activated CD8+ T-cells become cytotoxic T-lymphocytes (CTL), which destroy infected cells, and activated CD4+ T-cells help virus specific B-cells to produce antiviral antibodies and CD8+T-cells to become CTL.

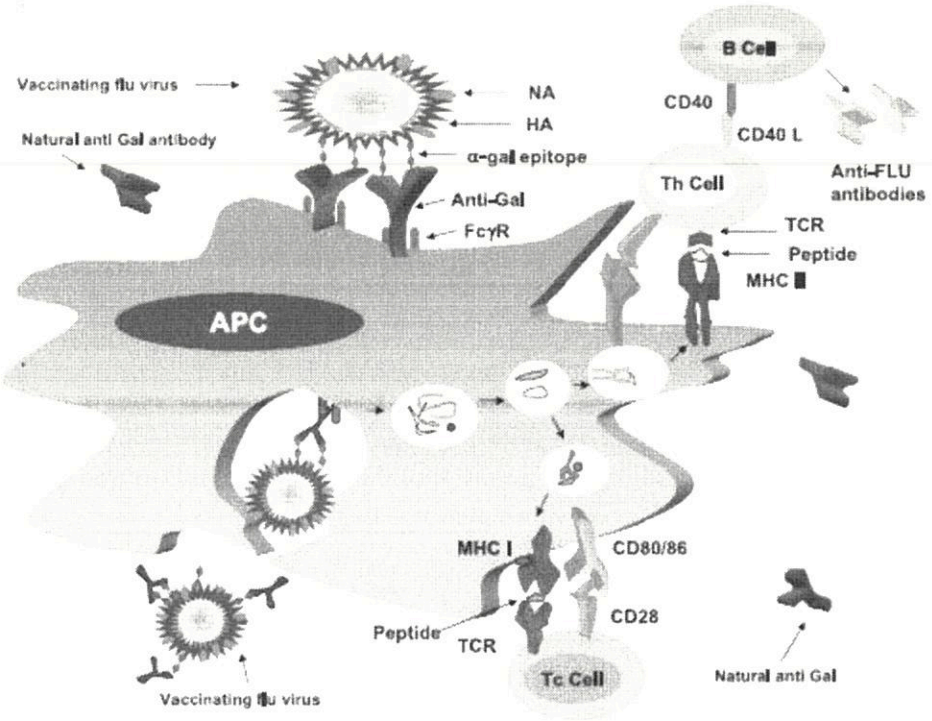

Figure 5: Anti-Gal-mediated targeting of vaccinating PR8αGal virus to APC. Inactivated PR8αGal virus with α-Gal epitopes (red diamonds) was injected as a vaccine. Anti-Gal binds to the α-Gal epitopes on the virus and opsonizes it. The Fc portion of anti-Gal interacts with FcγR on APC and induces uptake of the vaccine by the APC. The internalized virus undergoes processing in the endocytic vesicles and the cytoplasm. The viral immunogenic peptides are presented on MHC class I molecules for activation of $CD8^+$ CTL precursors (Tc cells) and on MHC class II molecules for activation of helper T cells (Th cells). Signal 2 provided by the APC also facilitates this activation. Activated Th cells provide help for the antibody response by B cells and for CTL activation. Activated Tc cells differentiate into CTL, which kill virus-infected cells. TCR, T-cell receptor. Adapted from Galili et al. [73].

The α1,3GT gene knockout (KO) mouse is one of view available nonprimate mammalian experimental model which produces anti-Gal and thus enables analysis of immunogenicity of anti-Gal opsonized vaccines [73]. The α1,3GT of the mouse was disrupted and do not have any α-Gal epitopes left. Instead, it produces anti-Gal antibodies after immunization procedures. The immune response to the PR8 (A/Puerto Rico/8/34-H1N1) influenza virus strain was studied in these KO mice. The results of the study show that a good method for

increasing the immunogenicity of inactivated influenza virus vaccines was contrived by synthesizing α-Gal epitopes on the multiple N-linked carbohydrate chains on envelope glycoproteins of the virus. The study indicates that the suboptimal uptake of vaccines that lack markers for recognition by APC at the vaccination site is the limiting factor in their immunogenicity.

1.5.4. Tumor Therapy

Many studies in experimental animal models and in humans have shown that tumor cells express a variety of tumor specific antigens. They emerge in tumor cells as a result of multiple mutations while development of the malignant tissue and most of the tumor antigens are specific to the tumor type and the person. Hence, the autologous tumor could be considered as a suitable source for vaccine material. Vaccinating these specific autologous tumor antigens is supposed to determine an immune response by anti-Gal and thus, a destruction of tumor cells [4].

Some of the tumor antigens have been characterized, e.g. carcinoembryonic antigen or MUC 1, and they are common to many types of tumors. These tumor antigens may also be expressed on normal cells and it is likely that this is the reason why the immune response to these antigens is usually very low. Furthermore, tumors elude the immune system by eliminating markers identifying them for uptake by APC [4].

Similar to viral vaccines, to achieve an effective uptake of tumor vaccines, it has to be effectively presented to APC and the vaccine has to express α-Gal epitopes. *Galili* and coworkers [74, 75] hypothesized that tumor cells or cell membranes can be submitted to in vitro synthesis of α-Gal epitopes by incubation in a solution containing neuramidase, recombinant α1,3GT and UDP-Gal. This autologous vaccine, after washing and irradiation, could be injected into the patient. Anti-Gal should bind to the α-Gal epitope and is supposed to induce an effective targeting of the vaccinating cells and cell membranes to APC at the vaccination site and eliciting a protective immune response against tumor antigens which are expressed on tumor cells remaining in the body. The

efficacy of these vaccines has been shown in an α1,3GT KO mice experimental model [76]. The outcome of this study demonstrated an induction of an immune response which protected the mice against challenge with live tumor cells and did not result in an autoimmune response.

In further studies, *Galili et al.* [77] developed a method to use anti-Gal for the destruction of visible lesions and their conversion into endogenous vaccines. These endogenous vaccines are able to target micrometastatic tumor cells and destroy them. This is possible with the aid of intratumoral injection of glycolipids carrying α-Gal epitopes (α-Gal glycolipids). These α-Gal glycolipids form micelles when they are dissolved in water. When these micellar glycolipids get injected into tumor lesions, they insert spontaneously into the tumor cell membranes and thus, the tumor express α-Gal epitopes and can interact with anti-Gal antibodies to induce activation of complement, a local inflammation and recruitment of APC for destruction. Furthermore, APC transport the internalized tumor antigens to lymph nodes for activation of tumor specific T cells. The efficacy of this treatment was shown in an experimental model of KO mice and cutaneous B16 melanoma lesions. The treatment with α-Gal glycolipids and the autologous tumor vaccines are currently in Phase I clinical trials.

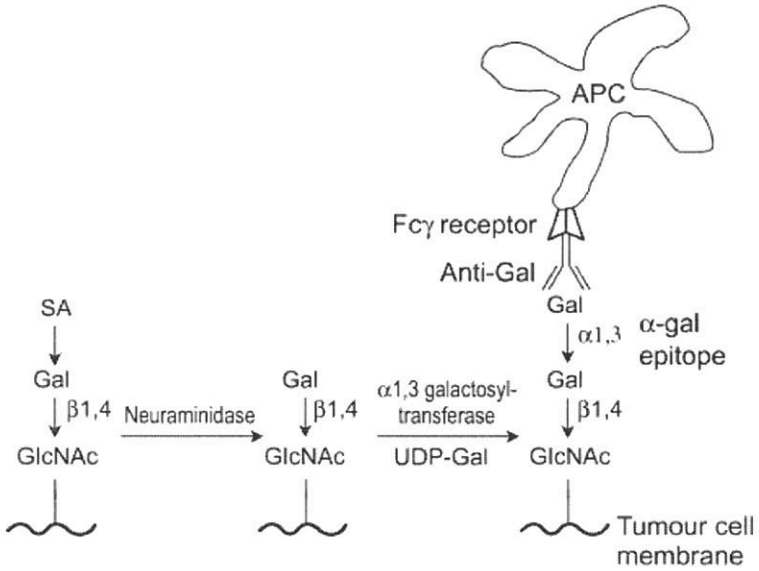

Figure 6: Processing of autologous tumour cell membranes to express α-Gal epitopes in order to achieve in situ anti-Gal mediated targeting to APC. Sialic acid (SA) is cleaved from the carbohydrate chains by neuraminidase to expose the penultimate N-acetyllactosamine residues (Galβ1-4GlcNAc-R). Recombinant α1,3GT transfers Galactose from the sugar donor uridinediphosphate-Galactose (UDP-Gal) to the exposed

N-acetyllactosamine residues to form α-Gal epitopes. Following the administration of the vaccinating tumor cells or cell membranes intradermally, anti-Gal of the vaccinated patient binds in situ to α-Gal epitopes on the vaccinating tumor cell membranes. The Fcγ receptors of APC at the vaccination site interact with the Fc portion of anti-Gal bound to the vaccinating cell membranes and induce uptake of the vaccine by the APC. Adapted from Galili et al.[78].

1.5.5. Food Allergy

According to the most accepted hypothesis, the anti-Gal antibodies are stimulated and produced throughout life because of the bacteria of the normal human flora.

Mañez et al. performed a study with baboons to investigate the efficacy of two different strategies to reduce the serum level of natural anti-Gal antibodies. Removal or neutralization of α-Gal antibodies has been achieved so far by several methods such as plasmapheresis ect., but the effect of these tries last only a few hours after the procedure ends. The study of *Mañez and colleagues* demonstrated that a removal of normal aerobic gram-negative bacteria from the intestinal flora with the antibiotic norfloxacin is more effective than immunosuppression with cyclophosphamide and steroids in reducing the level of Gal titers. Between week 5 and 9, the mean anti-Gal IgG level in the Norfloxacin group was significantly lower than in the Control group. At week 10 the anti-Gal IgG level started returning to the pretreatment level and reached it at week 20. No differences of anti-Gal IgM antibodies were found between those two groups in any of the 16 weeks of study period. Furthermore, no differences were found between the cyclophosphamide and the control group at any time and no changes were observed on the total level of any immunoglobulin isotype before, during or after treatments. However, they assume that the suppression of production of anti-Gal IgG is specific for this antibody because the level of total immunoglobulins did not change with the treatment. Thus, the hypothesis of the origin of anti-Gal antibodies gets fortified.

Another new and worth researching topic is the anti-Gal IgE increase in patients suffering from food allergies. *Platt-Mills et al.* [79] investigated whether IgE antibodies to α-Gal

are present in sera from patients who reported anaphylaxis or urticaria after eating beef, lamb or pork. They identified 24 patients with a consistent pattern of both skin testing and serum IgE antibody results. All 24 patients reported that episodes were associated with having eaten beef, pork or lamb 3 to 6 hours earlier.

This study is the first describing the connection between anti-Gal antibodies and food allergy and we are sure that several studies will follow soon.

1.5.6. Rationale and Aim of the Study

Alpha-Gal antibodies are the most abundant specific antibodies in the human circulation. *Nydegger and colleagues* [80] quantified anti-Gal antibodies in sera of 200 healthy persons of different age groups with ELISA technique. Their results showed a prominent interindividual variation of the different isotype titers which exceeded all, gender, age and histoblood group related changes. A genderrelated difference was found for anti-Gal IgM, but not for the IgG isotype.

To our knowledge, until presence only cross-sectional studies have been performed like the one of *Nydegger et al.* [80], but the longitudinal changes of healthy humans were not known. Therefore we performed a prospective, longitudinal investigation over 8 weeks.

The aim of the study was to show that anti-Gal titers in young healthy men and women are constant. This is important for the reliability of our work in consideration of ascendancies like illnesses and injuries. Furthermore, the results of the present study will provide a valuable basis for further research.

We wanted to measure the immunoglobulin subtypes IgM and IgG because they are the most investigated subtypes of α-Gal antibodies and they constitute an important basis for our research on this topic and help us to strengthen the validation of our former and future work [61, 65].

The anti-Gal IgA subtype was chosen as our study group is intensively doing research on IgA in different entities and it is the only subtype which is to be found in immediate proximity to α-Gal bearing bacteria.

The IgD class is the most unknown and unexplored antibody and so we involved IgD in our study because of our curiosity and the results of *Mosedale et al. [81]* who demonstrated that nearly every proband suffering from atherosclerosis displays high levels of anti-Gal IgD antibodies.

We included IgE in our longitudinal study as it plays a role in food allergy and thus, we are courious about this new and exciting implication concerning α-Gal. Furthermore, this thrilling topic is in center of research of our own and of our neighboring study group of *Bohle et al.*.

2. MATERIALS AND METHODS

2.1. Materials

2.1.1. Reagents

Gal-1.3-Galβ1-4GlcNAc-R (Dextra Labobaroties, Reading, UK)
Goat Anti-Human IgG-Fc Polyclonal Antibody, Horseradish Peroxidase (HRP) Conjugated (Bethyl, Montgomery, USA)
Mouse Anti-Human IgG Monoclonal Antibody (all subclasses), HRP Conjugated (Invitrogen, Carlsbad, USA)
3,3", 5,5" Tetramethylbenzidine (TMB) Liquid Substrate System for ELISA (Sigma-Aldrich, St. Louis, USA)
Sulfuric acid 2N (H2SO4) (Sigma-Aldrich, St. Louis, USA)
Phosphat-buffered saline (PBS)-/- GIBCO.(Invitrogen, Carlsbad, USA)
Isolectin GS´-IB4 from Griffonia simplicifolia, Alexa Fluor 488 conjugate, Molecular Probes, Eugene, USA

2.1.2. Chemical Solutions

Washing buffer

PBS-/- GIBCO™
0.05% Tween 20 (Bio-Rad, Hercules, USA)

Carbohydrate buffer

Aqua dest. (BBraun, Melsungen, D)
Na2CO3 (Merck, Darmstadt, D)
NaHCO3 (Merck, Darmstadt, D)
pH = 9.6

Blocking buffer

PBS-/- GIBCO™

0.05% Tween 20

0.01% Bovine Serum Albumin (BSA) (Sigma-Aldrich, St. Louis, USA)

Sample diluent

0.05% Tween 20

1mM EDTA (Sigma-Aldrich, St. Louis, USA)

0.25% BSA

0.02% Thimerosal (Sigma-Aldrich, St. Louis, USA)

15mM Na2B4O7 (Merck, Darmstadt, D)

120mM NaCl (Merck, Darmstadt, D)

pH = 8.5

Fixing buffer

0.1M phosphate buffer (Merck, Darmstadt, D)

4% formaldehyde (Sigma{Aldrich, St. Louis, USA)

pH = 7.4

2.2. Methods

2.2.1. Proband Selection

We examined sera of 19 healthy men and women, between 18 and 30 years old (w: n=5, m: n=14). The mean age of the probands was 24.4 years. Blood samples were taken once a week over 8 weeks in total. This has been done in accordance with the ethics-committee, medical University Vienna; a positive vote is present. The probands were recruited by notices posted on campus of the Medical University of Vienna.

Inclusion criteria: age <18, >30.

Exclusion criteria: chronic diseases of the respiratory or gastrointestinal tract; autoimmune diseases, immune deficiencies or malignancies; immune-suppressive medication, antibiotics or other drugs which manipulate immunoglobulin building; blood transfusions in the last eight weeks; coeval participation at another study.

Demographic data: We surveyed the following data: height (avg.: 177,4cm), weight (avg.: 69,6 kg), smoker or nonsmoker, allergies, medications, vegetarian, meat consume per week, yoghurt consume per week and previous diseases.

2.2.2. Enzyme-linked Immunosorbent Assay

Alpha-Gal-specific Antibodies

ELISA technique was used to measure anti-Gal ABs of all isotypes (IgM, IgG, IgA, IgE, IgD). Plates were coated with Galα1-3Galβ1-4GlcNac-BSA (Dextra Laboratories, Reading, UK) at 4° over night. After rinsing, blocking was done with blocking buffer (PBS-/- + 0,5% BSA + 0,05% Tween20). After another rinsing step and incubation with samples, detection antibodies were added (Goat anti–Human IgM Polyclonal Antibody, Goat anti–Human IgG Polyclonal Antibody, Goat anti–Human IgA Polyclonal Antibody, Goat anti–Human IgD Polyclonal Antibody, all HRP-conjugated (Bethyl Laboratories, Montgomery, TX, USA), and anti-Human IgE Antibody (). A color reaction was obtained with peroxidase reagent TMB (3.3′,5.5′-tetramethylbenzidine, Sigma-Aldrich, St. Louis, MO, USA) and optical density was read at 450nm using a Victor3 plate reader (1420 Multilabel Counter, PerkinElmer,Waltham, MA, USA). For IgE, tablets were used for color reaction and optical density was read at 405nm using a Victor3 plate reader.

As no human anti-Gal AB is commercially available, we are not able to express total anti-Gal Ig content. We used a dilution series of one sample serum on every ELISA plate as an internal standard and fitted the curves for inter-assay comparisons. For curve fitting, we used Origin 8.1 (OriginLabCorporation, Northampton, MA USA) and Excel 2003 (Microsoft Corporation, Redmond, WA, USA). In detail, we used the most accurate dilution series as a standard and fitted all other dilution curves on that. Consecutively, we fitted all measured OD values to these fitted curves.

Total Ig Isotypes

ELISA Kits were used to measure total content of Ig isotypes (Human ELISA IgG, IgM, IgA, IgD, IgE Quantitation Set, Bethyl Laboratories). The assay was performed following the instructions of the manufacturers' manual.

2.2.3. Statistical Analysis

Statistical analysis was performed using SPSS software (SPSS for Windows Version 15; SPSS Inc, Chicago, USA) and a p-value <0,05 was considered to be statistically significant. To evaluate the Gaussian distribution of our data we used the Kolmogorov-Smirnoff Test. The alteration of the specific anti-Gal antibodies between each time point was examined by doing repeated measurements and with the Dependent T-Test except from anti-Gal IgE, which was not normally distributed and therefore, we made use of the Wilcoxon Signed Rank Test. The alterations of the total immunoglobulin titer levels were not normally distributed as well and thus, the Wilcoxon Signed Rank Test was utilized. Correlations were calculated with the Pearson product-moment correlation coefficient.

3. RESULTS

3.1. Immunoglobulin G

3.1.1. Evaluation of alpha-Gal specific Ig G

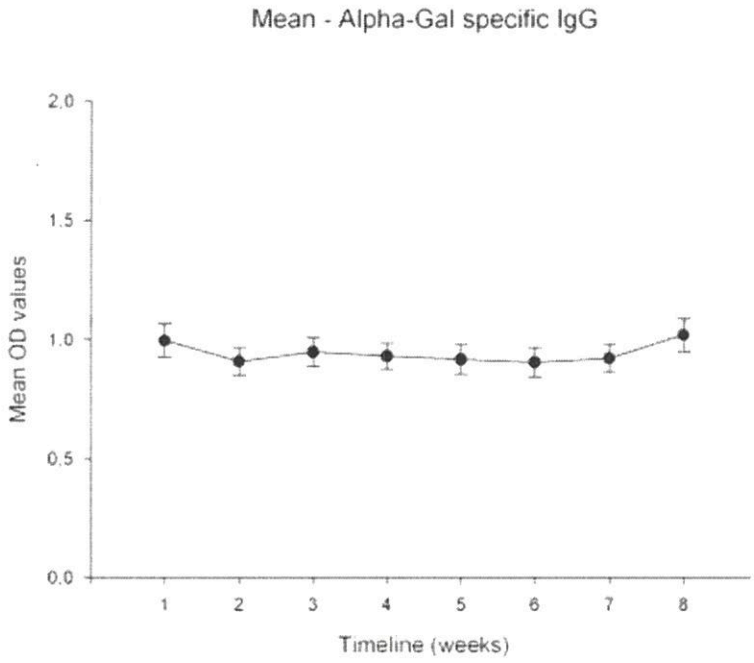

Figure 7: Each point represents the average of α-Gal IgG ELISA OD values at the respective time of measurement. Error bars ± standard error of the Mean.

We observed the alpha-Gal specific IgG antibodies for 8 weeks and determined significant valve changes between each time point. We found a significant change between week one to week two ($p<0,05$). Still, it can be stated that the anti-Gal IgG titer remained stable over the observed time frame.

No demographic data (age, height, sex, pack years, allergies, medication, vegetarian, meat/week, yoghurt/week, probiotics/week) correlated with the mean titer level of any proband.

3.1.2. Evaluation of total IgG titer levels

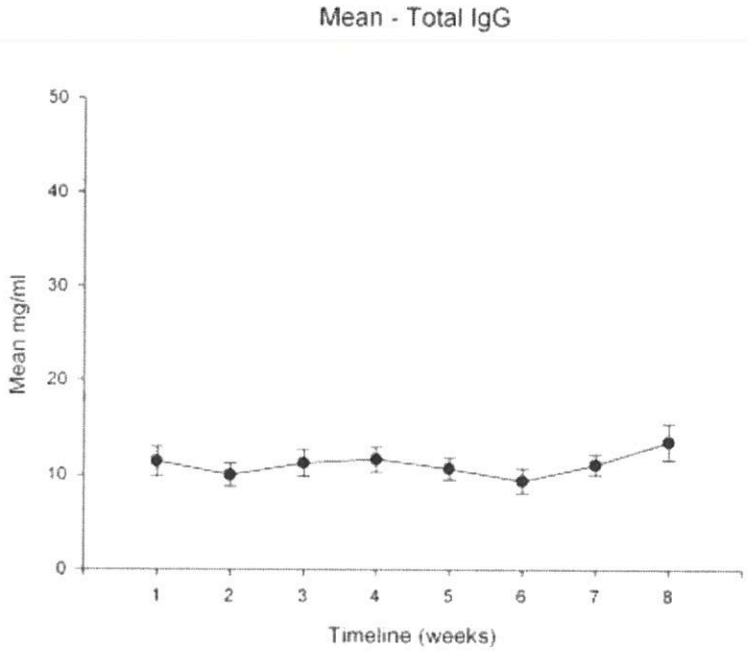

Figure 8: Each point represents the average of total IgG ELISA OD values at the respective time of measurement. Error bars ± standard error of the Mean.

Furthermore, we measured the total Immunoglobulin G content. We could examine a correlation between the anti-Gal IgG titer and the total IgG titer level but the titer changes of total IgG did not correlate with anti-Gal IgG titer alterations.

3.2. Immunoglobulin M

3.2.1. Evaluation of alpha-Gal specific IgM

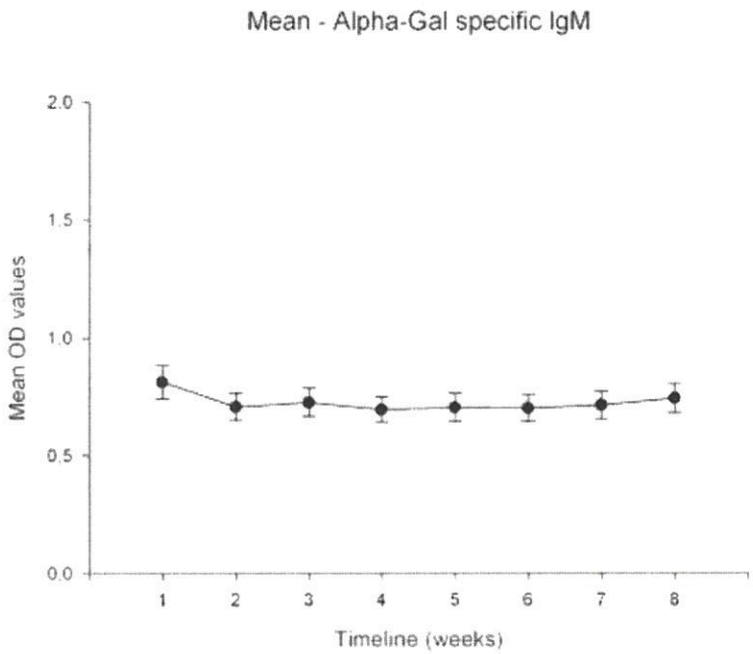

Figure 9: Each point represents the average of α-Gal IgM ELISA OD values at the respective time of measurement. Error bars ± standard error of the Mean.

We measured the alpha-Gal specific IgM antibodies and determined significant valve changes between each time point. We could not find any significant change apart from week one to week two ($p<0,05$). It can be stated that the anti-Gal IgM titer remained stable over the observed time frame.

Regarding the correlations with the demographic data (age, height, pack years, allergies, medication, vegetarian, meat/weak, yoghurt/week, probiotics/week) we could find a strong correlation between IgM and sex. No other correlations could be calculated.

3.2.2. Evaluation of total IgM titer levels

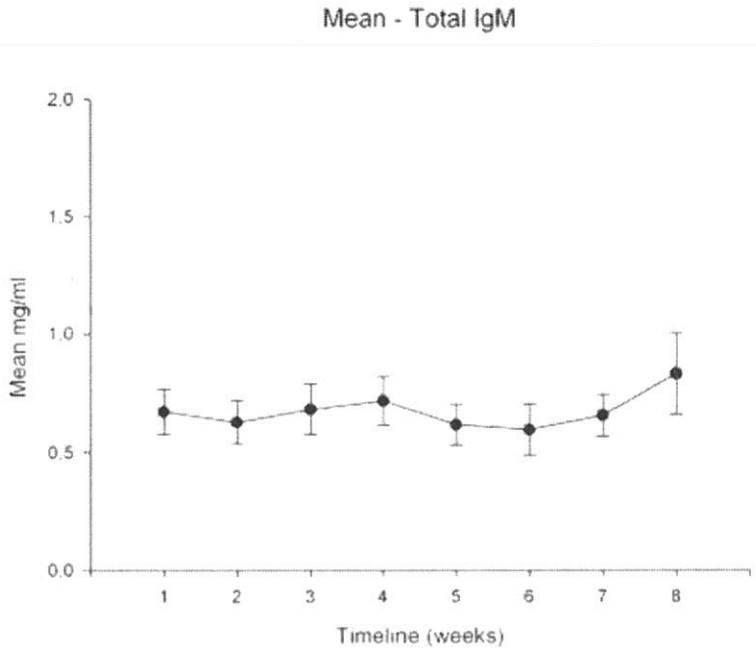

Figure 10: Each point represents the average of total IgM ELISA OD values at the respective time of measurement. Error bars ± standard error of the Mean.

We measured the total Immunoglobulin M content. We did not find any correlation between the total IgM titer level and the alpha-Gal titer level. The titer changes of the total IgM immunoglobulins did not correlate with anti-Gal IgM titer alterations.

3.3. Immunoglobulin A

3.3.1. Evaluation of alpha-Gal specific IgA

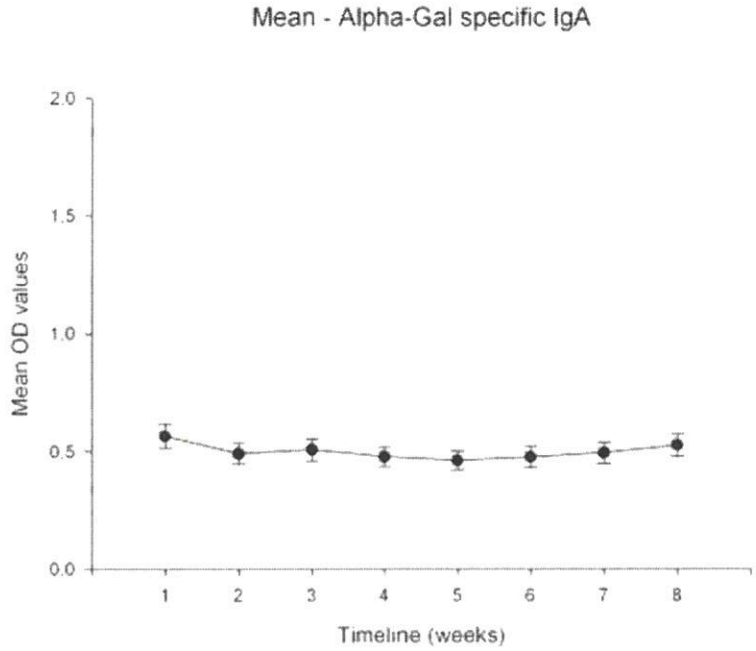

Figure 11: Each point represents the average of α-Gal IgA ELISA OD values at the respective time of measurement. Error bars ± standard error of the Mean.

We observed the alpha-Gal specific IgA antibodies and determined significant valve changes between each time point. Like the Anti-Gal IgG and IgM, we could find a significant change between the IgA time point one and time point two ($p<0,05$). Between week 2 and week 8, there were no significant changes and it can be stated that the anti-Gal titer remained stable as well.

No demographic data (age, height, sex, pack years, allergies, medication, vegetarian, meat/week, yoghurt/week, probiotics/week) correlated with the mean titer level of any proband.

3.3.2. Evaluation of total IgA titer levels

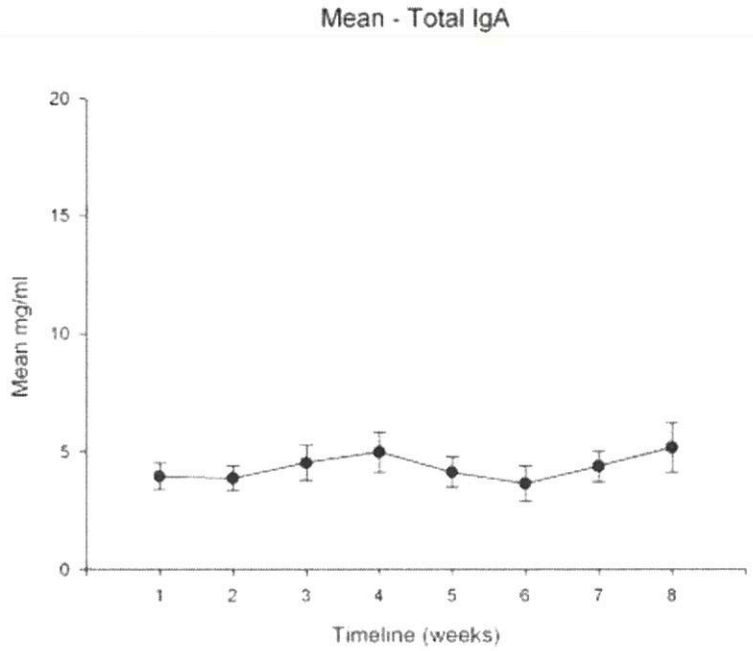

Figure 12: Each point represents the average of total IgA ELISA OD values at the respective time of measurement. Error bars ± standard error of the Mean.

We measured the total Immunoglobulin A content. We did not find any correlation between the total IgA titer level and the alpha-Gal titer level. The titer changes of the total IgA immunoglobulins did not correlate with anti-Gal IgA titer alterations.

3.4. Immunoglobulin E

3.4.1. Evaluation of alpha-Gal specific IgE

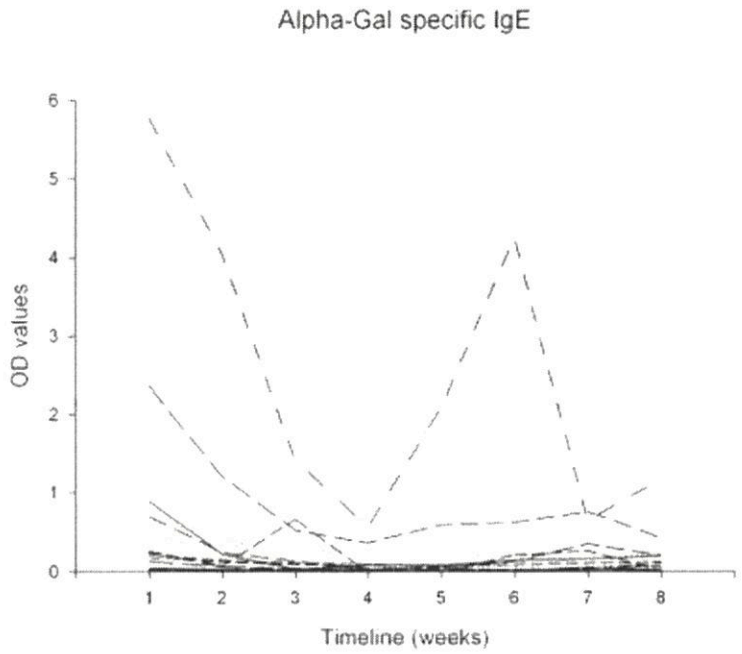

Figure 13: Progresses of the anti-Gal IgE AB concentrations of 20 probands during the 8 weeks.

Furthermore, we measured the alpha-Gal specific IgE antibodies and determined significant valve changes between each time point. Most of the probands were negative and one volunteer had to be excluded because of a newly diagnosed IgA-Nephritis. Coincidentally, he was the only proband who had extremely heightened anti-Gal IgE titer levels. We could find significant changes within the 8 weeks and it can be stated that the anti-Gal IgE titer did not remain stable over the observed time frame.

No demographic data (age, height, sex, pack years, allergies, medication, vegetarian, meat/week, yoghurt/week, probiotics/week) correlated with the mean titer level of any proband.

3.4.2. Evaluation of total IgE titer levels

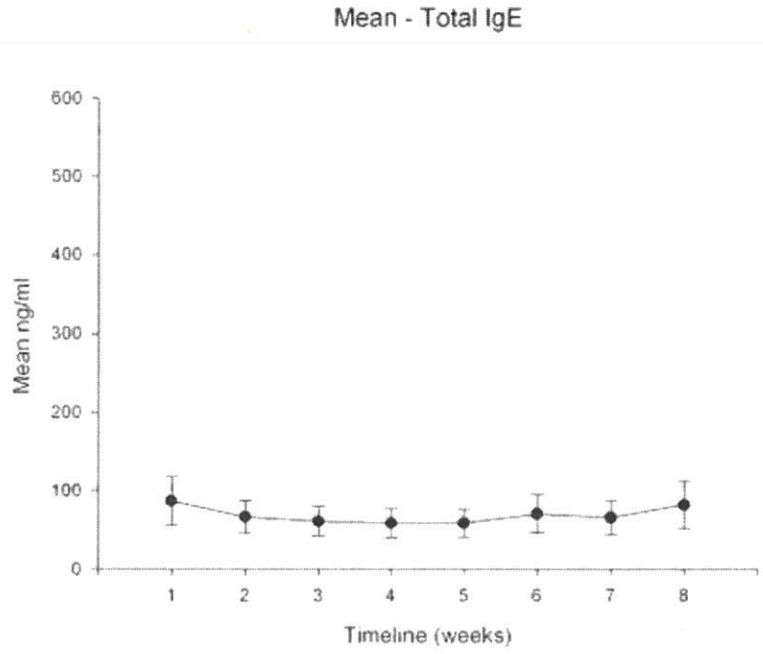

Figure 14: Each time point represents the average of total IgE ELISA OD values at the respective time of measurement. Error bars ± standard error of the Mean.

We measured the total Immunoglobulin E content as well. The anti-Gal IgE titer correlated with the total IgE titer but the titer changes of the total IgEs did not correlate with anti-Gal IgE titer alterations.

3.5. Immunoglobulin D

3.5.1. Evaluation of alpha-Gal specific IgD

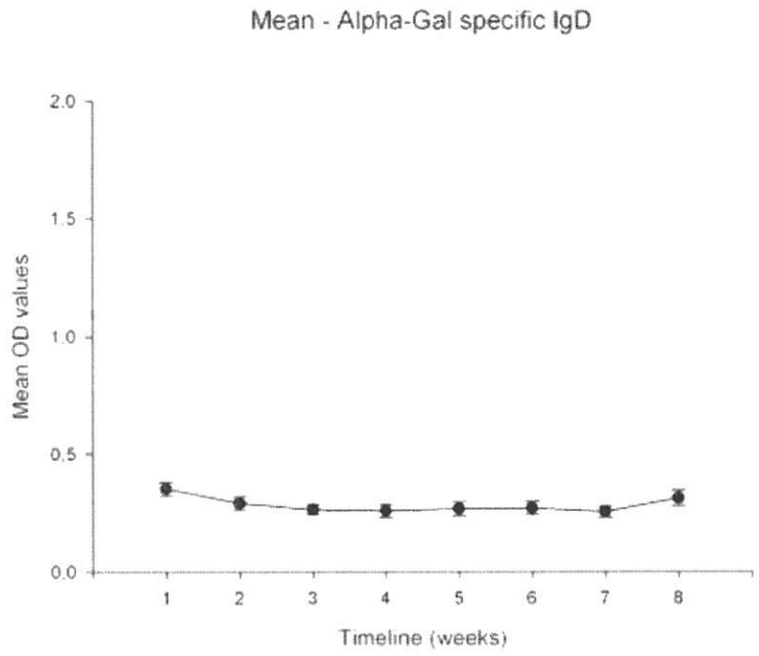

Figure 15: Each time point represents the average of α-Gal IgD ELISA OD values at the respective time of measurement. Error bars ± standard error of the Mean.

Lastly, we explored the alpha-Gal specific IgD antibodies and examined significant valve changes between each time point. We could not find any significant change apart from week one to week two ($p<0,05$). It can be said that the anti-Gal IgD titer remained stable over the observed time frame as well.

No demographic data (age, height, sex, pack years, allergies, medication, vegetarian, meat/week, yoghurt/week, probiotics/week) correlated with the mean titer level of any proband.

3.5.2. Evaluation of total IgD titer levels

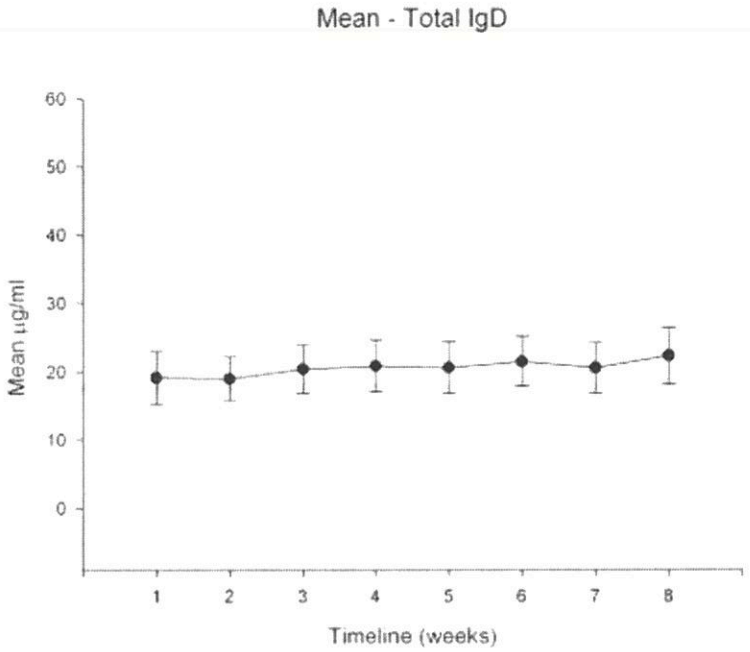

Figure 16: Each time point represents the average of total IgD ELISA OD values at the respective time of measurement. Error bars ±- standard error of the Mean.

We measured the total Immunoglobulin D content. We did not find any correlation between the total IgD titer level and the alpha-Gal titer level. The titer changes of the total IgDs did not correlate with anti-Gal IgD titer alterations.

4. DISCUSSION

4.1. Discussion of Results

In our study we measured the alpha-Gal specific Immunoglobulins, IgG, IgM, IgA, IgE and IgD. Except for anti-Gal IgE, all anti-Gal Ig titers remained stable over the observed time period of 8 weeks.

Furthermore, we compared our results with the demographic data and we chose age, height, sex, pack years, allergies, medication, vegetarian, meat/week, yoghurt/week and probiotics/week because of ongoing research of our own and other study groups. To the best of our knowledge, there are no correlations to be known yet. Even though we observed a small cohort in our study (n=19), we perceived a strong correlation of anti-Gal IgM and sex, so we could reproduce the results of *Nydegger et al. [80]* who reported the same correlation in their results.

Significant titer changes could be found in anti-Gal immunoglobulin isotype G, M, A and E between week one and week two. As we found no correlation with any observed data and after revalidation of our results on accuracy, no convincing hypothesis on these observed changes can be given. It cannot be totally excluded that the mathematical functions of the curve-fitting favoured this outcome.

In addition, we evaluated the total Immunoglobulin contents. The total Ig titer levels were more variant than the specific anti-Gal titer levels and thus, it is conjecturable that the most anti-Gal titers remain unaffected by overall Ig titer changes. Anti-Gal IgG and IgE correlated with the dynamic of the total IgG and IgE levels and thus, we are of the opinion that there is a direct link between anti-Gal IgE and IgG and their total Ig titers.

The IgE isotype turned out to be a special case. Most of the probands were negative. The single proband being highly positive for anti-Gal IgE had to be excluded from our study as he was diagnosed during our study period to suffer from IgA-Nephritis. The high anti-Gal IgE titer correlated with the total IgE titer. No demographic data observed correlated with the measured alterations of IgE titers. From our point of view, it is unlikely that the IgA-Nephritis influences the anti-Gal IgE titer. Moreover, it is probable that in this case, the high anti-Gal IgE titers are a coincidence. Gal exposition is believed to occur in the gastro- intestinal tract and thus, it is likely that an alpha-Gal IgE sensitization takes place there as well, parallel to other IgE sensitizations. Therefore, *Bohle and collegues*, our

neighboring study group, perform studies on food allergy like *Platt-Mills et al. [79]* who showed that people with meat-allergy show heightened levels of anti-Gal IgE.

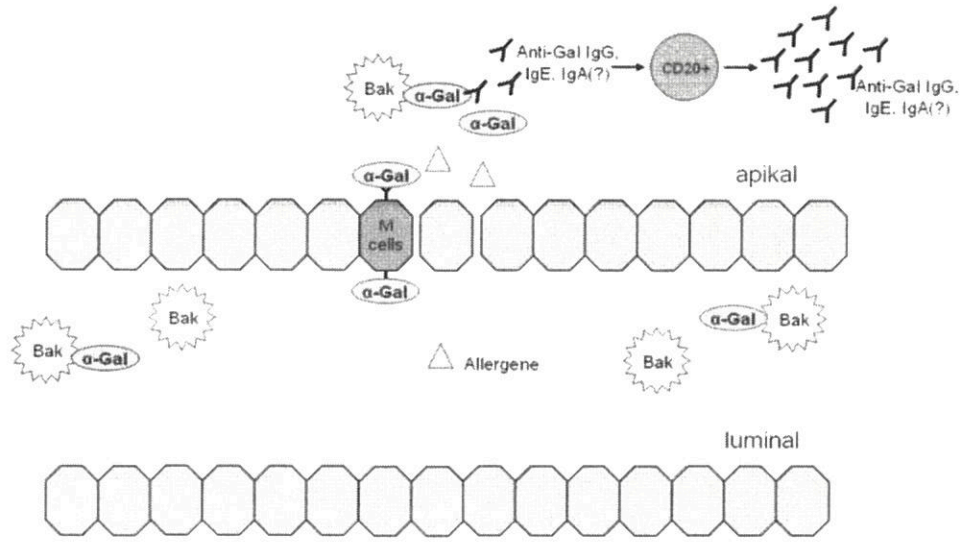

Figure 17: Schematic description of our hypothesis for significant higher anti-Gal titers in patients suffering from food allergy. Allergens and alpha-Gal are either penetrating the permeable intestinal epithelium, or are presented falsely to the lamina propria immune cells, or tolerance induction to these allergens/antigens is broken.

Other implications concerning anti-Gal immunoglobulins are inflammatory bowel diseases (IBD) like Ulcerative Colitis and Morbus Crohn. They are characterized through chronic inflammation in the gastrointestinal tract of unknown origin. The most frequent symptoms of both diseases are pain and diarrhea.

The commensal flora is usually tolerated in the gut, but it is likely that it is a major trigger for the chronic inflammation in IBD as well. The hypotheses for this false immune response are comparable to those that are meant to cause food allergy reactions, thus being a hyperpermeable epithelium, false antigen presentation, missing regulatory T cell activity or missing induction of anti-inflammatory feedbacks [82, 83]. From our point of view, it is highly favourable that α-Gal plays a role in that scenario.

The immunoglobulin isotype D is mostly unexplored. *Mosedale et al.* [81] described anti-Gal IgD antibodies for the first time. They measured the IgD levels in patients with and without severe atherosclerosis and identified a number of interesting differences in the levels of anti-carbohydrate antibodies between patients and controls. In our study, every healthy volunteer was anti-Gal IgD positive and the levels stayed constant over the 8 weeks. The anti-Gal IgD isotype is a very promising research topic and we are curious about future study results concerning Gal and atherosclerosis.

Immunoglobulin A stayed constant within the time period as well. Our study group is focusing on that isotype in current studies because we consider IgA to be of special importance to the Gal/anti-Gal homeostasis as secretory IgA stays in continuous contact with Gal-bearing bacteria. Therefore, we are planning to measure anti-Gal IgA in human fluids, such as tears, saliva, and fluid from colon lavages to correlate these values with the systemic anti-Gal IgA titers. Since different human fluids are searchable in different entities we expect a lot of these future results and promising preliminary data are already at hand.

The isotypes IgG remained constant too. This was very important for us, in relation to our former bioprosthesis study results to strengthen their validation. Our hypothesis that the anti-Gal antibodies stay constant, amplifies the results of *Ankersmit et al.* [61] and *Mangold et al.* [65]. A binding statement can be made by saying anti-Gal antibodies are constant and thus, α-Gal epitopes must be present on implanted bioprosthetic heart valves and must be a trigger for anti-Gal IgM and IgG production, otherwise anti-Gal titers would not have significantly changed right after implantation. In our longitudinal study a slight dynamic of anti-Gal IgG titers takes place and they correlate with the total IgG titer levels. Therefore, it is important to set strict inclusion and exclusion criteria while measuring IgG in consideration of illnesses or other causes which affect the total IgG titer levels.

The anti-Gal IgM titer levels were not variant as well. The specific anti-Gal IgM antibodies correlated with the sex as in the study of *Nydegger et al.* [80] who explains the fact that women have significantly higher levels of anti-Gal IgM with the hypothesis that women have a higher risk of developing autoimmune diseases like the Graves`disease than men and thus, higher titers of anti-Gal IgM antibodies. However, they emphasize that it is not established yet if the anti-Gal antibodies are a consequence or a cause of these autoimmune diseases. At this point it is to mention that in 1991, *Galili* and colleagues

[84] tested the α-Gal epitope on human normal and autoimmune thyroid cells as they suggested that an interaction between anti-Gal and aberrantly expressed Gal-epitopes on thyroid cells may contribute to the initiation of autoimmune thyroid disorders. The Gal epitopes were assessed with a sensitive radioimmunoassay and the epitopes were found both on normal and diseased thyroid cells. Although the concentration of these epitopes on Graves`disease thyroid membranes was somewhat higher that that observed in normal gland no significant differences could be found. According to our opinion it is to expect that anti-Gal IgM titer are higher in women than in men but there is no sufficient explanation for this hypothesis what on the other hand constitutes a promising research topic for the future.

4.2. Outlook

The anti-Gal antibodies are the most highly abundant antibodies in human circulation. To the best of our knowledge, this is the first description of a longitudinal evaluation of all anti-Gal immunoglobulin isotypes.

The antibodies as well as the antigens are well accessible as α-Gal can be easily stained, α-Gal knockout mice exist and potentially every kind of isotype is to be found. Therefore, we are of the opinion that this is a great model for immunehomeostasis. Unfortunately, a commercially available human antibody is not purchasable until present maybe this is more or less associated with the previous dominance of some special study groups. It is to be hoped that the vibrant research of many smaller study groups will change this condition. We assume that the evaluation of α-Gal in different entities will elucidate how the interaction works and whether there are inhibiting antibodies against anti-Gal antibodies or not. Anyway, we are curious about our own and other upcoming study results concerning α-Gal. With our presented data in this study, we created an important basis to evaluate the reliability and mandatory statements can be made as the anti-Gal titers in young healthy adults are mainly constant.

5. ABBREVIATIONS

α-Gal	Galα1-3-Galβ1-4GlcNAc-R
α1,3GT	β-Galactosyl α1-3-Galactosyltransferase
AB	Antibody
ADCC	antibody dependent cellular cytotoxicity
APC	antigen-presenting cells
BSA	bovine serum albumine
CLSM	confocal laser scan microscopy
CPH	ceramide pentahexosidase
CTL	cytotoxic T lymphocytes
ELISA	enzyme-linked immunoabsorbent assay
FET	formaldehyde, ethanol and Tween 80
GA	glutaraldehyde
hDAF	human decay accelerating factor
HRP	horseradish peroxidase
IgM,E,A,D,G	Immunoglobulin M,E,A,D,G
KO	knockout
MAC	membrane attack complex
MHC	major histocompatibility complex
MMP	matrix-metalloproteinase
NK cells	natural killer cells
PBS	phosphate buffered saline

SEM	Standard Error of the Mean
TMB 3,3',5,5'	Tetramethylbenzidine

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7. LIST OF FIGURES

8. APPENDIX

Curriculum Vitae

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Education	1990 – 1994	**Primary School** Sacre Coeur Riedenburg, Bregenz, Austria
	1994 – 2002	**Bundesrealgymnasium** Gallusstraße, Bregenz, Austria
	1999 – 2000	Social Work within the Project "**Public Service**", Gallusstraße, Bregenz, Austria
	2002	**Cambridge Certificate of Advanced English**, Sidney, Australia
	2003 – 2010	**Medical University of Vienna,** Austria
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Medical clerkships	08/2006	**Department of Public Health**, Dr.Jungblut, Bregenz, Austria
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	02/2010	**Department of Internal Medicine**, Hospital of Dornbirn, Austria

Further Activities	2001	Catering Walch, Festspiele Bregenz, Austria
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