The role of mannose binding lectin in infectious complications of paediatric hemato-oncologic diseases

Ph.D. Thesis

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LIST OF PUBLICATIONS

Publications providing the basis of the dissertation

I. **Fekete F**, Fadgyas B, Papp É, Szilágyi Á, Prohászka Z, Müller B, Kovács G. The role of mannose binding lectin on fever episodes in pediatric oncology patients. Pathol Oncol Res. 2016 Jan;22(1):139-43. doi: 10.1007/s12253-015-9992-x. Epub 2015 Oct 3. PMID: 26433879.

II. Dobi M, Szilágyi Á, Csuka D, Varga L, Prohászka Z, Bereczki C, Kovács G, **Fekete F.** The Role of Mannose-binding Lectin in Infectious Complications of Pediatric Hemato-Oncologic Diseases. Pediatr Infect Dis J. 2021 Feb 1;40(2):154-158. doi: 10.1097/INF.0000000000002919. PMID: 33433161.

1. INTRODUCTION

The tumours of the blood-forming organs account for more than one third of all malignant tumours among children. Modern treatment of childhood malignancies has markedly changed, leading to a higher life expectancy. Over the past 20 years, the possibilities of oncological therapy have improved significantly, leading to a better prognosis. Currently, the five-year survival rate is more than 70-80% in cases of paediatric tumours. However, infectious complications still remain a serious problem, as they considerably worsen mortality and morbidity. The mortality is still significant, as the chemotherapy-induced immunosuppression increases susceptibility to infections, which contributes to about 10–20 % of mortality in paediatric oncology.

Chemotherapy often induces neutropenia and leukopenia, and therefore impairs cellular functions of the adaptive and innate immune systems. Hence, appropriate function of the humoral immune defence mechanisms such as the complement system is essential in protection against infections in hemato-oncological patients.

1.1. Complement system and lectin pathway

The complement system is an important part of the innate immune system that serves as a first line of defence against foreign and altered host cells. This is an essential component of the ancient immune response to infections caused by a wide variety of pathogens.

The complement system consists of more than 50 small proteins that are synthesized by the liver. Some of these proteins circulate in the plasma and tissue fluids; some bind to cell membranes. The circulating proteins are in an inactive proenzyme formed in the plasma, and are activated by a triggered-enzyme cascade.

The complement system is activated through three different pathways on pathogen surfaces: the classical, alternative and lectin pathways. The three pathways each result in the formation of a shared C3 convertase, the activation of the central element of the complement cascade. The C3 cleaving initiates the splitting of C5 and forms the C5 convertase, which finally binds the terminal portion of the complement cascade (C6, C7, C8, and C9).

Complement activation triggers the immune functions, leads to destruction of pathogens or abnormal host cells through opsonisation and phagocytosis, activates a cell-killing membrane attack complex, and modulates the inflammation by attracting macrophages and neutrophils.

Products of the complement cascade also have an important role in modulating aspects of humoral and cell-mediated immunity via interactions with B cells and T cells.

The lectin pathway is activated through the mannan-binding lectin (MBL) and a the ficolins and collectins. The serum MBL protein recognises the carbohydrate patterns on microbiological surfaces, and forms a complex with mannose-binding lectin-associated serine protease (MASP1, MASP2). The complex cleaves C4 and C2 to form the C3 convertase.

MBL binds with high affinity to several microorganisms, which are frequent pathogens in haematological departments and are frequent causes of severe sepsis, such as Gram-negatives enterobacteriacea, several types of *Streptococcus*, *Staphylococcus aureus*, and *Candida albicans*.

The serum functional MBL concentration is mainly genetically determined. The MBL protein is encoded by the MBL2 gene (10q11.2-q21) which consists of four exons. The promoter region of the gene contains a single nucleotide polymorphism (SNP) at position-221, denoted Y/X in the literature. In the first exon, the gene may contain three single nucleotide polymorphisms causing amino acid substitutions in the protein. The three variant alleles are at codons 54 (Gly54Asp), 57 (Gly57Glu), and 52 (Arg52Cys). They are termed B, C, and D respectively, and any of these variants on a chromosome is referred to as the 0 allele, while the wild type allele without any polymorphic variant is named A. Thus, the occurrence of either of the amino-acid changes causes disturbance in the structure of the collagen-like domain and decreased stability of the higher-order forms, resulting in a markedly reduced functional MBL level and decreased activation of the lectin pathway. Variant MBL oligomers bind with lower affinity to the carbohydrate patterns on microbiological surfaces; therefore their occurrence results in a reduced functional MBL level.

An increasing amount of data suggests an important role for MBL level in immunosuppressed conditions among adults. In the case of immunosuppression resulting from bone marrow inhibition following chemotherapy, it is becoming increasingly accepted that carrying MBL polymorphisms may predispose adults to more frequent and severe infections. However, contradictory data have been published concerning childhood malignancies under immunosuppressed conditions. What role MBL may play is still unclear, despite research having been conducted since the late 1990s.

2. AIM OF THE THESIS

2.1 We studied children who were diagnosed with malignant diseases and treated between 2001 and 2008 at the 2nd Department of Paediatrics of Semmelweis University in Budapest in a retrospective study.

We examined:

2.1.1. we studied the distribution of MBL level-determining polymorphisms,

2.1.2. we compared the frequency of MBL2 alleles in children with vs. without malignancies,

2.1.3. we examined the possible role of polymorphisms influencing MBL serum level on the incidence, frequency, and duration of febrile neutropenia (FN) in hemato-oncological patients.

2.2. We studied children treated between 2009 and 2012 with hemato-oncological diseases at the 2nd Department of Paediatrics of Semmelweis University, Budapest and at the Department of Haematology of Heim Pál Children's Hospital, Budapest in a prospective study. We examined:

2.2.1. the role of polymorphisms causing low MBL levels in the frequency of febrile neutropenic episodes,

2.2.2 whether the MBL genotype affects the severity of infections during chemotherapy,

2.2.3. how the survival rate without infection after the beginning of chemotherapy is related to the MBL genotype,

2.2.4. the relationship between MBL-MASP2 complex activity and MBL genotype,

2.2.5. the association between polymorphisms resulting in low MBL levels and activation of the MBL-MASP2 complex in children suffering from hemato-oncological diseases,

2.2.6. several characteristics of febrile neutropenic episodes occurring within eight months of the beginning of therapy.

3. MATERIAL AND METHODS

Fifty-four patients (24 girls, 30 boys) diagnosed with malignant diseases and treated between 2001 and 2008 at the 2nd Department of Paediatrics of Budapest's Semmelweis University were enrolled into our retrospective clinical study. Inclusion criteria were hemato- oncological disease and an age of 18 years or younger at the date of diagnosis.

The diagnoses of enrolled participants were: acute lymphocytic leukaemia (ALL) (N = 30); acute myeloid leukaemia (AML) (N = 2); Hodgkin's disease (N = 7); non-Hodgkin lymphoma (NHL) (N = 9), and osteosarcoma (N = 6). Each patient received chemotherapy according to protocols ALL (IC) BFM 95/2002, AML BFM 98, COSS 96, Interfant 98, NHL BFM 95 or HD 95. Chemotherapy was the only treatment modality used in the study population.

To assess the frequency of the MBL2 polymorphisms in an age-matched population, 53 children without malignancies were enrolled as controls with the following diagnoses: phimosis, preputial adhesion, hernias (inguinal, umbilical and abdominal), pectus excavatum, major labial adhesion, acute appendicitis, acute gastroenteritis, celiac disease, carpal ganglion; fractures, verrucas, gland mycosis, varicocele or testicular hydrocele.

3.1.2. Prospective study

Ninety-seven children treated between 2009 and 2012 with hemato-oncological diseases at the 2nd Department of Paediatrics of Semmelweis University, Budapest and at the Department of Haematology of Heim Pál Children's Hospital, Budapest were enrolled into our prospective clinical study. The inclusion criteria included a newly diagnosed hemato-oncological disease and patients under 18 years of age at the time of diagnosis. The diagnoses of participants were acute lymphoid leukaemia (ALL) in 76 cases, acute myeloid leukaemia (AML) in 10 cases, and non-Hodgkin lymphoma (NHL) in 11 cases. Each patient received myeloablative chemotherapy, according to current protocols ALL (IC) BFM 2002/2009, AML BFM 98 and NHL BFM 95, respectively.

Both studies were approved by the National Ethical Committee (TUKEB 180/2007), and parents or guardians of all participants gave informed consent.

3.2.1. Retrospective study

Fever episodes that occurred during chemotherapy or shortly after treatment were followed up for two years after the diagnosis of patients with hemato-oncologic disorders. Febrile

neutropenic episode (FN) was defined as an axillary temperature exceeding 38°C and a granulocyte count under 0.5G/ l. Several parameters were recorded during each episode, such as the date of first and last day of fever, and certain clinical parameters (WBC, Neutrophils and CRP) determined at the onset of the episode, at the time of blood culture test, and on the first day of normal body temperature. In the case of positive blood culture, the identified microbe, its antibiotic resistance, and the treatment (antimicrobial and/or cytokine) were also registered.

3.2.2. Prospective study

The children were followed for a period of eight months dating from their diagnosis. The time of diagnosis was established by the results of histopathological findings. Patient characteristics, such as sex, age, tumour type, stage of disease, time of diagnosis, applied therapy, and mortality were collected from patient documentation. Febrile neutropenia (FN) was defined as an axillary temperature greater than 38°C and a granulocyte count less than 0.5 G/l. Several parameters of each febrile neutropenic episode occurring during the follow-up period were also recorded, such as the date of the first and last day of FN, certain clinical parameters (WBC, neutrophil, CRP), antimicrobial treatment, the time of blood culture and, in case of a positive blood culture, the identified microbe and antibiotic resistance.

In both studies, EDTA-anticoagulated blood samples were obtained for genomic DNA preparation using a salting-out procedure. Genotyping of MBL2 C (rs1800451), D (rs5030737), and Y/X (rs7096206) polymorphisms was carried out by real-time PCR with commercially available TaqMan® SNP Genotyping Assays (Applied Biosystems, CA, USA), while the B allele (rs1800450) was determined by PCR-RFLP.

For the measurement of MBL-MASP2 complex activity, native blood serum samples were used. Activation of the MBL-MASP2 complex was evaluated by enzyme-linked immunosorbent assay (ELISA) from blood serum obtained at the time of diagnosis and during an infection. This method is based on the C4-cleaving ability of the complex, described previously by *Presanis et al.* (2004), with some modifications (*Csuka et al.* 2010).

Data were evaluated with SPSS 13.0 (SPSS Inc., Chicago IL) and GraphPad Prism 4 (GraphPad Software Inc., San Diego, CA) software. Since most variables were non-normally distributed, non-parametric tests were applied. The Mann-Whitney U and the Kruskal-Wallis tests were used to compare two independent groups, and categorical data were analysed by the Pearson and χ^2 tests. The survival rate without infection was established by the Kaplan-Meier curve.

Multiple logistic regression analysis was applied, adjusted to the underlying disease, age, and sex of the patient as well as the applied chemotherapy protocol. The difference between MBL-MSP2 activity at the time of the diagnosis and during an infection was calculated by t-test. All tests were two-tailed, and statistical significance for p-values was considered less than 0.05.

4. RESULTS

The average age of the fifty-four enrolled patients (24 girls, 30 boys) at diagnosis was 9.4 years (range 3 months - 17 years). Allele frequencies of the studied SNPs of the MBL2 gene were compared in the groups of children with and without hemato-oncologic disorders. There were no significant differences in the allele frequencies in either the promoter or the exon 1 polymorphisms of this gene (Y, X, exon-1 A, B, C, D allels).

In our retrospective study, patients were assessed in three groups according to the expected serum level of MBL protein encoded by the carried genotype as reported by *Garred et al.* Group 1: patients carrying genotypes (YA/YA and YA/XA) encoding normal MBL level; group 2: patients with genotypes associated with low protein levels (XA/XA and YA/0); and group 3: MBL-deficient (XA/0 and 0/0) subjects.

As the incidence of infections and their treatment is different in distinct childhood malignancies, the ratio of different diseases was evaluated in the three groups of patients according to the carried MBL2 genotype. The difference among all groups was not significant ($p = 0.85$).

The analysis of the features of febrile neutropenia during the first two years following diagnosis in three genotype groups has revealed a shorter time interval between diagnosis and the first episode in individuals with low MBL level (Group 2) and in MBL-deficient patients (Group 3) than in subjects with genotypes encoding normal MBL level (Group 1). However, this difference was not significant ($p = 0.196$)

There was a trend ($p = 0.052$) that patients with a lower expected MBL level based on the MBL2 genotype have a longer average duration of FN, indicating an inverse relationship between MBL level and duration of FN. Individuals with genotypes associated with lower MBL levels had slightly higher ratio of febrile days during chemotherapy in the first two years following diagnosis, but this difference was not significant ($p = 0.690$). The frequency of FN episodes was similar among the genotype groups (median 1–1.25 FN/year).

In the following analyses, patients carrying the variant allele of exon 1 polymorphism (A/0, 0/0) and those homozygous for the promoter allele associated with lower MBL expression level (XA/XA) were merged (groups 2 and 3). The average duration of fever episodes was significantly shorter (p = 0.035) in those carrying the AA genotype and maximum one X allele (YA/YA and YA/XA) than in patients with genotypes associated with lower functional MBL level (group 2 and 3). The median (IQ range) of average fever episode length was 3.7 days (0–5.4) in group 1 and 5.0 days (3.8–6.6) in the merged group of 2 and 3.

Next, we performed a multiple logistic regression analysis in order to assess the strength of the association among MBL2 genotype groups and the average duration of FN (dichotomized at the median: ≤4 days vs. >4 days). The carrier state of genotypes associated with low or deficient functional MBL level was found to be a significant risk factor for longer average duration (>4 days) of fever episodes after adjustment for the diagnosis (OR (95 % confidence interval), 1.84 (1.04–3.25), p = 0.037) or the applied chemotherapy protocol (OR: 1.86 (1.05–3.28), p = 0.033) or the duration of chemotherapy (days) (OR: 3.34 (1.06–10.56), p = 0.040) as possible confounding variables.

We evaluated 97 patients, of which 54 were boys and 37 were girls, and the mean age was 8.03 ± 4.43 years at the time of diagnosis. The patients were followed for 8 months after the beginning of therapy. During the study period 12 patients died, and 6 of them died during the follow-up period, thus in the end, data from 91 patients were analysed.

We determined the genotype of the enrolled patients. Genotype distribution aligned with the Hardy–Weinberg equilibrium, and allele frequencies corresponded to the frequencies described in the Caucasian population.

Patients were divided into two groups based on genotype classification established in previous studies by *Frakking et al.* The first group included patients with low expected MBL level coding genotypes (YA/0, XA/0, 0/0, XA/XA), while the other group consisted of patients with normal expected MBL level coding genotypes (YA/YA, YA/XA). First, we studied whether certain diseases occur with the same frequency in both genotype groups. No significant difference was found (p=0.31), so the involvement of patients with different diseases is unlikely to have distorted the results of the study.

We then analysed the correlation between the characteristics of febrile neutropenic episodes occurring during the follow-up period and the MBL2 genotype groups.

The total number of febrile neutropenic episodes was significantly higher (p=0.0016) and the total length of febrile neutropenic days was significantly longer (p=0.0112) in the group with genotypes encoding lower MBL level than in patients with genotypes encoding normal MBL level. In line with these findings, there was a trend observed in individuals with genotypes associated with lower MBL level having longer average duration of febrile neutropenic episodes, but this difference was not significant. Time intervals between the diagnosis and the first febrile neutropenic episode was found to be shorter in patients with lower expected MBL level (p=0.0018).

Regarding the other parameters recorded (white blood cells, C-reactive protein, identified microbe), no significant difference was found between the two groups of genotype.

We assessed the likelihood of patients contracting infections during the follow-up period. We studied the length of the period until the first febrile neutropenia, i.e. the period without infection, in the two genotype groups. According to the Kaplan-Meier survival analysis, patients carrying genotypes coding normal MBL level have a higher chance for a longer period without febrile neutropenia (Log-rank test p = 0.0029).

We also examined the cofactors influencing infections with a multivariate Cox logistic regression model adjusted for the diagnosis, age, or applied chemotherapy. A-genotype carrying variant alleles could be a risk factor for infections in the first eight months with a hazard ratio of 1.649 (95% CI 1.014-2.681) (p=0.044). These patients are one and a half times more likely to contract an infection with febrile neutropenia than patients with a normal MBL level.

We analysed the correlation between the MBL-MASP2 complex activation and the MBL2 genotype. From the pre-chemotherapy samples available from 64 patients we obtained the expected result that the MBL2 polymorphisms considerably determine the MBL-MASP2 complex activation. In patients with the lower MBL level coding genotype, the activation level of the MBL-MASP2 complex is significantly lower (p <0.00001).

Both a pre-chemotherapy sample and a sample obtained during a febrile neutropenic episode were available for 42 patients, enabling us to study the changes in MBL-MASP2 activation during a febrile neutropenic episode. The activation decreased significantly during infections in patients with low MBL level coding genotypes. In this group, 81% of patients displayed

reduced or unaltered MBL-MASP2 activation, while in the normal MBL level coding group this ratio was 60%.

5. DISCUSSION

An increasing amount of data suggests an important role for MBL level in immunosuppressed conditions among adults, however contradictory data have been published concerning childhood malignancies.

In 2007 we began a retrospective study on the role of MBL SNPs in infections following chemotherapy in a study of paediatric oncological patients. Our study evaluated the influence of MBL2 gene polymorphisms on the incidence, frequency, and duration of febrile neutropenia in hemato-oncological patients. Our results showed that genotypes encoding high MBL level are associated with shorter-duration fever episodes in the first two years after the diagnosis of malignancy. Frequency of variant alleles of Y/X and A/0 polymorphisms in our patients was similar to that found in the general population. In our study, oncologic paediatric patients were compared with non-oncologic age-matched controls, and no difference was found in the allele distribution.

Analysing the characteristics of fever episodes in the first two years after the diagnosis of malignancy, we have found that patients carrying high MBL level coding genotypes (YA/YA and YA/XA) had shorter average duration of febrile neutropenia than individuals with genotypes coding for lower MBL serum levels (XA/XA, XA/0, YA/0 and 0/0). Differences were also found in time interval between the diagnosis and the first fever episode and the ratio of days with fever during chemotherapy among patients grouped by MBL2 genotypes, but none of these were significant. Therefore we continued our study and designed a prospective study.

The prospective study explored the relationship between the presence of MBL2 gene polymorphisms, the level of the MBL-MASP2 complex activation, and the increased risk of infection with febrile neutropenia in children suffering from hemato-oncological disease. We analysed the correlation between the incidence, frequency, and duration of febrile neutropenic episodes, and the expected MBL level based on MBL2 gene polymorphisms and MBL-MASP2 complex activation.

We found a correlation between the presence of MBL2 polymorphisms and the incidence of infections in children suffering from hemato-oncological diseases. The total number of febrile neutropenic episodes during the first eight months after the beginning of chemotherapy was

significantly higher in the group with genotypes encoding lower MBL level. In line with this finding, the total number of febrile neutropenic days was higher during the follow-up period. The average length of the febrile neutropenic episodes was not significantly different in the two groups, showing that the severity of infections is not influenced considerably by MBL level, presumably because of the intensive medication applied. The chance of a longer period without infection is more likely in patients with normal MBL level. In these patients the chance of suffering from an infection during the follow-up period is lower or, if they contract an infection, it typically occurs later. We considered the age and the applied chemotherapy through a Cox regression analysis, which supported the positive and predictive effect of the MBL2 genotype on the infections.

In accordance with these studies, we decided to analyse the MBL-MASP2 complex activation together with the MBL2 polymorphisms in pediatric hemato-oncologic patients. The MBL-MASP2 complex activation assay is a specific and sensitive method, which can eliminate all influential factors, and give information solely about the MBL-MASP2 complex function. The benefit of this method is receiving more information about lectin pathway and the functional activity of the MBL-MASP2 complex. As expected, genotype of the MBL2 polymorphisms onsiderably influenced the complex activity: in patients carrying a variant allele, the MBL-MASP2 activation was significantly lower. Moreover, we found a correlation between the decrease in complex activation during the febrile neutropenia and the genotype groups A possible reason for the decrease of the MBL-MASP2 complex activation during an infection could be the consumption of these molecules. This decreased activation has probaly also role in the susceptibility to infection.

In the literature, several studies discuss the role of MBL in diseases co-occurring with an immunosuppressive state, especially in patients with chemotherapy-induced neutropenia. Although research has been ongoing since the 1990s, the obtained results are contradictory. We have found 10 articles since 1999 which sought answers to the above questions. Five of these studies feature discussions which assume the effect of MBL on infection incidence and severity, while six research papers contradicted this assumption and dismissed the role of MBL.

Frakking et al. also published a comprehensive review in 2011 which attempted to clarify the reason behind these conflicting findings. They systematically searched for articles in the main databases (Embase, Medline, Cochrane Central Register) between 1966 and April 2010 which discuss the role of MBL in paediatric oncologic patients. They concluded that the contradictory

results of the examined studies might be explained by several clinical and methodological inconsistencies. Another possible reason may be that none of these studies examined the question as a multivariate risk analysis. The results of this systematic review showed that the MBL is probably not an independent risk factor for susceptibility to or severity of infection in paediatric oncologic patients. However, these results are refutable; therefore a clinically relevant study with an unified definition would be necessary to explain the role of MBL allowing for other risk factors.

Despite the contradictory results reported concerning the role of MBL, the efficiency and safety clinical trials of substitution therapy have begun in parallel with genetic testing. Currently two types of MBL preparations are in clinical phase trials: plasma delivered and human recombinant MBL. The phase I clinical trial, analysing safety and pharmacokinetics, found neither clinical nor laboratory changes. The biological activity, safety, and stability were similar in the two different preparations. Therefore the results are promising, yet a phase II/III randomized, placebo-controlled, double-blind clinical trial is still necessary to determine the clinical efficacy of MBL therapy.

In summary, our results support the importance of the MBL molecule in infectious complications of paediatric hemato-oncological patients, but further analysis would be necessary to confirm these results and to study other molecules of complement pathways that may influence the development of infections and could explain previous contradictory results.

Careful evaluation of all available data is of utmost importance in analysing the role of complement factors in immunosuppressed patients, as inter-individual differences, which may influence infection risk, are not limited to single gene variations but rather to a combination of genetically determined predispositions that can also be affected by acquired defects caused by the applied chemotherapeutic drugs. Some of the possible important molecules are the ficolins and collectins, as well as other heretofore unknown and undiscovered molecules.

The aim of these studies is to create an individual, personalized therapy based on genetic predisposition. Our study could help to define those patients who have susceptibility and risk for infection during chemotherapy. And the long-term benefit would be to determine those patients who may benefit from prophylactic MBL therapy or from infection prophylaxis by antibiotics that could be applied simultaneously with myeloablative chemotherapy to prevent infections. Further studies are necessary to examine the complement molecules and find new influencing factors, and to finally analyse this complicated and very complex system.

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