Low levels of natural IgM antibodies only partly explain susceptibility to neoehrlichiosis

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Abstract

Background: Neoehrlichiosis is an infectious disease caused by the tick-borne bacterium "*Candidatus* Neoehrlichia mikurensis". Splenectomy and rituximab (anti-CD20) therapy are risk factors for severe neoehrlichiosis, a condition characterized by fever and vascular complications such as deep vein thrombosis. The principal aim of this study was to examine if neoehrlichiosis patients had low levels of natural IgM antibodies and/or were hypogammaglobulinemic, and if such deficiencies were associated with asplenia and development of vascular complications.

Methods: Neoehrlichiosis patients (n = 9) and control subjects (n = 10) were investigated for total serum levels of IgG, IgA, and IgM, and levels of natural IgM antibodies directed against pneumococcal polysaccharides (6B, 14) by ELISA and against the malondialdehyde acetaldehyde epitope of oxidized LDL by a chemoluminescence immunoassay. The multivariate method Projection to Latent Structures (PLS) was applied to examine the relationships between levels of natural IgM antibodies, splenectomy, and vascular complications, respectively.

Results: Half of the neoehrlichiosis patients lacked or had very low levels of natural IgM antibodies directed against pneumococcal polysaccharides and malondialdehyde acetaldehyde compared with 1/10 of the age-matched control subjects. One neoehrlichiosis patient and one control subject each were hypogammaglobulinemic. The levels of natural antibodies to the two pneumococcal serotypes were positively associated to one another, and negatively associated to the development of deep vein thrombosis according to the multivariate analyses. Natural IgM antibody levels were not directly coupled to splenectomy.

Conclusions: Neither hypogammaglobulinemia nor depletion of natural IgM antibodies alone predisposes for severe neoehrlichiosis. This indicates that specific antibodies are likely to be key defense mechanisms in neoehrlichial infection.

Introduction

Candidatus Neoehrlichia mikurensis (*Ca.* N. mikurensis) is an emerging tick-borne pathogen of the *Anaplasmataceae* family that can infect human beings (1-3). It gives rise to the infectious disease neoehrlichiosis, which in severe cases may present as a febrile illness that does not respond to broad-spectrum antibiotics, frequently accompanied by vascular and thromboembolic complications such as deep vein thrombosis, pulmonary embolism, transitory ischemic attacks and arterial aneurysms (4). Diagnosis of this strict intracellular bacterium presently relies on PCR as it does not grow in blood culture flasks, nor are there any serological assays available (5). The novelty of this microbe and the fact that it escapes detection by routine microbiological diagnostic methods probably explains why there are only 30-some published cases of neoehrlichiosis worldwide despite the fact that *Ca.* N. mikurensis is widely spread among ticks and rodents in Europe and Asia (5).

The knowledge regarding what constitutes an efficient immune response to this new pathogen is scant. However, most patients afflicted by the more serious variant of neoehrlichiosis have been older and immunocompromised (4). As a rule, the patients have had an underlying hematologic malignancy such as chronic lymphocytic leukemia or malignant lymphoma, and/or a systemic rheumatic or autoimmune disease (4). A common theme for these diseases is that they are caused by clonal expansion of B lymphocytes and may feature hypogammaglobulinemia and/or impaired antibody production either directly, or secondary to immunosuppressive therapy. In addition, a high frequency of neoehrlichiosis patients have been splenectomized, and/ or treated with the B cell-depleting monoclonal anti-CD20 antibody rituximab (4; 6). Together, this indicates that B cells and antibodies may be important protective immune mechanisms to combat *Ca.* N. mikurensis infection.

The aim of this study was to investigate the role of natural IgM antibodies in neoehrlichiosis. Unfortunately, it is not possible to study anti-neoehrlichial B cell or antibody responses directly, as *Ca.* N. mikurensis has not yet been cultivated nor has its genome been sequenced. As a consequence, it has not been possible to set up specific ELISA, IFA or ELISPOT assays for serological analyses. We hypothesized that natural IgM antibodies might be an important host defense mechanism in neoehrlichiosis since polyreactive IgM antibodies produced by splenic B1 cells protect against murine

ehrlichiosis (7) and splenectomy is a risk factor for severe neoehrlichiosis. *Ca.* N. mikurensis is not believed to be encapsulated (8), implying that the importance of the spleen in neoehrlichial host defense does not lie in its ability to synthesize opsonizing anti-carbohydrate IgG antibodies. Natural IgM antibodies form a central part of innate immunity by virtue of their broad anti-microbial specificity (9-11). Unlike specific IgM antibody responses, the generation of natural IgM antibodies is not antigen-driven. In fact, natural IgM antibodies are present in cord blood. Supposedly, self-antigens drive their production, which explains why they also recognize stressed, damaged or dead host-derived cells (12; 13). Polyreactivity, i.e., the ability to bind several unrelated antigens that share epitope structures, is an essential feature of natural antibodies (7).

To test whether a shortage of natural IgM antibodies is a risk factor for contracting neoehrlichiosis, we measured the levels of natural IgM antibodies in the sera of patients diagnosed with neoehrlichiosis that could bind to an epitope of oxidized LDL named malondialdehyde acetaldehyde (13), expressed on apoptotic eukaryotic cell surfaces, and to pneumococcal polysaccharides (14), expressed on bacterial cell walls, respectively. We also quantified the total serum levels of IgG, IgA and IgM to evaluate if neoehrlichiosis patients were hypogammaglobulinemic. Finally, the data were analyzed using the multivariate method of pattern recognition "Projection to Latent Structures" to see if an association between natural IgM levels, splenectomy and vascular complications could be identified.

Materials and methods

Study subjects

Nine patients diagnosed with severe *Ca.* N. mikurensis infection in Sweden were investigated. Eight of the patient cases (SE01-SE12) have been described earlier (4; 15). All patients had febrile illness. Clinical data are summarized in Table 1. Sera collected from ten age- and gender-matched subjects sent to the Clinical Microbiology Laboratory at Sahlgrenska University Hospital in Göteborg, Sweden, for analysis of Borrelia-specific IgM and IgG antibodies (Chemiluminescence immunoassay, Liaison, DiaSorin S.p.A., Saluggia, Italy) were used as controls (Table 2). Ethical permission was granted by the local Ethical Review Board of Göteborg, Sweden.

Total IgG, IgA, and IgM in serum

Serum immunoglobulin quantification was done by nephelometry at the accredited Laboratory of Clinical Immunology, Sahlgrenska University Hospital, Göteborg, Sweden. Reference values are based on a Swedish population.

Natural IgM antibody levels to oxidized LDL epitope malondialdehyde acetaldehyde

The LDL fraction (density 1.019–1.063g ml^{-1}) was isolated from human plasma by sequential density-gradient centrifugation and modified by malondialdehyde acetaldehyde (MAA) as previously described (16). Natural IgM to MAA in serum samples was determined by a chemiluminescence immunoassay. In short, antigens (5 µg ml^{-1}) MAA-LDL, MAA-BSA (Merck, Darmstadt, Germany), MDA-BSA and 0.5% (w/v) fish gelatin (Sigma, St Louis, MO, USA) dissolved in 0.27 mM EDTA in PBS were immobilized overnight in 96-well microtiter plates at 4°C. The wells were thrice in 0.27 mM EDTA in PBS. Nonspecific binding sites were blocked with 0.5% fish gelatin in 0.27 mM EDTA/PBS for 50 min at room temperature. Serum samples (1:100) were incubated for 1 h at room temperature. When measuring the total IgM concentration in plasma, 5 µg ml^{-1} of anti-human-IgM (Sigma) in 0.27 mM EDTA/PBS was immobilized to microtiter plates and purified human IgM (Sigma) was used as a standard. Serum samples from the patients and healthy controls were diluted

1:10 000 for determination of the total IgM concentration. Alkaline phosphatase-conjugated anti-human IgM (Sigma) was used as a secondary antibody and LumiPhos 530 as a substrate in the assay. The chemiluminescence was measured as relative light units (RLU) with a Wallac Victor^3 multilabel reader (Perkin Elmer, Waltham, MA, USA).

Natural IgM antibody levels to pneumococcal polysaccharide

Serum was assayed for IgM antibodies to pneumococcal serotypes 6B and 14 at the University College London Institute of Child Health's WHO Reference Laboratory for Pneumococcal Serology, by adapting a previously described IgG ELISA protocol (http://www.vaccine.uab.edu/ELISA%20Protocol.pdf). In short, serum samples were mixed with an absorbent that contains C-polysaccharide and 22F capsular polysaccharide to neutralize antibody binding to contaminants normally present in pneumococcal polysaccharides used for coating ELISA plates. Bound IgM antibodies were detected with anti-human IgM goat antibody conjugated with ALP at 1/3000 dilution (Sigma) for 2 h, at RT, followed by the addition of substrate *p*-nitrophenyl phosphate. Absorbance was read at 405 nm and 690 nm. Antibody concentrations were extrapolated from double point dilutions by using a standard human anti-pneumococcal reference serum, lot 89-SF.

Statistical analyses

The non-parametric Wilcoxon signed rank test was used to compare groups with a statistical significance level of $P < 0.05$, using Graph Pad Prism software 6.0 (San Diego, CA). Correlations were estimated by the Spearman correlation test. The multivariate method for pattern recognition "Projection to Latent Structures" (PLS) was applied to identify associations between levels of natural antibodies and other laboratory and clinical data. This technique merges features from principal component analysis and multiple linear regression, which can be used to identify association patterns between selected query variables (Y) and sets of analysis variables (X). Models can be generated to assess to what degree a set of variables can explain the covariance between Y-variable(s) and the analysis or predictor variables X (17).

Results

The neoehrlichiosis patients had a mean age of 65 (range 54-78), and seven out of nine were men (Table 1). The majority of the patients (7/9) suffered from a vascular complication, in most of the cases deep vein thrombosis, and less frequently pulmonary embolism, transitory ischemic attack or vascular aneurysm (Table 1). All but one had received immunosuppressive therapy, including rituximab in one third of the cases (Table 3). More than half of the patients were asplenic, either following splenectomy or due to inborn splenic aplasia. Anonymous age- and gender-matched persons with suspected *Borrelia burgdorferi s.l.* infections were selected as controls (mean age 66; range 54-79). The reasoning behind this choice of control subjects was that both *Ca.* N. mikurensis and *Borrelia spp.* are tick-borne bacterial species, and that the aging immune system and gender are factors that need to be taken into account in infectious host defense (18; 19).

Hypogammaglobulinemia

Since all of the neoehrlichiosis patients were immunocompromised, we first investigated if they had sub-normal levels of immunoglobulins (Table 3). One of the neoehrlichiosis patients (SE12) had serum IgG, IgA, and IgM levels below the reference interval, as did one of the control subjects (BO09). In addition, one of the neoerhlichiosis patients had serum IgG just below the cut-off level, but did not fall below the definition of IgG deficiency of < 6 g/L (Table 3). All in all, the majority of the neoehrlichiosis patients had normal levels of serum IgG (6/8), serum IgA (7/8) and serum IgM (8/9), as did the controls (9/10), for all three isotypes (Fig. 1).

Natural IgM antibodies to pneumococci

Analysis of natural IgM antibody levels to the pneumococcal serotypes 6B (of low antigenicity) and 14 (of high antigenicity) revealed that half of the assayable neoehrlichiosis patients (3/6) had undetectable levels of serum IgM to serotypes 6B and 14, respectively (Fig. 2 and Table 3). In comparison, 1/10 control subjects lacked natural IgM antibodies to serotype 14 and 2/10 had undetectable or very low levels of IgM to serotype 6B (Fig. 2).

Natural IgM antibodies to malondialdehyde acetaldehyde epitope

Half of the neoehrlichiosis patients also had markedly reduced levels of natural IgM antibodies to the oxidized LDL epitope malondialdehyde acetaldehyde (MAA). Hence, 4/9 assayable neoehrlichiosis patients had very low levels of antibodies compared with the age-matched control subjects (Fig. 3).

Multivariate analyses

Lastly, we examined if study subjects having high levels of natural IgM of one specificity tended to have high levels of the other specificities, i.e. if the levels of IgM to serotypes 6B, 14 and MAA were positively associated with one another. We also evaluated if the levels of natural IgM antibodies co-varied with age, sex, splenectomy, immune suppressive therapy, rituximab therapy, hypogammaglobulinemia, and development of vascular complications. The multivariate method of pattern recognition PLS was chosen to address these questions. The parameter "Levels of natural IgM antibodies to pneumococcal serotype 14" was set as the query Y-variable, and its relation to the following X-variables was assessed: levels of natural IgM to serotype 6B, levels of natural IgM to serotype MAA, total levels of serum IgG, IgA, IgM, immunosuppressive therapy (yes or no), rituximab therapy (yes or no), having a spleen (yes or no), and development of vascular complications (deep vein thrombosis, pulmonary embolism and/or transitory ischemic attacks). It was found that natural IgM antibodies to serotype 14 were positively associated with levels of natural IgM antibodies to serotype 6B, and negatively associated with development of vascular complications, deep vein thrombosis in particular (Fig. 4A). In addition, the multivariate analysis revealed a possible positive association between the levels of IgM to MAA with total serum IgG levels among the neoehrlichiosis patients. This was partly confirmed by univariate correlation analyses. A positive association was seen between the levels of natural IgM to serotype 14 and 6B (Fig. 4B), but it did not quite reach statistical significance, most likely because of too few study subjects ($n = 6$). A statistically significant positive association was shown between the levels of natural IgM to MAA-LDL and total serum IgG (Fig. 4C). We could not do univariate contingency analyses of the possible inverse relationship between having high levels of natural IgM antibodies to serotype 14 and the risk of developing vascular complications because of limited number of evaluable study subjects ($n = 6$). Nevertheless, it may be seen that the

three patients with undetectable natural IgM to serotype 14 (SE02, SE10, SE12; Table 1 and 3) all had contracted deep vein thrombosis. These same patients also had very low levels of natural IgM to MAA-LDL (Table 1 and 3).

It was not meaningful to do contingency analyses of the relationship between splenectomy and levels of natural IgM antibodies due to the limited number of patients. However, it was seen that out of the 6 neoehrlichiosis patients, 1/3 patients with high IgM levels to serotype 14 was splenectomized compared with 1/3 patients with low levels of antibodies to serotype 14. Similarly, half of the patients with high levels of antibodies to MAA were splenectomized (2/4) and half of those with low MDA levels were splenectomized (2/4). Together, this suggests that having or not having a spleen does not have a major influence on the levels of natural IgM antibodies.

Discussion

The observation that splenectomy and rituximab therapy seem to be independent risk factors for the development of severe neoehrlichiosis (4) led us to examine the hypotheses that shortage of natural IgM antibodies and/or hypogammaglobulinemia might feature among patients afflicted by severe neoehrlichiosis. Hypogammaglobulinemia could be discarded as a major protective mechanism as only one of the eight neoehrlichiosis patients had clearly reduced levels of all three immunoglobulin isotypes. This patient had not been treated with rituximab. Rituximab is a monoclonal antibody directed against CD20, which is expressed on the surface of most maturational stages of B cells except for plasma cells. Consequently, rituximab does not deplete the body of existing long-lived antibody-producing plasma cells, which explains why IgG deficiency only affects a fraction of rituximab-treated patients, roughly every third patient (20; 21). In contrast, IgM deficiency appears to be more common after rituximab treatment, affecting an estimated two thirds of treated patients (20). Irrespective of this, IgM deficiency was not prominent among the studied neoehrlichiosis patients.

Half of the neoehrlichiosis patients lacked natural IgM antibodies. Our expectation was that all the patients in our cohort of immunosuppressed neoehrlichiosis patients would have been deficient in natural IgM antibodies since patients infected with the related pathogen *Ehrlichia chaffeensis* develop high titers of polyreactive IgM antibodies, implying that this might be a crucial immune defense mechanism (7). Our finding of a near-significant correlation between IgM seroreactivity to the two pneumococcal serotypes among the neoehrlichiosis patients might indicate polyreactivity. Unfortunately, we could not assess if there existed a correlation between IgM reactivity to MAA and pneumococcal polysaccharide due to the small number of assayable serum samples. The limited number of patients is one of the shortcomings of the study, although it should be emphasized that there are only thirty-some published cases of neoehrlichiosis worldwide. An intriguing finding was the apparently negative association between levels of natural IgM antibodies to pneumococcal serotype 14 and development of vascular complications, deep vein thrombosis in particular. Clot formation is a primitive form of infectious defense and might reflect an inability of the host to curb the infection.

An interesting observation was that 3/9 neoehrlichiosis patients were diagnosed with B-cell lymphoproliferative non-Hodgkin lymphomas, e.g. B cell chronic lymphocytic leukemia and diffuse large B cell lymphoma, diseases characterized by CD5+ B cell clones with dysregulated natural IgM production. In fact, a majority of B-CLL patients have B cell clones that express IgM B-cell receptors specific for oxLDL epitopes (22-24). The clones are often frozen in an anergic, resting (G0 stage of cell cycle) and do not release IgM antibodies until activated by innate receptor signals such as TLR9 ligands, which includes un-methylated bacterial CpG-rich DNA (22). However, patient SE01 presented with extreme levels of natural IgM to the oxidized LDL epitope MAA (160 RLU x 10^{-3}) and elevated total serum IgM values of 7.1 mg/ml, which might indicate that the leukemic clone secreted IgM anti-oxLDL appearing as an M-component.

The spleen did not seem to be of major importance for the generation of natural IgM in this study cohort as shortage of natural IgM was equally frequent in the splenectomized as in the non-splenectomized group of patients. It is far from clear where natural IgM are produced in the human body. In mice, B1 cells located in the spleen are the major producers of natural IgM, but other sites of production such as the bone marrow, body cavities and regional lymph nodes may also be engaged (9). The B1 subset of natural antibody-producing cells has not been defined in humans, but splenic marginal zone B cells have been proposed to be possible B1 counterparts: A subset of the human marginal zone B cells have polyreactive B cell receptors that can bind a variety of microbial structures, akin to the toll-like receptors and generate T cell independent antibody responses (25). Moreover, DiGeorge patients have with pulp atrophy of the spleen and decreased levels of marginal zone-like B cells in the circulation together with reduced levels of natural IgM antibodies (26).

Altogether, our results suggest that asplenia probably constitutes a risk factor for neoehrlichiosis patients because the spleen is where specific IgM and IgG memory B cells are located (27; 28), rather than where natural IgM antibodies are produced. Severe neoehrlichiosis has only been described in older patients with underlying systemic rheumatic or hematologic disease, not in persons splenectomized because of trauma, and it is the former group of patients that appears unable to compensate for the removal of the spleen by establishing memory B cells in other body sites (29). Similarly, rituximab therapy has a dramatic effect on the levels of circulating memory B cells, which

recover much more slowly, if at all, after termination of therapy compared with naïve B cells (30). Hopefully, we will be able to test the hypothesis that loss of specific IgM memory B cells is a crucial risk factor for severe neoehrlichiosis in the near future using specific immune assays once bacterial antigens or cultivated bacterial are available.

Table 1. Clinical data of neoehrlichiosis patients

Patient ID	Sex	Age	Diagnosis	Vascular complication
SE01	Male	77	B-Chronic lymphocytic leukemia	DVT, PE, TIA
SE02	Male	75	B-Chronic lymphocytic leukemia	DVT
SE03	Female	67	Systemic lupus erythematosus	TIA
SE05	Male	54	Hereditary gout, psoriasis	Aneurysm
SE06	Male	59	Diffuse large B cell lymphoma	None
SE09	Male	78	Rheumatoid arthritis	None
SE10	Male	55	Granulomatosis with polyangiitis	DVT
SE12	Male	57	Pre-B-Acute lymphocytic leukemia	DVT
SE13	Female	65	Autoimmune hemolytic anemia	DVT, PE

DVT = Deep vein thrombosis; PE = Pulmonary embolism; TIA = transitory ischemic attacks.

Table 2. Control subjects

ID	Sex	Age	Serum antibodies to Borrelia (AU/mL)	
			IgM (Ref <25)	IgG (Ref <15)
BO01	Male	54	7.6	<5
BO02	Male	54	15	1012
BO03	Male	58	<2	<5
BO04	Male	60	14	954
BO05	Male	71	16	12
BO06	Male	72	9.6	50
BO07	Male	79	2.6	<5
BO08	Male	74	<2	874
BO09	Female	67	11	<5
BO10	Female	67	11	39

Table 3. Serum levels of natural IgM Abs in neoehrlichiosis patients in relation to asplenia and immunosuppressive therapy

Patient ID	Immunosuppression			Total serum		Pneumococcal serotype		Oxidized LDL epitope
	Splen-ectomy	Ritux[a]	IS[b]	IgM* mg/mL	IgG mg/mL	6B µg/mL	14 µg/mL	MAA[c] RLU[d]x10^{-3}
SE01	Yes	No	Yes	7.1	10	NA[e]	NA	160
SE02	Yes	No	Yes	1.9	7.5	**<0.15**	**<0.15**	**3.5**
SE03	Yes	No	No	1.2	14	NA	NA	16
SE05	No	No	Yes	1.0	19	0.78	0.52	16
SE06	Yes	Yes	Yes	0.50	NA	NA	NA	8.6
SE09	No	Yes	Yes	0.41	8	0.48	1.1	**3.0**
SE10	No	Yes	Yes	0.45	6.5	**<0.15**	**<0.15**	**5.1**
SE12	No	No	Yes	0.27	3.0	**<0.15**	**<0.15**	**0.69**
SE13	Yes	No	Yes	0.96	8.4	1.1	0.68	15

a) Rituximab therapy

b) Any type of immunosuppressive therapy

c) MAA, malondialdehyde acetaldehyde

d) RLU, relative light units

e) NA, not assessed

Figure 1

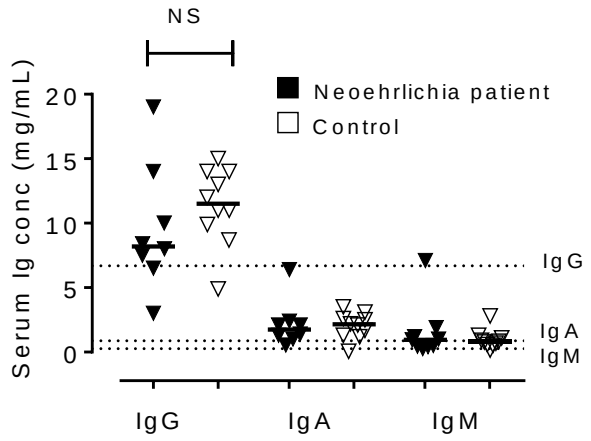


Fig. 1 Total serum IgG, IgA, and IgM levels among neoehrlichiosis patients and control subjects. Each symbol denotes one individual. Horizontal bars indicate medians. Dashed line shows lower reference value for serum IgG (6.7 g/L), IgA (0.88 g/L) and IgM (0.27 g/L), respectively. The Mann-Whitney test was used for statistical comparisons.

Figure 2

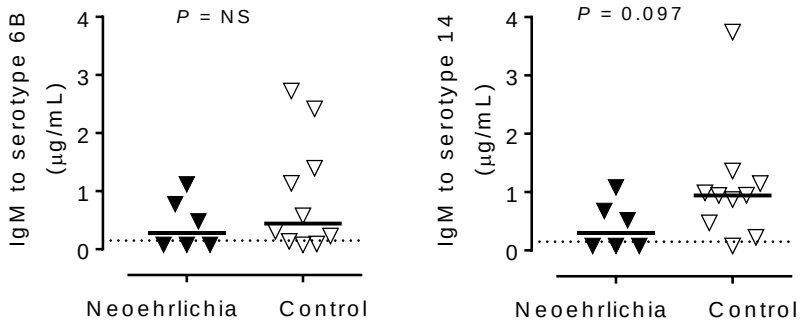


Fig. 2 Levels of natural IgM antibodies to pneumococcal polysaccharides among neoehrlichiosis patients and control subjects. Each symbol denotes serum IgM level to the indicated pneumococcal serotype derived from one individual. Horizontal bars indicate medians. Dashed line shows the detection limit of IgM antibodies = 0.15 µg/mL. The Mann-Whitney test was used for statistical comparisons.

Figure 3

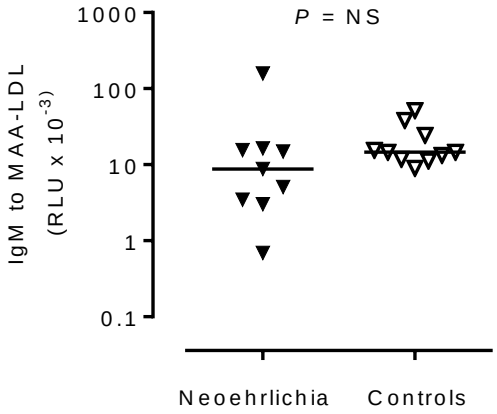
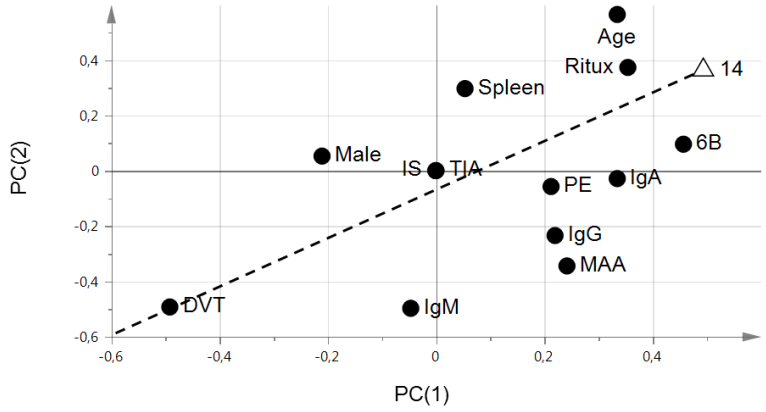


Fig. 3 Serum levels of natural IgM antibodies to the oxidized LDL epitope malondialdehyde acetaldehyde (MAA) in patients with neoehrlichiosis and healthy controls. Natural anti-MAA IgM antibodies in serum samples of neoehrlichiosis patients (n = 9) and age- and gender-matched controls (n = 10) were determined by a chemiluminescence-based immunoassay and expressed as "Relative light units" (RLU). Horizontal bars indicate medians. (P = 0.24; Mann-Whitney test).

Figure 4

A



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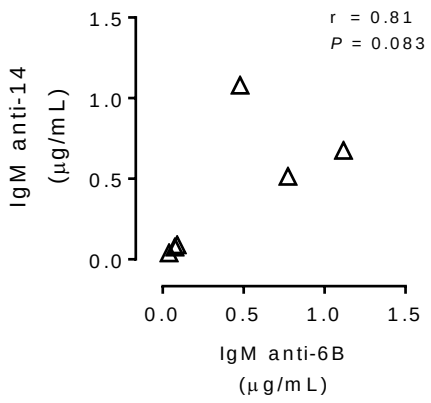

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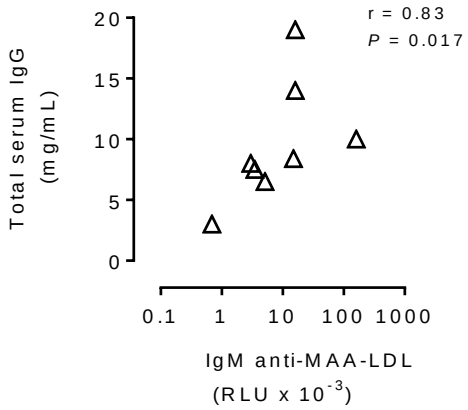

Fig 4. The levels of natural IgM antibodies to pneumococcal serotype 14 are positively associated with natural IgM antibody levels to serotype 6, and negatively associated to occurrence of deep vein thrombosis in patients with neoehrlichiosis. (**A**) The multivariate method of pattern recognition Projection to Latent Structures (PLS) was employed. The selected Y-variable was "levels of natural IgM to pneumococcal serotype 14", indicated by a triangle labelled "14". The analyzed X-variables were deep vein thrombosis (DVT), levels of natural IgM antibodies to malondialdehyde acetaldehyde (MAA), male sex, immunosuppressive therapy (IS), transitory ischemic attacks (TIA), total serum IgM levels (IgM), having a spleen (spleen), total serum levels of IgG, IgA, and IgM, pulmonary embolism (PE), age, rituximab therapy (Ritux), and levels of natural IgM antibodies to pneumococcal serotype 6B (6B). Parameters that are closely located to the Y-variable in the graph are positively associated with the Y-variable, whereas variables that are distant from the category marker along the diagonal line are negatively associated with the Y-variable. The quality of the generated model is indicated by R2Y, which estimates the amount of variance in Y that is explained by the X-variables (0.97) and the validity of the model is indicated by the Q2Y-value (0.61). PC(1) and PC(2) indicates that the model is composed of two principal components (**B**) Correlation between natural IgM levels to serotype 14 and serotype 6B in neoehrlichiosis patients (*n* =6). (**C**) Correlation between total serum

IgG and levels of natural IgM against malonacetate aldehyde-LDL in neoehrlichiosis patients combined (n = 9). r = Spearman correlation coefficient.

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