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.

ABBREVIATIONS

A, G, C, T: Nucleotides (A-Adenine, G-Guanine, C-Cytosine, T-Thymine)

ALL: acute lymphocytic leukaemia

AML: Acute myeloid leukaemia

BFM: Berlin-Freiburg-Münster Study Group

C1-C9: 1-9 complement component

CNS: Coagulase-Negative Staphylococcus species

CRD: Carbohydrate-recognition domain

CRP: C - reactive protein

DNS: Deoxyribonucleic acid

EDTA: Ethylen-ediaminetetraacetic acid

ELISA: Enzyme linked immunosorbent assay

FN: Febrile neutropenia

HL: Hodgkin lymphoma

ICU: intensive care unit

MAC: Membrane attack complex

MASP1, 2, 3: Mannose binding lectin associated protein 1, 2, 3

MBL: Mannose binding lectin

MOF: multiorgan failure

Neu: neutrophil count

NHL: Non-Hodgkin lymphoma

PAMP: Pathogen-associated molecular pattern,

PCR: Polymerase chain reaction,

pdMBL: plasma delivered mannose binding lectin

rMBL: recombinant mannose binding lectin

SDS: Sodiumdodecylsulphate

SNP: Single nucleotide polymorphism

WBC: white blood cell

SPSS: Statistics is a powerful statistical software (IBM)

1. INTRODUCTION

1.1. **Childhood tumours**

The tumours of the blood-forming organs account for more than one third of all malignant tumours among children[1]. 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[1,2]. 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[3,4].

Chemotherapy often induces neutropenia and leukopenia, and therefore impairs cellular functions of the adaptive and innate immune systems[5]. Hence, appropriate function of the humoral immune defence mechanisms such as the complement system is essential in protection against infections in hemato-oncological patients[6].

1.2. **Complement system**

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 members of the complement cascades are protease enzymes, which cleave and activate the next enzyme, resulting in the amplification of every subsequent enzymatic reaction. Several triggers can stimulate the system, leading to robust and efficient proteolytic cascades[7-9].

1.2.1. **Complement activation**

The complement system is activated through three different pathways on pathogen surfaces. The pathways differ from the molecules that initiate the complement cascade, but lead to the

activation of the same effector molecules. These three different pathways are referred to as the classical, alternative, and lectin pathways[10].

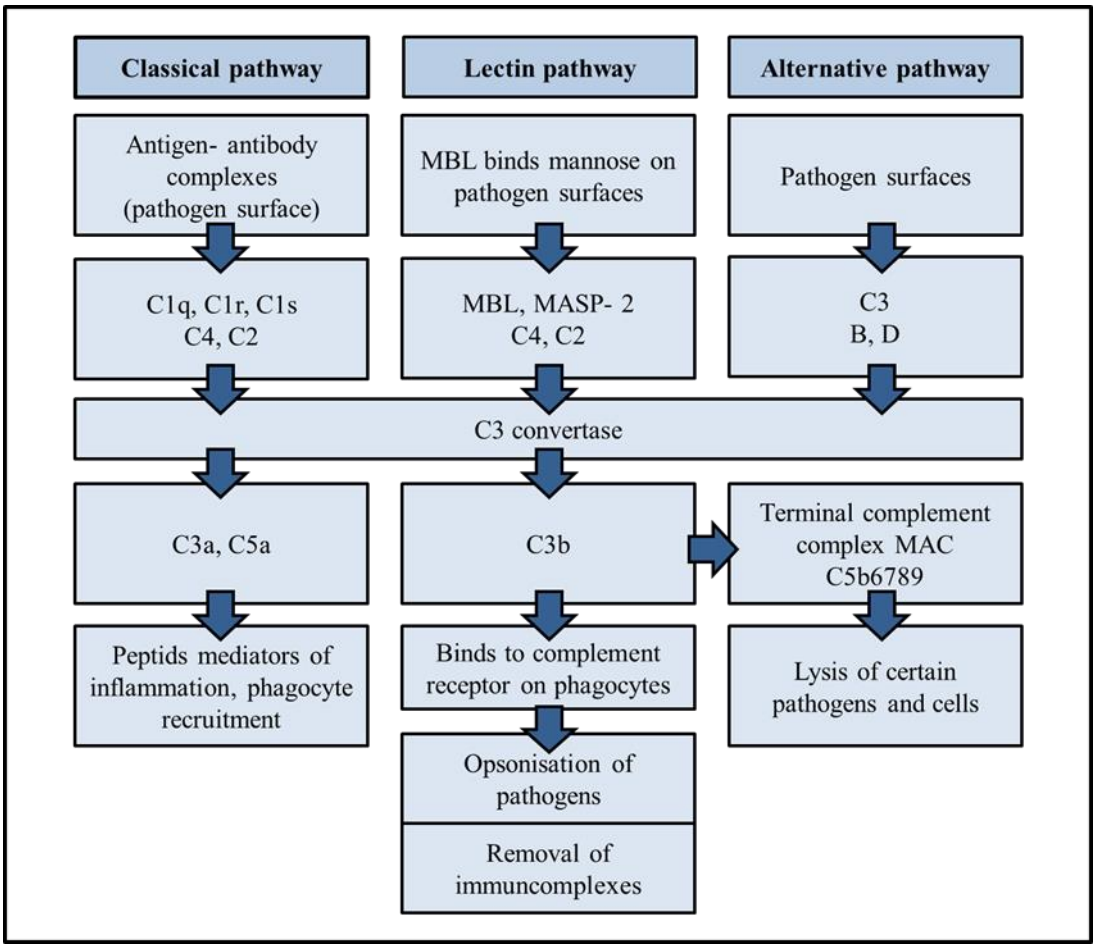

Figure 1 Complement cascade, Janeway's Immunobiology (Garland Science 2005)[10]

The classical pathway is initiated by antibody-antigen complexes binding to C1q molecules. The C1q recognise the Fc region of antibody isotypes IgG or IgM on the surface of the bacteria and viruses, binding to apoptotic cells and acute phase proteins. The inactive C1 complex consists of the C1q, C1r, and C1s molecules. The next step is the cleaving of C4 and then C2 to form two large fragments – C4b and C2b – finally leading to the generation of C3 convertase[7,8].

The alternative pathway is triggered by the C3b protein directly binding to the microbe surface through foreign materials and tissue damage. C3b binds to B factor and activates C3 convertase (C3bBb), regulated by the D, H, and I factors[7,8].

The mannan-binding lectin pathway is similar to the classical pathway[11]. Mannose-binding lectin (MBL) is an acute-phase protein – one of the most important elements of the lectin

pathway. 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 C4b2a[8-10].

The classical pathway activation is an immunoglobulin-mediated process. The adequate function of the adaptive immune response, such as T and B cell function, is necessary to detect and bind to non-self antigens, and finally results in the activation of the cascade.

The lectin pathway activation is independent of cell count, cell function, and immunoglobulin formation; the complement itself can bind to pathogens after detecting their pathogen-associated molecular patterns (PAMPs) and can immediately activate the cascade[7,8,12].

1.2.2. **Complement terminal phase**

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 (MAC), 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[7,8,13].

1.3. **Mannose-binding lectin**

MBL is a member of the family of Ca^{2+} dependent collagenous lectins. MBL is an essential protein of the humoral innate immune system. The serum MBL protein recognises the carbohydrate patterns on microbiological surfaces, and activates the complement system independent of the C1 complex.

MBL is a multimeric protein and consists of identical polypeptide chains, each containing the same parts: C-terminal, calcium-dependent carbohydrate-recognition domain (CRD); a short, a-helical, hydrophobic neck region, a collagenous region containing 19 Gly- Xaa-Xaa triplets, and a cysteine-rich N-terminal region. Three polypeptide chains form a triple helix, and these formations link to each other via the N-terminal cysteine-rich region. The linkage of 2-6 subunits results in the final structure of MBL, forming dimers into hexamers.

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) (Figure 2). 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[14-16].

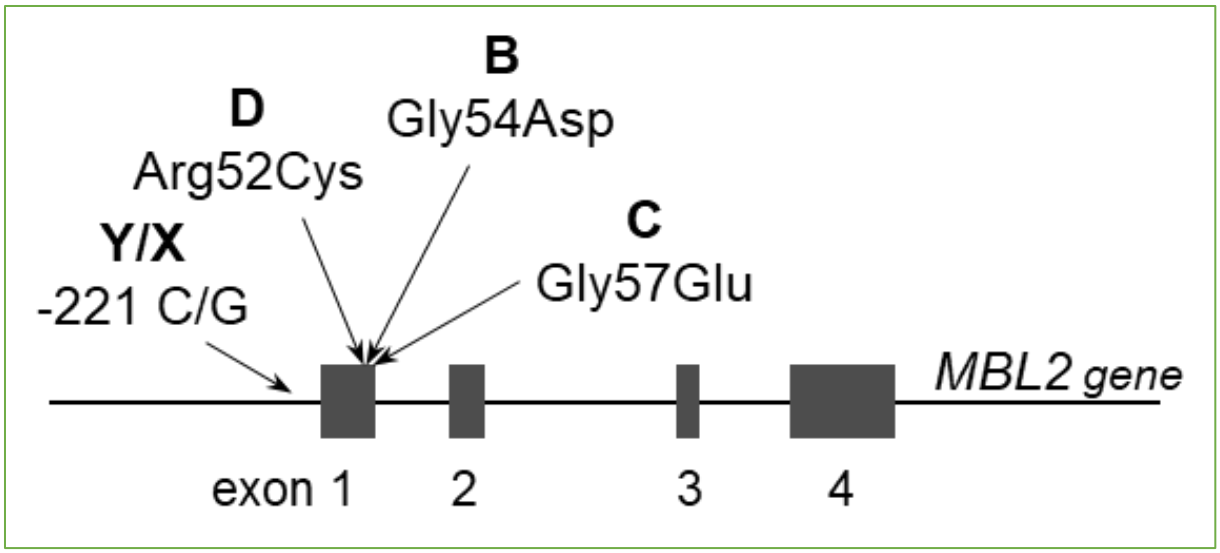

Figure 2 MBL gene structure

If the MBL gene contains the wild type allele, the expressed polypeptide chains join together in the form of a triple helix. (Figure 3). From these subunits, a tulip bouquet-like oligomer structure arises with further linkage. The three SNPs are located in the collagen-like domain, which is responsible for the oligomerization that is essential for activation of complement. 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.

The serum functional MBL level in heterozygotes is much lower, and may be extremely low in homozygotes compared to subjects carrying the wild type genotype. The polymorphisms of the promoter region can also influence the serum MBL level; the highest effect is attributed to the X/Y variation[11,14-17] .

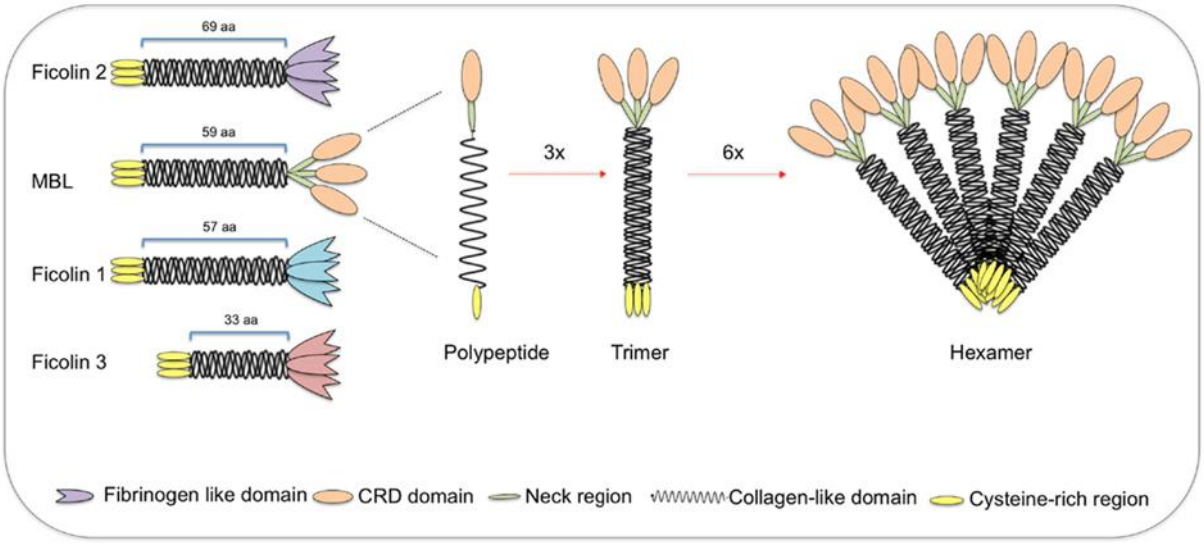

Figure 3 Structural subunits of mannan-bindinglectin (MBL) and ficolins, Source: Beltrame MH et al.: The lectin pathway of complement and rheumatic heart disease, Front Pediatr. 2015 Jan[18]

1.4. **Mannose binding lectin and mannose binding lectin associated protein**

However, when the different forms of MBL are artificially bound via an antibody to a polystyrene surface, MASP2 interacts with variant MBL forms as well, and complement activation may be detected, though markedly reduced compared to normal MBL. Thus, reduced serum concentration, disruption of MBL-MASP2 interactions in addition to changes in the oligomeric structure, and reduced binding to carbohydrate ligands in variant MBL compared with normal MBL all probably account for the biological phenotype in MBL-deficient individuals. Moreover, besides MBL, differences in MASP2 protein concentration and functional variations of the MASP2 gene may also contribute to the variability in the functional activity of the complex. For instance, the MASP2 +359G variant was reported to abolish the formation of the MBL-MASP2 complex, and heterozygotes were found to present approximately half MASP2 concentration in serum[19]. Therefore, instead of studying the MBL level, measuring the MBL-MASP2 complex activity is more informative about the functionality of the lectin pathway[17,20,21].

There are additional MBL-associated serine proteases. The MASP1 gene is located on chromosome 3q27-8 (*Takada et al.,* 1995) and contains 18 exons. The role of MASP1 in the activation of lectin pathway is controversial. The MASP1 protein probably modulates the function of the MASP2 protein and the activation of the lectin pathway. *Héja et al.* confirmed in 2015 the crucial role of the MASP1 protein. When they inhibited the MASP1 protein, the

activation of MASP2 was thereby decreased, and the activation of C2a – the molecule responsible for C3 convertase formation – was damaged.

Alternative splicing of the pre-mRNA encoding MASP1 results in two other products: MASP3 and MAp44. Both proteins have also regulator effect on complement cascade activation and the terminal pathway function[17,21,22].

1.5. **Ficolins and collectins**

The ficolins and collectins, discovered approximately 20 years ago, are other key elements of the lectin pathway activation. Ficolin-1 (M-ficolin), Ficolin-2 (L-ficolin), Ficolin-3 (H-ficolin), and collectin-11 also recognize the carbohydrate patterns on microbiological surfaces (pathogen associated molecular patterns, or PAMP) and form multimolecular complexes: mannose-binding lectin-associated serine protease 2 (MASP2)[4,22-25]. Ficolins consist of homogenous subunits. Three monomers assemble to form a trimeric structure held via disulphide bonds, and these units oligomerize to larger multimers. The roles and level-determining factors of ficolins are not clearly identified. Recently, many reports have shown that dysfunction or abnormal expressions of ficolins may play crucial roles in infectious and inflammatory diseases. Some single nucleotide polymorphisms in ficolin genes were discovered and also seem to have a role in the susceptibility to infections[26-28].

1.6. **MBL and infections**

Several studies have explored the role of MBL in the susceptibility and severity of infections. *Neth et. al* studied in 2000 the binding of purified MBL to pathogens isolated from immunocompromised children. Table 1. below shows their results[29]. 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*[22,29-31].

Table 1 MBL affinity to microorganisms (Neth et. al., 2000)[29]

High affinity	**Moderate affinity**	**Variable affinity**
Neisseria meningitidis	Group B *Streptococcus*	*Escherichia coli*
Staphylococcus aureus	*Streptococcus pneumoniae*	*Klebsiella* species
Group A *Streptococcus*	*Enterococcus* species	
Candida albicans	*Staphylococcus epidermidis*	
Aspergillus fumigatus	*Haemophilus influenzae*	
Cryptococcus neoformans	*Pseudomonas aeruginosa*	

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.[32-35]

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

3.1. **Patients**

3.1.1. **Retrospective clinical study**

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 with an average age of 6.9 years (range 1–17 years) 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. **Study design**

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.

3.3. **Genotyping**

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 .

3.4. **MBL-MASP2 complex activity**

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 (Figure 4.). This method is based on the C4-cleaving ability of the complex, described previously by *Presanis et al.* (2004)[21], with some modifications (*Csuka et al.* 2010)[36].

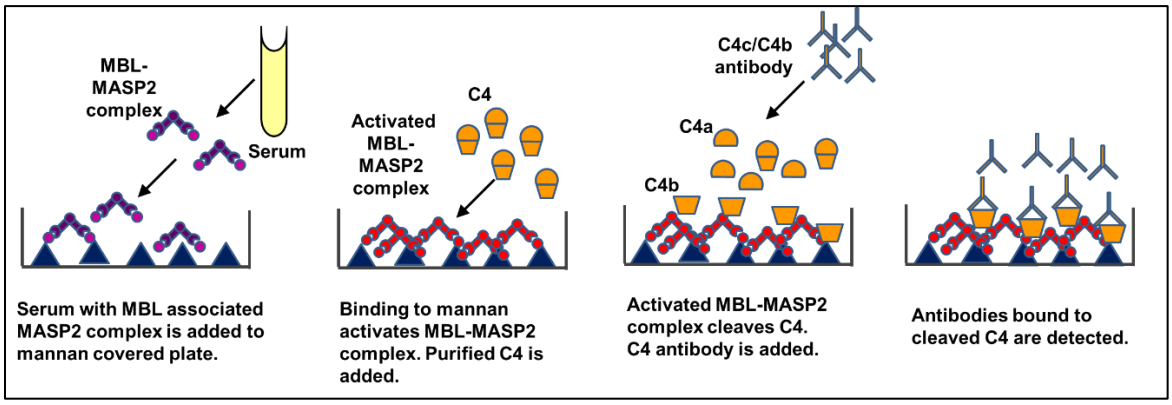

Figure 4 MBL-MASP2 activation-ELISA

3.5. **Statistical analysis**

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

4.1. Retrospective study

4.1.1. Patient characteristics

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 (Table 2). There were no significant differences in the allele frequencies in either the promoter or the exon 1 polymorphisms of this gene.

Table 2 Allele frequencies of MBL2 polymorphisms in children with and without hemato-oncologic disorders

Allele		**Children with hemato-oncologic disorder**	**Children without hemato-oncologic disorder**
Promoter region	**Y allele**	78.7%	85.8%
	X allele	21.3%	14.2%
Exon-1	**A allele**	79.6%	76.5%
	B allele	11.1%	16%
	C allele	1.9%	1.8%
	D allele	7.4%	5.7%

4.1.2. Genotype groups

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[15,37].

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 (Table 3). The difference among all groups was not significant (p = 0.85).

Table 3 Frequencies of different diseases in the three groups of patients formed according to the carried MBL2 genotype

	Genotypes associated with normal MBL level (YA/YA and YA/XA)	**Genotypes associated with low MBL level (XA/XA and YA/0)**	**Genotypes associated with MBL deficiency (XA/0 and 0/0)**
ALL	16 *51.6%*	10 *59%*	4 *66.6%*
AML	2 *6.5%*	0 *0%*	0 *0%*
Osteosarcoma	4 *12.9%*	2 *12%*	0 *0%*
Hodgkin's disease	4 *12.9%*	2 *12%*	1 *16.7%*
NHL	5 *16.1%*	3 *17%*	1 *16.7%*
Sum	31 *100%*	17 *100%*	6 *100%*

4.1.3. **Febrile neutropenic episodes characteristic**

The analysis of the features of febrile neutropenia during the first two years following diagnosis in three genotype groups (Table 4) 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 in Tables 3 and 4). 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 between 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.

Table 4 Data on fever episodes experienced by patients in the first 2 years after diagnosis in the three MBL2 genotype groups

	N	**Duration between diagnosis and the first fever episode (days) (median (IQ range))**	**Average length of fever episodes (days) (median (IQ range))**	**Ratio of days with fever during chemotherapy (median (IQ range))**
YA/YA, YA/XA	*31*	*53 (12-730)*	*3.7 (0-5.4)*	*2.9 (0-6.7)*
XA/XA, YA/0	*17*	*38 (22.5-161)*	*4.5 (3.4-6.2)*	*3.2 (1.7-5.9)*
XA/0, 0/0	*6*	*23.5 (3.2-85.8)*	*5.3 (4.5-8.7)*	*3.5 (1.9-6.1)*

4.2. **Prospective study**

4.2.1. **Patients:**

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.

4.2.2. **Genotype groups**

By genotyping the enrolled patients, the following minor allele frequencies were found: 13.3, 1.6, 8.8, and 17.0 for the MBL2 B, C, D, and X alleles, respectively. Genotype distribution aligned with the Hardy–Weinberg equilibrium, and allele frequencies corresponded to the frequencies described in the Caucasian population (Table 5).

Table 5 Allele and genotype frequency in the studied group

Allele	***Number of the alleles***	***Frequency of the alleles***	***Caucasian population ****	***Genotype***	***Number of the genotype***	***Genotype frequency***
A allele	*139*	*76.4%*	*76.3%*	***YA/YA***	*34*	*37.4%*
B allele	*24*	*13.2%*	*15.0%*	***YA/XA***	*14*	*15.4%*
C allele	*3*	*1.6%*	*1.6%*	***XA/XA***	*2*	*2.2%*
D allele	*16*	*8.8%*	*7.1%*	***A/0***	*39*	*42.8%*
Y allele	*151*	*82.3%*	*78.3 %*	***0/0***	*2*	*2.2%*
X allele	*31*	*17.7%*	*21.7 %*	***Hardy-Weinberg equilibrium: p <0.05***		

Patients were divided into two groups based on genotype classification established in previous studies by *Frakking et al*[38]. 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 (Table 6).

Table 6 Frequencies of different diseases in the three groups of patients formed according to the carried MBL2 genotype

Diagnosis of the patients	***Patients with normal expected MBL serum level***	***Patients with low expected MBL serum level***	***P value***
	YA/YA, YA/XA	***XA/XA, A/0, 0/0***	
ALL	*42*	*31*	
AML	*3*	*5*	
NHL	*3*	*7*	***p= 0.31***
	48	***43***	

4.2.3. **Febrile neutropenic episodes characteristic**

We then analysed the correlation between the characteristics of febrile neutropenic episodes occurring during the follow-up period and the MBL2 genotype groups (Table 7.). FN episode was defined as an axillary temperature greater than 38°C and a granulocyte count less than 0.5 G/l. 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).

Table 7 Characteristics of fever episodes experienced by patients in the first 8 months after diagnosis in the two MBL2 genotype groups

Characteristics of FN, median (IQ range)	***MBL2* genotype groups**		**P value (Mann-Whitney test)**
	Patients with normal expected MBL serum level (YA/YA, YA/XA) n=48	**Patients with low expected MBL serum level (XA/XA, A/0, 0/0) n=43**	
The total number of febrile episodes	*1.0 (0.25 - 3.0)*	*3.0 (1.0- 4.0)*	*0.0016*
The average length of febrile episodes (days)	*5.0 (0.25-7.0)*	*6.0 (3.0-8.0)*	*0.1532*
The total length of febrile episodes (days)	*8.5 (0.25-15.3)*	*14.0 (5.0-31.0)*	*0.0112*
The number of days until the first FN	*73.5 (30.25-241.0)*	*23.0 (13.0-74.0)*	*0.0018*

4.2.4. **Survival analysis**

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) (Figure 5).

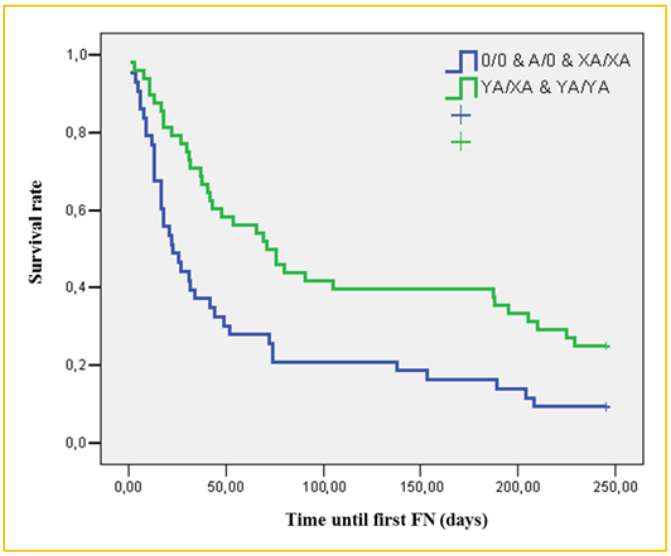

Figure 5 Survival analysis: Kaplan-Meier survival analysis. FN-free survival of patients with high (green line) or low (blue line) MBL level coding genotypes during the first 8 months from the beginning of chemotherapy

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.

4.2.5. **Clinical parameters during the febrile neutropenic episodes**

Certain clinical parameters (WBC, neutrophil, CRP) were monitored in the patients for each infectious episode during the follow-up period. As these measurements were performed several times during an episode, we chose to record for each FN episode:

- the number of days with an axillary temperature greater than 38°C,

- the number of days with granulocyte count less than 0.5 G/l,

- the lowest WBC count,

- the lowest neutrophil count,

- the highest CRP level,

- blood culture tests results.

As expected, patients had leukopenia in almost all FN episodes: WBC was lower than $3x10^9$cells/l in 195 episodes, and higher than 7 $x10^9$cells/l in the remaining 4 episodes. When the FN episodes were categorized according to the lowest WBC levels, the numbers were found to be similar in the four groups (Table 8).

The number of episodes with a neutrophil count lower than 0.1 G/l differed significantly between the genotype groups, however, data are not available on the length of the <0.1 G/l neutropenic periods.

For CRP, we chose the 8 mg/dl value as a cut-off point based on the study of *Nath et al.* (PMID: 28504925) As expected, CRP was higher than this value in a great portion of all FN episodes (170/199, 85%) and no difference was found between the genotype groups in the number of FN episodes with a CRP value below the cut-off point (Table 9).

Table 8 WBC level during the FN episodes

Lowest WBC in the FN episode (x10^{9}cells/l)	**FN episodes (n=77) of 48 patients with normal expected MBL serum level (YA/YA, YA/XA)**		**FN episodes (n=122) of 43 patients with low expected MBL serum level (XA/XA, A/0, 0/0)**	
WBC <1	*67*	*87.01%*	*102*	*83.61%*
1≤ WBC <2	*8*	*10.39%*	*11*	*9.02%*
2≤ WBC <3	*1*	*1.30%*	*6*	*4.92%*
3≤ WBC	*1*	*1.30%*	*3*	*2.46%*

Table 9 CRP level and neutrophil count in the two genotype groups

	***MBL2* genotype groups**		**P value (Mann-Whitney test)**
	Patients with normal expected MBL serum level (YA/YA, YA/XA) n=48	**Patients with low expected MBL serum level (XA/XA, A/0, 0/0) n=43**	
The number of FN episodes with a lowest neutrophil count less than 0.1 G/l	*1.0 (0.0 - 2.0)*	*2.0 (1.0- 4.0)*	*0.007*
The number of FN episodes where the highest CRP level is lower than 8 mg/dl	*0.0 (0.0 - 0.0)*	*0.0 (0.0 - 1.0)*	*0.159*

Altogether, 20.1% (40/199) of blood culture tests were positive for various pathogens (13 Coagulase-negative *Staphylococcus*, 6 *Streptococcus* spp, 5 *Micrococcus* spp, 2 *Staphylococcus aureus*, 2 *Pseudomonas aeruginosa*, 2 *Klebsiella pneumoniae*, 1 *E. coli*, 1 *Corynebacterium* spp, 1 ESBL-producing bacterium, and 7 patients with multiple pathogens). We compared the microbes identified in the normal MBL group to those detected in the low expected MBL group, but no considerable difference was detected.

4.2.6. **MBL-MASP2 complex activation**

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 ($p < 0.00001$) (Figure 6).

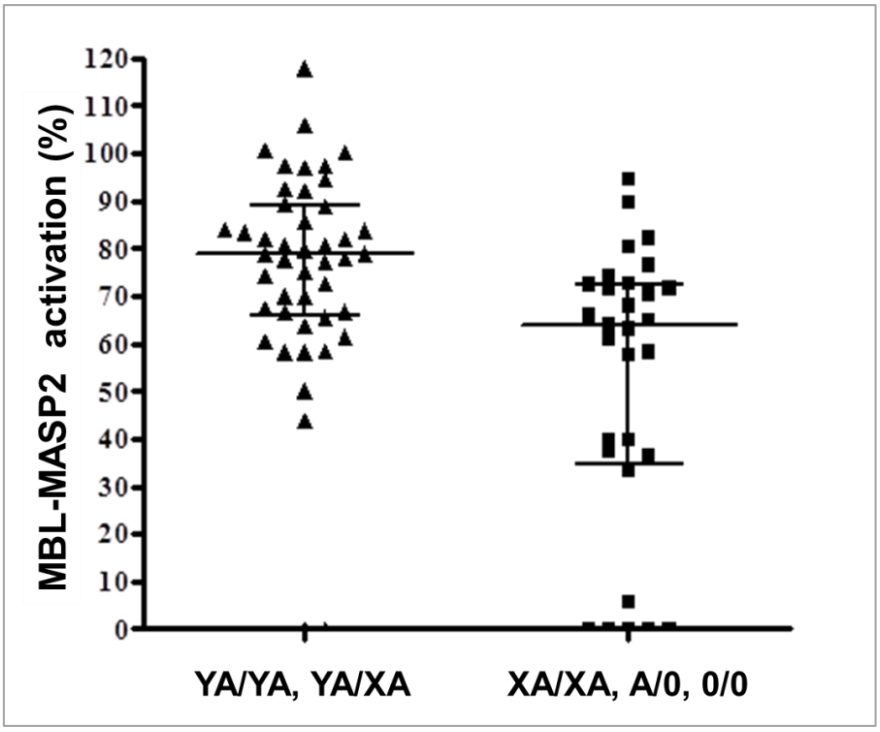

Figure 6 MASP2 complex activation at the time of diagnosis. Activation level of the MBL-MASP2 complex in patients with high (triangles) or low (squares) MBL level coding genotypes measured in samples collected at time of diagnosis. Solid lines represent medians and interquartile ranges. MBL-MASP2 indicates mannose-binding lectin-associated serine protease 2.

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 (Table 10). 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%.

Table 10 MBL-MASP2 complex activation at diagnosis and during a febrile neutropenic episode

	MBL-MASP2 activation (%), median (IQ range)	
	Patients with normal expected MBL serum level (YA/YA, YA/XA) n=20	**Patients with low expected MBL serum level (XA/XA, A/0, 0/0) n=22**
Time of diagnosis	*82.9 (70.1-98.8)*	*59.7 (16.7-72.1)*
During FN	*85.7 (54.1-98.7)*	*42.1 (0-64.7)*
paired t-test	*p = 0.123* *(60% reduced or unchanged)*	*p = 0.006* *(81% reduced or unchanged)*

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 21.3 and 20.4 – similar to that found in the general population. A previous paper reported that MBL2 variant alleles occur significantly more frequently in children with ALL compared to healthy individuals, however only adults comprised the control group[39]. 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 examination.

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 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[38,40-43], while six research papers contradicted this assumption and dismissed the role of MBL[44-48]. In one case the result was surprising: *Schlapbach et al*. found a controversial correlation between MBL level and infection[42].

Table 11 summarizes the characteristics of the ten studies, analysing the points at which a significant differences can be observed.

The possible reasons of these contradictory results are as follows:

a. Study characteristics

Three of these studies examined the role of MBL in a retrospective study; seven studies were prospective. The disadvantage of the retrospective studies is that the patients who were dying during the study period were not included in the studies.

We assume that the variant allele carrying genotype predisposes to severe infections. The mortality among children receiving chemotherapy is higher partly due to severe infection. Consequently, certain patients with severe infections could have been excluded from the study.

Therefore, this method may distort the results. *Rubnitz et al*., *Zehnder et al*. didn't find a correlation between the low MBL coding genotype and the infectious complications. In one case, the results were surprising: *Schlapbach et al*. found increased risk for infection in patients with serum MBL level 100-999 μg/l compared with patients with very low (<100 μg/l) or high (>1000 μg/l) serum MBL level.

Therefore we planned our second study as a prospective, cohort study. The prospective cohort study is suitable for assessing the consequences of various biological, mostly pathological processes.

Table 11 List and type of publications studying the role of MBL in children suffering from hemato-oncologic disease

Publication	***Type of study***	***Number of examined patients***	***Diagnoses of the examined patients***	***Poly-morphisms***	***Classification of MBL level***
Lehrnbecher et al. 1999[45]	*Prospective cohort study*	*n=56*	*ALL,AML, lymphoma, solid tumour*	*no data*	*no data*
Neth et al. 2001[41]	*Prospective cohort study*	*n=100*	*ALL, AML, NHL, MDS, neuroblastoma*	*-B, C, D, -Y/X, -P/Q*	*low (<1000 μg/l) normal (>1000 μg/l)*
Lausen et al. 2005[46]	*Prospective cohort study*	*n=137*	*non-B ALL*	*-B, C, D, -Y/X*	*not studied*
Frakking et al. 2006[48]	*Prospective cohort study*	*n=110*	*Hematologic disease lymphoma, solid tumour*	*-B, C, D, -Y/X, -P/Q -H/L*	*low (<1000 μl) normal (>1000 μl)*
Schlapbach et al. 2007[42]	*Retrospective cohort study*	*n=94*	*ALL, AML, lymphoma, solid tumour*	*not studied*	*normal (>1000 μg/l) low (100-999 μg/l) extremely low (<100μg/l)*
Rubnitz et al. 2007[44]	*Retrospective cohort study*	*n=91*	*ALL, AML*	*not studied*	*low (<500 μg/l) normal (>500 μg/l)*
Zehnder et al. 2009[47]	*Retrospective cohort study*	*n=372*	*ALL, AML, lymphoma, solid tumour*	*not studied*	*MBL, MASP2 level measure*
Frakking et al. 2011[38]	*Prospective cohort study*	*n=220*	*ALL, AML, lymphoma, solid tumour*	*-B, C, D, -Y/X, -P/Q -H/L*	*low (<200 μg/l) normal (>200 μg/l)*
Ghazi et al. 2012[43]	*Prospective cohort study*	*n=75*	*ALL*	*not studied*	*low (<1000 μl) normal (>1000 μl)*
Dommett et al. 2013[40]	*Prospective cohort study*	*n=220*	*ALL, AML, lymphoma, solid tumour*	*-B, C, D, -Y/X, -P/Q -H/L*	*normal (>1000 μg/l) low (100-999 μg/l) extremely low (<100μg/l)*

b. Patients' characteristics

The number and the diagnoses of the enrolled patients were highly varied. *Lehrnbecher* and colleagues examined 56 patients, while *Zehnder et al*. enrolled 372 children in their study.

The diagnoses of the examined patients were different in each study. For example, *Lausen et al.* solely enrolled children suffering from B-cell acute lymphoblastic leukaemia; the other studies enrolled children suffering from lymphoma, leukaemia, and solid tumours. The type of solid tumours could be Wilms tumour, Ewing sarcoma, retinoblastoma, and all tumours which occur in childhood, as found in previous studies.

We enrolled in our studies patients with different hemato-oncological diseases. The different chemotherapy protocols likely have varying effects on bone marrow damage and the degree of immunosuppression, but the low incidence of hemato-oncological disease does not allow the statistical analysis of one patient group. We studied whether certain diseases occur with the same frequency in both genotype groups. No significant difference was found, therefore the involvement of patients with different diseases probably did not distort the results of the study.

c. MBL deficiency

The definition of MBL deficiency was different in each study. In some studies, MBL deficiency was defined as low serum MBL concentration measured by the ELISA technique; in other studies the serum functional deficiency was defined based on the low MBL level coding genotype.

However, in experimental studies it was found that the opsophagocytosis was damaged below 500 μg/l. *Ghazi et el.* defined the low level of MBL concentration at below 1000 μg/l. *Schlapbach et al.* created three groups in which the ranges of MBL level were >1000 μg/l, 1000 μg/l-100 μg/l, and <100 μg/l.

The definition of MBL2 gene polymorphisms was also different. In some studies the SNP in the structure gene was examined; in other studies the promoter region was included. Finally, the created genotype groups were also very heterogeneous.

In our study, we aimed to examine a wide range of MBL polymorphisms. We determined the MBL2 gene B allele (rs1800450) C (rs1800451), D (rs5030737) and Y/X (rs7096206) polymorphisms by the real-time PCR technique. Further, instead of studying simply the MBL level, we found the measure of the MBL-MASP2 complex activity to be more informative about

the functionality of the lectin pathway. This technique is a sensitive specific method, which gives information exclusively about the MBL-MASP2 complex activity and eliminates all other influencing factors.

Although inherited MASP2 deficiency occurs very rarely, the protein concentration can show variability, and therefore should not be ignored. As expected, genotype of the MBL2 polymorphisms considerably 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 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. The MBL marks pathogens as an opsonin, leading to the chemically modified molecules having stronger interactions to cell surface receptors on phagocytes and thus enhancing phagocytosis. The explanation of the difference between the two genotype groups could be that in patients with normal genotype the consumption is less significant or the expression of the protein is more inducible. Therefore when the protein level decreases, the production could compensate the consumption.

d. Outcome

The studies analysed the infectious outcomes in different ways. Some of these examined the frequency and severity of infections and the length of the febrile neutropenic episodes. Others analysed the occurrence of sepsis, bacteraemia, or invasive fungal diseases; admission to an intensive care unit; survival rate; or survival time. These different aspects raise the heterogeneity of the studies (Table 12).

In our study, we describe the infectious episodes with several parameters, such as the number of infectious episodes, the total and average numbers of the febrile neutropenic days, and the number of the days until the first neutropenic episode. We also attempted to determine the chances of children with different genotypes developing an infectious episode in the first eight months of chemotherapy. Further, certain clinical parameters (WBC, neutrophil, CRP) were monitored in the patients for each infectious episode during the follow-up period.

Table 12 Results of publications studying the role of MBL in children suffering from hemato-oncologic disease

Publication	Outcome	FN characteristics	Groups	Result
Lehnbecher et al. 1999[45]	-FN -Bacteraemia -Fungemia -Virus infection	axillary T >38,5°C or >38,0°C two times<4 h neu <1000/µl	no data	no correlation
Neth et al. 2001[41]	-FN number/length -Bacteraemia -Fungal infection	axillary T >38,5°C or >38,0°C two times <12h neu <1000/µl	AA vs. A0+00	low MBL: more and longer FN
Lausen et al. 2005[46]	-Infection -Pneumonia -Bacteraemia -Fungemia	T> 38,5°C neu <500/µl	MBL level: high (YA/YA,YA/XA) intermediate (YA/0, XA/XA) low (XA/0, 0/0)	no correlation
Frakking et al. 2006[48]	-FN number -Bacteraemia -Sepsis -Admission to ICU	ear T> 38,5°C neu <500/µl severe neu <100/µl	MBL level: high (YA/YA,YA/XA) intermediate (YA/0, XA/XA) low (XA/0, 0/0)	no correlation
Schlapbach et al. 2007[46]	-FN -Bacteraemia -severe bact. inf. -virus infection	axillary T> 38,5°C > 2h or once >39°C neu <500 µl	normal (>1000 µg/l) low (100-999 µg/l) extremely low (<100µg/l)	normal and extremely low MBL level higher risk to FN
Rubnitz et al. 2007[44]	-FN -Bacterial infection -Fungal infection	no data	low (<500 µg/l) normal (>500 µg/l)	no correlation
Zehnder et al. 2009[47]	-time to the first FN -survival time	no data	no data	no correlation
Frakking et al. 2011[38]	-time to the first FN -survival time -sepsis, MOF	no data	MBL level high (YA/YA,YA/XA) intermediate (YA/0, XA/XA) low (XA/0, 0/0)	low MBL: time to the firs FN shorter; sepsis, MOF, death: higher risk
Ghazi et al. 2012[43]	-FN number/ severity	ear T> 38,5°C neu <500 µl severe neu <100 µl	low(<1000 µl) normal(>1000 µl)	low MBL: severe infection, more major infection
Dommett et al. 2013[40]	-FN number/length -Bacteraemia -Fungal infection	T>38°C, 4h T> 38,5°C, 2 two times neu <1000/µl	AA vs. A0+00	low MBL causing genotype group: severe infection, more major infection

e. Definition of febrile neutropenic episodes

Most of the studies examined the occurrence of infectious complication and characteristic parameters. Each study used a different definition of the febrile neutropenic episodes in terms of fever temperature and the length of the episodes.

Lausen et al. took into account when the body temperature exceeded 38.5°C. *Neth et al.* gave a much more precise definition. They defined febrile neutropenic episode less strictly, when the

axillary body temperature was more than 38.5°C measured at least once, or more than 38.0°C measured at least twice within 12 hours.

The neutropenia definition was also variable, with the neutrophil count in the blood lower than either 500/μl or 1000/μl.

Frakking et al. also published a comprehensive review in 2011 which attempted to clarify the reason behind these conflicting findings[49]. 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. The reviewers extracted from the articles the design and characteristics of studies, study group, tumour type, method of MBL analysis, definition of MBL deficiency, definition of outcome, methods used to detect infection, follow-up, and risk factor analysis. 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. In the immune response of younger children, innate immunity outweighs adaptive immunity; as a result, the role of MBL is re-evaluated depending on age. The tumour type and intensity of chemotherapy could also be risk factors as the administration of certain chemotherapeutic drugs appears to be directly related to functional complement defects. 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 a unified definition would be necessary to explain the role of MBL allowing for other risk factors.

Increasing evidence suggests that besides MBL, variability of other constituents of the complement pathways or their combinations may also influence the occurrence of infections in immunocompromised patients. For instance, deficiency of MASP2, ficolin-3, as well as single or combined deficiencies of MBL2 and ficolin-2 were reported to be associated with an increased risk of infections and prolonged duration of febrile neutropenic episodes in leukemic children[20,28,50].

5.1. **MBL substitution therapy**

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[51-55].

In 2014, *Keizer et al.* published an overview of literature about MBL replacement studies, and the possibilities of MBL substitution therapy[56]. He found five clinical studies: two of these were case reports, the rest were phase I/II pdMBL clinical studies. The early studies verified the safety and efficacy of pdMBL infusions in MBL-deficient individuals. The activated MASPs could form a complex with C1-inh leading to ineffective lectin pathway activation. The recombinant MBL is produced by the *Escherichia coli* or other insect cells, but the oligomer does not form the specific higher oligomers resulting a suboptimal restoration of LP functional activity.

Limited information is available regarding the safety and efficacy of rMBL. In 2006, *Petersen et al.* examined MBL-deficient healthy adult males in a placebo-controlled double-blind study. After the substitution therapy the MBL levels were in the therapeutic range, and there were no adverse events during or after the therapy[57].

Brouwer et al. examined the effect of MBL therapy *in vitro* and found an increase in complement activation and opsonophagocytosis after plasma-delivered MBL substitution therapy. However, the opsonophagocytosis recovery was suboptimal, and the function increased after repeated MBL infusions. [58]

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.

5.2. **Summary**

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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ORIGINAL ARTICLE

The role of mannose binding lectin on fever episodes in pediatric oncology patients

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Abstract Despite significant changes in pediatric oncological therapy, mortality is still high, mainly due to infections. Complement system as an ancient immune defense against microorganisms plays a significant role in surmounting infections, therefore, deficiency of its components may have particular importance in malignancies. The present paper assesses the effect of promoter (X/Y) and exon 1 (A/0) polymorphisms of the *MBL2* gene altering mannose binding lectin (MBL) serum level in pediatric oncological patients with febrile neutropenia. Furthermore, frequency distribution of *MBL2* alleles in children with malignancies and age-matched controls was analysed. Fifty-four oncohematological patients and 53 children who had undergone pediatric surgery were enrolled into this retrospective study. No significant differences were found in the frequency of *MBL2* alleles between the hemato-oncologic and control group. The average duration of fever episodes was significantly shorter ($p = 0.035$) in patients carrying genotypes (AY/AY and AY/AX) that encode normal MBL level, compared to individuals with genotypes associated with lower functional MBL level (AX/AX, AY/0, AX/0, or 0/0) (days, median (IQ range) 3.7(0–5.4) vs. 5.0(3.8–6.6), respectively). In conclusion, our data suggest that *MBL2* genotypes may influence the course of febrile neutropenia in pediatric patients with malignancies, and may contribute to clarification of the importance of MBL in infections.

Keywords MBL · Polymorphism · Febrile neutropenia · Oncohematology

Introduction

Modern treatment of childhood malignancies have been markedly changed leading to a higher life expectancy; the overall 5-year survival rate is 70–80 %. Nonetheless, the mortality is still significant, as the chemotherapy-induced immunosuppression increases susceptibility to infections, which contributes to about 10–20 % of mortality in pediatric oncology.

As a sequel of treatment, pediatric oncology patients may often become neutropenic, leukopenic or pancytopenic that emphasizes the importance of innate immune defense against microbes. The complement system, activated through the classical, alternative or lectin pathways, is an essential component of the ancient immune response to infections caused by a wide variety of pathogens. The lectin pathway can be initiated by a circulating protein called mannose binding lectin (MBL) that binds to carbohydrates found on the surface of many pathogens [1, 2]. MBL binds with high affinity to microbes often detected in hematology departments and can cause severe sepsis, such as diverse *Candida* species, *group A Streptococci* or *Staphylococcus aureus* with specific antibiotic resistance (MRSA) [3].

The MBL protein is encoded by the *MBL2* gene (10q11.2-q21) comprising 4 exons. The promoter region of the gene contains a single nucleotide polymorphism (SNP) at position −221 denoted as Y/X in the literature. The most widely studied

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Table 1 Allele frequencies of *MBL2* polymorphisms in children with and without hemato-oncologic disorders

Allele		children with hemato-oncologic disorder	children without hemato-oncologic disorder
Promoter	Y	78.7 %	85.8 %
	X	21.3 %	14.2 %
Exon-1	A	79.6 %	76.5 %
	B	11.1 %	16 %
	C	1.9 %	1.8 %
	D	7.4 %	5.7 %

variations of the gene are three polymorphisms in the first exon causing aminoacid substitutions in the protein. The wild type allele without any polymorphic variant is named A, while the alleles with amino acid changes at codon 54 (Gly54Asp), 57 (Gly57Glu) or 52 (Arg52Cys) are termed as B, C or D, respectively and any of these variants on a chromosome is referred to as a "0 allele". Serum concentration of functional mannose binding protein shows close correlation with the genotype of *MBL2* polymorphisms. The wild type *A* allele is associated with normal plasma level, while all variant alleles (*B*, *C* and *D*) have a dominant effect lowering the level of functional MBL. Polymorphism of the promoter region also influences the circulating MBL level in particular the variant allele (X) is associated with lower MBL expression [4, 5].

A growing body of evidence suggests that functional MBL deficiency may be associated with an increased risk of infections especially in malignancies; however, contradictory results have also been reported [6, 7]. Present paper deals with the possible role of polymorphisms influencing MBL serum level on the incidence, frequency and duration of febrile neutropenia (FN) in oncohematological patients. Moreover, frequency of *MBL2* alleles was compared in children with vs. without malignancies.

Materials and Methods

Fifty-four patients (24 girls, 30 boys) diagnosed with malignant diseases and treated between 2001 and 2008 at the 2nd Department of Pediatrics of the Budapest Semmelweis University were enrolled into our retrospective clinical study. Inclusion criteria were oncohematological disease and age of 18 years or younger at the date of diagnosis. The average age at diagnosis was 9.4 years (range 3 months-17 years). The diagnoses of enrolled participants were: acute lymphocytic leukemia (ALL) (N = 30); acute myelocytic leukemia (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 and 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 of average age of 6.9 years (range 1–17 years) without malignancies were enrolled as controls with following diagnoses: phymosis; adhesion of preputium; 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. The study was approved by the National Ethical Committee (TUKEB 180/2007), and parents or guardians of all participants gave informed consent.

Fever episodes occurred during chemotherapy or shortly after treatment were followed up for 2 years after the diagnosis of patients with hemato-oncologic disorders. Febrile neutropenic episode (FN) was defined as an axillary temperature exceeding 38 °C for at least 2 days and granulocyte count under 0,5G/l. Several parameters were recorded during each episode, such as the date of first and last day of fever, certain clinical parameters (WBC, Neutrophils and CRP) determined at the onset of the episode, at the time of hemoculture test, and on the first day of normal body temperature. In case of positive hemoculture, the identified microbe, its antibiotic resistance and the treatment (antibiotic and/or citokin) were also registered.

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 [8]. In our study patients were assessed into three groups according to the expected serum level of MBL protein encoded by the carried genotype as repoterted by Garred et al. [5, 9] 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 goup 3: MBL-deficient (XA/0 and 0/0) subjects.

Statistical analysis was performed with the GraphPad Prism 4.0 (GraphPad Software Inc., San Diego, CA) and SPSS 13.0 (SPSS Inc., Chicago, IL) software. Categorical data were analyzed by the χ^2 test and between-group differences were evaluated by the Mann-Whitney or Kruskal-Wallis tests. Multivariate analyses were performed by multiple logistic regression adjusted to the diagnosis of the patients or the applied chemotherapy protocol.

Results

Allele frequencies of the studied SNPs of the *MBL2* gene were compared in the groups of children with or without hemato-oncologic disorders (Table 1). There were no significant

Table 2 Frequencies of different diseases in the three groups of patients formed according to the carried *MBL2* genotype

	Genotypes associated with normal MBL level (YA/YA and YA/XA)	Genotypes associated with low MBL level (XA/XA and YA/0)	Genotypes associated with MBL-deficiency (XA/0 and 0/0)
ALL	16	10	4
	51.6 %	*59 %*	*66.6 %*
AML	2	0	0
	6.5 %	*0 %*	*0 %*
Osteosarcoma	4	2	0
	12.9 %	*12 %*	*0 %*
Hodgkin's disease	4	2	1
	12.9 %	*12 %*	*16.7 %*
NHL	5	3	1
	16.1 %	*17 %*	*16.7 %*
Sum	31	17	6
	100 %	*100 %*	*100 %*

differences in the allele frequencies in either the promoter, or the exon 1 polymorphisms of this gene.

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 (Table 2). The difference between all groups was not significant ($p = 0.85$).

The analysis of the features of febrile neutropenia during the first 2 years following the diagnosis in 3 genotype groups (Table 3), have 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, that indicates 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 2 years following the diagnosis, but this difference was not significant ($p = 0.690$). 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 (group 2 and 3 in Tables 2 and 3). 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 between *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.

Table 3 Data on fever episodes experienced by patients in the first 2 years after the diagnosis in the three *MBL2* genotype groups

	N	Duration between diagnosis and the first fever episode (days) (median (IQ range))	Average length of fever episodes (days) (median (IQ range))	Ratio of days with fever during chemotherapy (median (IQ range))
YA/YA, YA/XA	31	53 (12–730)	3.7 (0–5.4)	2.9 (0–6.7)
XA/XA, YA/0	17	38 (22.5–161)	4.5 (3.4–6.2)	3.2 (1.7–5.9)
XA/0, 0/0	6	23.5 (3.2–85.8)	5.3 (4.5–8.7)	3.5 (1.9–6.1)

Discussion

Our study evaluated the influence of *MBL2* gene polymorphisms on the incidence, frequency and duration of febrile neutropenia in oncohematological patients. Our results showed that genotypes encoding high MBL level are associated with shorter duration of fever episodes in the first 2 years after the diagnosis of malignancy.

Frequency of variant alleles of Y/X and A/0 polymorphisms in our patients was 21.3 and 20.4 that is similar to that found in the general population. A previous paper reported that *MBL2* variant alleles occur significantly more frequently in children with ALL compared to healthy individuals, however only adults comprised the control group [10]. In our study oncologic pediatric patients were compared with non-oncologic age-matched controls and no difference was found in the allele distribution.

Analyzing the characteristics of fever episodes in the first 2 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.

Previous studies analyzing the role of MBL in infections in children with cancer showed contradictory results. Neth et al. found that the median duration of febrile neutropenic episodes was longer in MBL deficient children receiving chemotherapy than in patients with normal MBL coding genotypes [11]. Similarly, other studies also showed association between low concentrations of MBL or low-producing *MBL2* genotypes and serious infections related to chemotherapy [12–15], while a recent paper showed that MBL deficiency was associated with decreased event-free survival in children with cancer [16]. However, several reports failed to find relationship between the incidence or duration of fever episodes and MBL levels in patients with different malignancies [17–21]. Another recent paper retrieving data from six cohorts studies failed to identify MBL deficiency as an independent risk factor for febrile neutropenia or infection in pediatric oncology patients [22]. Although inconsistent results have been published, MBL therapy for MBL deficient immunocompromised patients is an area of ongoing research [23–25]. Phase I and II trials have already been performed with plasma-derived MBL in small populations and as recombinant human MBL has recently become available, more resultes are expected to be published in the near future.

We are aware of the limitations of our study, namely low sample size and the heterogenous patient group in terms of diagnosis. However, as the multivariate analyis including diagnosis or the applied chemotherapy has confirmed that low MBL level coding genotypes confer risk of longer FN episodes, our results may contribute to clarify previous controversial findings. Further studies on the role of MBL as a clinical prognostic factor in febrile neutropenia are needed to verify present results on a larger population of pediatric oncologic patients and to assign candidates for MBL replacement therapy.

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Conflict of Interest The author(s) declare that they have no conflict interests.

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The Role of Mannose-binding Lectin in Infectious Complications of Pediatric Hemato-Oncologic Diseases

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Abstract: The complement system is essential for protection against infections in oncologic patients because of the chemotherapy-induced immunosuppression. One of the key elements in the activation of the complement system via the lectin pathway is the appropriate functioning of mannose-binding lectin (MBL) and mannose-binding lectin-associated serine protease 2 (MASP2) complex. The objective of our study was to find an association between polymorphisms resulting in low MBL level and activation of the MBL-MASP2 complex. Also, we aimed at finding a connection between these abnormalities and the frequency and severity of febrile neutropenic episodes in children suffering from hemato-oncologic diseases. Ninety-seven patients had been enrolled and followed from the beginning of the therapy for 8 months, and several characteristics of febrile neutropenic episodes were recorded. Genotypes of 4 *MBL2* polymorphisms (-221C/G, R52C, G54D, G57E) were determined by real-time polymerase chain reaction. Activation of the MBL-MASP2 complex was evaluated by enzyme-linked immunosorbent assay at the time of diagnosis and during an infection. The number of febrile neutropenic episodes was lower, and the time until the first episode was longer in patients with normal MBL level than in patients with low MBL level coding genotypes. The MBL-MASP2 complex activation level correlated with the MBL genotype and decreased significantly during infections in patients with low MBL level. Our results suggest that infections after immunosuppression therapy in children suffering from hemato-oncologic diseases are associated with the *MBL2* genotype. Our results may contribute to the estimation of risk for infections in the future, which may modify therapeutic options for individuals.

Key Words: complement system, pediatric hemato-oncologic diseases, neutropenia, mannose-binding lectin

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The tumors of the blood-forming organs account for more than one-third of all malignant tumors among children. [1] Over the past 20 years, possibilities of oncologic therapy have improved significantly leading to a better prognosis and higher life expectancy. Currently, the 5-year survival rate is more than 70%–80% in case of pediatric tumors.[1,2] However, infectious complications still remained a serious problem, as they considerably worsen mortality and morbidity.[3,4]

Chemotherapy often induces neutropenia and leukopenia and therefore impairs cellular functions of the adaptive and innate immune systems.[5] Hence, appropriate function of the humoral immune defense mechanisms such as the complement system is essential in protection against infections in hemato-oncologic patients.[6]

The complement system is activated through the classical, alternative, and lectin pathways. Mannose-binding lectin (MBL) is an acute-phase collectin, one of the most important elements of the lectin pathway.[7,8] The serum MBL protein recognizes the carbohydrate patterns on microbiologic surfaces, forms a complex with mannose-binding lectin-associated serine protease (MASP2) and activates the proteolytic cascade system, which facilitates the elimination of microorganisms.[9–13]

MBL binds with high affinity to several microorganisms, which are frequent pathogens in hematologic departments and are frequent causes of severe sepsis, such as Gram-negatives enterobacteriacea, several types of Streptococcus, *Staphylococcus aureus*, and *Candida albicans*.[14–17]

The serum functional MBL concentration is mainly genetically determined.[18] The MBL protein is encoded by the *MBL2* gene (10q11.2-q21) comprising 4 exons. The promoter region of the gene contains a single nucleotide polymorphism at position -221 denoted as Y/X in the literature. In the first exon, the gene may contain 3 single nucleotide polymorphisms causing amino acid substitutions in the protein. The 3 variant alleles are at codons 54 (Gly54Asp), 57 (Gly57Glu), and 52 (Arg52Cys). They are termed B, C, or 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. The 3 SNPs are located in the collagen-like domain, which is responsible for oligomerization that is essential for activation of complement. Thus, 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, and therefore results in markedly reduced functional MBL level and decreased activation of the lectin pathway. Serum functional MBL level in heterozygotes is much lower and may be immensely low in homozygotes compared with subjects carrying the wild-type genotype. The polymorphisms of the promoter region can also influence the serum MBL level; the highest effect is attributed to the X/Y variation.[19,20]

While increasing data suggest an important role of MBL level in immunosuppressed conditions among adults, contradictory data have been published concerning childhood malignancies.[21–23] Therefore, in 2007, our research group started a study on the role of *MBL2* SNPs in infections following chemotherapy in a retrospective study of pediatric oncologic patients. Having analyzed infectious periods of 54 patients, no significant differences were found; however, a tendency for more severe infections was detected in patients carrying a variant allele of *MBL2*. As a result, a prospective study comprising more patients was initiated.[24]

The aim of this prospective cohort study was to evaluate the role of polymorphisms causing low MBL level in the total number and severity of febrile neutropenic episodes and to find an association between polymorphisms resulting in low MBL level and activation of the MBL-MASP2 complex in children suffering from hemato-oncologic diseases.

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MATERIALS AND METHODS

Ninety-seven children treated between 2009 and 2012 with hemato-oncologic diseases at the Second Department of Pediatrics of the Semmelweis University, Budapest, and at the Department of Hematology of Heim Pál Children's Hospital, Budapest, were enrolled into our prospective clinical study. The inclusion criteria included a newly diagnosed hemato-oncologic disease and patients under 18 years of age at the time of diagnosis. The diagnoses of participants were acute lymphoid leukemia in 76, acute myeloid leukemia in 10, and non-Hodgkin lymphoma in 11 cases. Each patient received myeloablative chemotherapy, according to current protocols, acute lymphoid leukemia (IC) BFM 2002/2009, acute myeloid leukemia BFM 98, and non-Hodgkin lymphoma BFM 95, respectively. The study was approved by the National Ethical Committee, and a written informed consent from parents or guardians was obtained.

Febrile neutropenia (FN) was defined as an axillary temperature greater than 38°C and granulocyte count less than 0.5 G/L. The children were followed for a period of 8 months dating from the diagnosis. The time of diagnosis was established by the results of histopathologic findings. Patient characteristics, such as sex, age, tumor type, stage of disease, time of diagnosis, applied therapy, and mortality, were collected from patient documentation. Several parameters of each febrile neutropenic episode occurred during the follow-up period were also recorded, such as the date of the first and last day of FN, certain clinical parameters (white blood cells, neutrophil, C-reactive protein), antibiotic treatment, the time of hemoculture and, in case of a positive hemoculture, the identified microbe, and antibiotic resistance.

Blood samples were collected at the time of diagnosis and during the first 2 febrile neutropenic episodes. DNA was isolated from EDTA-anticoagulated whole blood samples using salting-out procedure. Commercially available TaqMan® SNP Genotyping Assays (Applied Biosystems, Carlsbad, CA) were used for genotyping of *MBL2* B (rs1800450), C (rs1800451), D (rs5030737), and Y/X (rs7096206) polymorphisms by real-time polymerase chain reaction.

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 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,[25] with some modifications.[26]

Data were evaluated with the SPSS 13.0 (SPSS Inc., Chicago IL) and the GraphPad Prism 4 (GraphPad Software Inc., San Diego, CA) software. Since most variables were nonnormally distributed, nonparametric tests were applied. The Mann-Whitney *U* test was used to compare 2 independent groups, and categorical data were analyzed by the Pearson test. The survival rate without infection was established by the Kaplan-Meier curve, and multiple logistic regression analysis was applied adjusted to the underlying disease, age, and sex of patients. 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 <0.05.

RESULTS

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 chemotherapy. During the study period, 12 patients died, 6 of them during the follow-up period; thus, in the end, data from 91 patients were analyzed.

By genotyping the enrolled patients, the following minor allele frequencies were found, 13.3, 1.6, 8.8, and 17.0 for the *MBL2* B, C, D, and X alleles, respectively. Genotype distribution aligned with the Hardy–Weinberg equilibrium and allele frequencies corresponded to the frequencies described in the Caucasian population. Patients were divided into 2 groups based on genotype classification established in previous studies by Frakking et al.[27] The first group included patients with low-expected MBL level coding genotypes (YA/0, XA/0, 0/0, XA/XA), while the other group comprised 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 involvement of patients with different diseases probably did not distort the results of the study.

Then we analyzed the correlation between the characteristics of febrile neutropenic episodes occurred during the follow-up period and the *MBL2* genotype groups (Table 1). 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 were 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 genotype genotypes.

Further, 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, ie, the period without infection in the 2 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, $P=0.0029$) (Fig. 1).

We also examined the cofactors influencing infections with 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 8 months with a hazard ratio of 1.649 (95% confidence interval: 1.014–2.681) ($P=0.044$). These patients are 1½ times more likely to contract an infection with febrile neutropenia than patients with normal MBL level coding groups.

Finally, we analyzed the correlation between the MBL-MASP2 complex activation and the *MBL2* genotype. From the prechemotherapy samples available from 64 patients, we obtained the expected result that the *MBL2* polymorphisms considerably determine the MBL-MASP2 complex activation. In patients with lower MBL level coding genotype, the activation level of the MBL-MASP2 complex is significantly lower ($P<0.00001$) (Fig. 2). Both prechemotherapy sample and a sample obtained during a febrile neutropenic episode were available in 42 patients that enabled us to study the changes in MBL-MASP2 activation during a febrile neutropenic episode (Table 2). 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%.

DISCUSSION

This study explored the relationship between the presence of *MBL2* gene polymorphisms, the level of the MBL-MASP2 complex

TABLE 1. Characteristics of Fever Episodes Experienced by Patients in the First 8 Months After the Diagnosis in the 2 *MBL2* Genotype Groups

Characteristics of FN, Median (IQR)	*MBL2* Genotype Groups: Patients With Normal Expected MBL Serum Level (YA/YA, YA/XA) n = 48	*MBL2* Genotype Groups: Patients With Low-expected MBL Serum Level (XA/XA, A/0, 0/0) n = 43	*P* (Mann-Whitney test)
The total number of FN episodes	1.0 (0.25–3.0)	3.0 (1.0–4.0)	0.0016
The average length of FN episodes (d)	5.0 (0.25–7.0)	6.0 (3.0–8.0)	0.1532
The total length of FN episodes (d)	8.5 (0.25–15.3)	14.0 (5.0–31.0)	0.0112
The number of days until the first FN	73.5 (30.25–241.0)	23.0 (13.0–74.0)	0.0018

FN indicates febrile neutropenia; IQR, interquartile range.

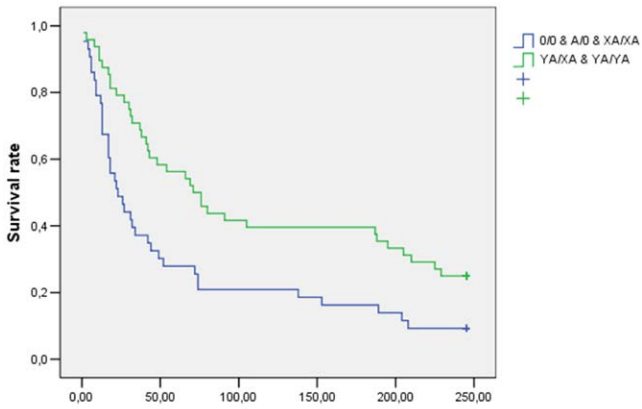

FIGURE 1. Kaplan-Meier survival analysis. FN-free survival of patients with high (green line) or low (blue line) MBL level coding genotypes during the first 8 months from the beginning of chemotherapy. MBL indicates mannose-binding lectin. full color online

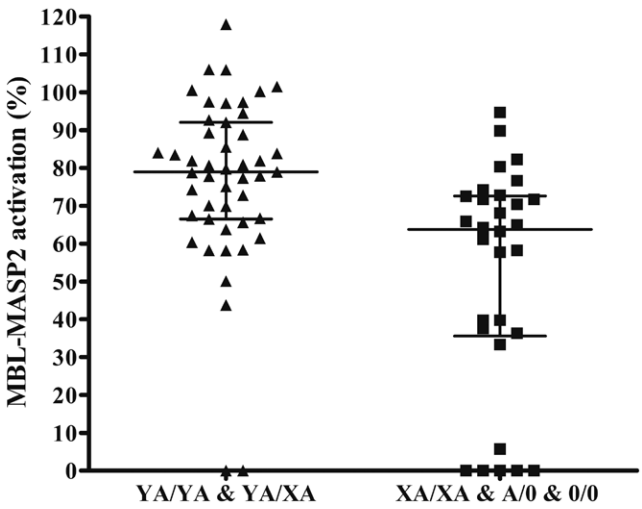

FIGURE 2. MBL-MASP2 complex activation at the time of diagnosis. Activation level of the MBL-MASP2 complex in patients with high (triangles) or low (squares) MBL level coding genotypes measured in samples collected at time of diagnosis. Solid lines represent medians and interquartile ranges. MBL-MASP2 indicates mannose-binding lectin-associated serine protease 2.

activation, and the increased risk of infection with febrile neutropenia in children suffering from hemato-oncologic disease. We analyzed the correlation between the incidence, the frequency, and duration of febrile neutropenic episodes, and the expected MBL level based on *MBL2* gene polymorphisms and MBL-MASP2 complex activation.

In the literature, several studies discuss the role of MBL in diseases cooccurring with 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 11 articles since 1999, which were seeking answers to the above-mentioned questions. Five of these studies feature discussions which assume the effect of MBL on infection incidence and severity,[27–31] while 5 research papers contradicted this assumption and dismissed the role of MBL.[32–36] In 1 case, the results were surprising. Schlapbach et al[29] found increased risk for infection in patients with serum MBL level 100–999 µg/L compared with patients who have very low (<100 µg/L) or high (>1000 µg/L) serum MBL level.

Frakking et al[27] 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 2010 April, which discuss the role of MBL in pediatric oncologic patients. The reviewers extracted from the articles the design and characteristics of studies, study group, tumor type, method of MBL analysis, definition of MBL deficiency, definition of outcome, methods used to detect infection, follow-up, and risk factor analysis. They concluded that the contradictory results of the examined studies might be explained by several clinical and methodologic inconsistencies. Another possible reason may be that none of these studies examined the question as a multivariate risk analysis. In the immune response of younger children, innate immunity outweighs adaptive immunity, as a result, the role of MBL is reevaluated depending on age. The tumor type and intensity of chemotherapy could be also risk factors as administration of certain chemotherapeutic drugs appears to be directly related to functional complement defects.[37] 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 pediatric oncologic patients. However, these results are refutable; therefore, a clinically relevant study with a unified definition would be necessary to explain the role of MBL, allowing for other risk factors.

We tried to plan and carry out our study considering the above-mentioned aspects. We found a correlation between the presence of *MBL2* polymorphisms and the incidence of infections in children suffering from hemato-oncologic diseases. The total number of febrile neutropenic episodes was significantly higher in the group with genotypes encoding lower MBL level during the first 8 months after the beginning of chemotherapy. In line with this finding, the number of the total febrile neutropenic days was higher

TABLE 2. MBL-MASP2 Complex Activation at Diagnosis and During a Febrile Neutropenic Episode

	MBL-MASP2 Activation (%), Median (IQR)	
	Patients With Normal Expected MBL Serum Level (YA/YA, YA/XA) n = 20	Patients With Low-expected MBL Serum Level (XA/XA, A/0, 0/0) n = 22
Time of diagnosis	82.9 (70.1–98.8)	59.7 (16.7–72.1)
During FN	85.7 (54.1–98.7)	42.1 (0–64.7)
paired t-test	$P = 0.123$ (60% reduced)	$P = 0.006$ (81% reduced or unchanged)

FN indicates febrile neutropenia; IQR, interquartile range; MBL indicates mannose-binding lectin; MBL-MASP2, mannose-binding lectin-associated serine protease 2.

during the follow-up period. The average length of the febrile neutropenic episodes was not significantly different in the 2 groups, showing that the severity of infections is not influenced considerably by the 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 to suffer 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.

Increasing evidence suggest that, besides MBL, variability of other constituents of the complement pathways or their combinations may also influence the occurrence of infections in immunocompromised patients. For instance, deficiency of MASP2, ficolin-3 as well as single or combined deficiencies of MBL2 and ficolin-2 were reported to be associated with an increased risk of infections and prolonged duration of febrile neutropenic episodes in leukemic children.[38–40]

In accordance with these studies, we decided to analyze 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. Although the inherited MASP2 deficiency occurs very rarely, the protein concentration can show variability, and therefore it should not be ignored. As expected, genotype of the *MBL2* polymorphisms considerably 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. The MBL marks pathogens as an opsonin leading to the chemically modified molecules having stronger interactions to cell surface receptors on phagocytes and thus enhancing phagocytosis. The explanation of the difference between the 2 genotype groups could be that in patients with normal genotype, the consumption is less significant or the expression of the protein is more inducible. Therefore, when the protein level decreases, the production could compensate the consumption.

Despite the contradictory results reported concerning the role of MBL, the efficiency and safety clinical trials of substitution therapy have started parallel to the genetic testing. Currently, 2 types of MBL preparations are in clinical phase trials: plasma delivered and human recombinant MBL. The phase I clinical trial, analyzing safety, and pharmacokinetics found neither clinical nor laboratory changes. The biologic activity, safety, and stability were similar in the 2 different preparations.[41–44]

Brouwer et al examined the effect of MBL therapy in vitro, and they found an increase in complement activation and opsonophagocytosis after plasma delivered MBL substitution therapy. However, the opsonophagocytosis recovery was suboptimal, the function increased after repeated MBL infusions. 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 the MBL therapy.[45]

In summary, our results support the importance of the MBL molecule in infectious complications of pediatric hemato-oncologic 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 analyzing the role of complement factors in immunosuppressed patients as interindividual differences, which may influence infection risk are not limited to single-gene variations but rather a combination of genetically determined predispositions that can also be affected by acquired defects caused by the applied chemotherapeutic drugs. The long-term benefit of these studies 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.

Pediatric hemato-oncologic patients carrying low MBL level coding genotypes are prone to infections following chemotherapy as indicated by higher frequency of febrile neutropenia and shorter infection-free survival. Besides, in these patients, MBL-MSP2 activation level showed marked decrease in infections, supporting the importance of a well-functioning MBL pathway in immunocompromised patients.

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