Mannose-binding lectin serum levels and (Gly54asp) gene polymorphism in recurrent aphthous stomatitis: A case-control study

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Abstract
Objectives: Dysregulation of the immune response appears to play a significant role in recurrent aphthous stomatitis (RAS) development. The main objective of this case-control study is to investigate the blood levels of mannose-binding lectin (MBL) and the frequency of the MBL2 gene (gly54asp) polymorphism in RAS patients, including 40 RAS patients and 40 healthy controls.

Methods: Serum MBL levels were determined by ELISA, while the PCR-restriction fragment length polymorphism was used in MBL2 genotyping.

Results: The median serum MBL level was significantly lower in the RAS group than in the control group (975 ng/mL (545–1320) vs. 1760 ng/mL (1254–2134); p < 0.001). The MBL levels were significantly lower in the BB genotype, whereas they were significantly higher in the wild type AA with a median of 525 and 1340 ng/mL, respectively (p =0.005). The B allele was expressed in significantly higher percentages of RAS patients than in controls. There was no significant association between MBL serum levels (p=0.685) or MBL2 codon 54 genotypes (p=0.382) with the type of ulcers.

Conclusion: There was an association between low MBL serum levels and the variant allele B of the MBL2 (gly54asp) gene, and the susceptibility to RAS. As a result, potential novel therapeutic options for RAS patients with MBL deficiency should be investigated.

Keywords
Codon 54, mannose binding lectin, oral ulcer, polymorphism, recurrent aphthous stomatitis

Date received: 27 July 2021; accepted: 16 November 2021
Introduction

Recurrent aphthous stomatitis (RAS), or recurrent oral ulcers (ROU) is the most prevalent oral mucosal disorder, characterized by recurrent painful ulcers in the non-keratinized oral mucosa (numerous episodes per year with more than one ulcer per episode). It affects up to 25% of the general population and can be manifested clinically in one of three forms: mild, major, or herpetiform. The minor subtype is the most prevalent (85% of all RAS), and it heals spontaneously within 7–10 days with no scars. The etiology of RAS is unknown, despite its elevated incidence. Hormonal factors, hematologic diseases, genetic predisposition, and immunologic disorders (autoimmunity, immunodeficiency), as well as local trauma, viral or bacterial infections, vitamin deficiencies, emotional stress, and chronic inflammatory bowel diseases (Crohn’s disease and ulcerative colitis), have been identified as local and systemic risk factors. RAS-induced severe pain, discomfort, and difficulties with eating and speaking can reduce the quality of life, particularly in patients who have recurrent attacks regularly. Since the etiology of RAS is still undetermined, there is currently no specific therapy. Indeed, the fundamental goal of RAS therapy is to alleviate pain and minimize wound healing time while also attempting to diminish the frequency and the acute attacks of the disease.

The mannose-binding lectin (MBL) is a component of the innate immune system and a member of the collectin family of complement system proteins. It plays a crucial role in first-line host defense by its antimicrobial properties and binds to particular sugar groups on various pathogens, initiating opsonization and phagocytosis. It can also modulate inflammatory responses. MBL deficiency appears to be a clinically significant predisposing factor to RAS, particularly in the presence of immunodeficiency or when the immune system is stressed.

The Human MBL-encoding gene, namely MBL2, is a four-exon gene with three introns found in chromosome 10 (10q11.2–q21). The MBL2 gene wild type allele is designated as an A allele. Three allelic variants in MBL2 exon 1 at codons 52, 54, and 57 have been named D (arginine to cysteine), B (glycine to aspartic acid), and C (glycine to glutamic acid) variant alleles, respectively, and grouped as an O allele. Varied ethnic groups have different frequencies of these alleles. All three alleles are found in Caucasians, whereas alleles C and D are extremely uncommon in Asians. The B allele, in which aspartic acid replaces the wild-type A glycine, is the most prevalent variant among other MBL polymorphisms.

Genetic polymorphisms in the MBL2 gene can interfere with the protein assembly, causing low serum levels of MBL, impairing its biological function, enhancing susceptibility to infectious diseases, and mediating various autoimmune as well as inflammatory disorders. This study aimed to investigate MBL serum levels and (gly54asp - rs1800450) polymorphism in RAS patients to evaluate whether MBL can be used as a future therapeutic target in these patients.

Methodology

Study settings and subjects

The study was carried out in compliance with the ethical criteria established by the Ethics Committee of the Faculty of Medicine, Ain Shams University and was approved by the same committee. All procedures were explained to all participants, and informed consent was obtained from them or their guardians.

The sample size was justified using 11 Pass Program for sample size calculation, setting power at 80% and α-error at 0.05, and according to Inanc et al. 2005, a sample size of 37 subjects per group was sufficient to achieve the study objective.

A total sample of 40 RAS patients and 40 healthy control subjects were included in this case-control study from November 2020 to August 2021. They were recruited from the Allergy and Clinical Immunology and Pediatric Clinics, Faculty of Medicine, Ain Shams University, while laboratory work was carried out at the Medical Microbiology and Immunology Department, Faculty of Medicine, Zagazig University and Clinical Pathology Department, Faculty of Medicine, Ain Shams University.

All participants were subjected to a detailed medical history, thorough clinical examination and laboratory investigations (including evaluation of serum levels of iron, ferritin, folate, thiamin (vitamin B1), riboflavin (vitamin B2), vitamin B6, vitamin B12, and zinc) to determine risk factors that include a family history of RAS, autoimmune or secondary conditions. Participants were also assessed for genital ulcers as well as any cutaneous, ocular, or rheumatological complaints.

All RAS patients had to have recurrent, numerous oral ulcers despite good oral hygiene. They were classified into; minor RAS, with superficial ulcers in the anterior part of the mouth, about 4–5 mm in diameter; major RAS, with ulcers similar in appearance to minor RAS but >10 mm in diameter; or herpetiform ulceration, with multiple (5–100) and small (1–2 mm) ulcers that can be coalescent. Patients with any possible underlying cause of oral aphthae were excluded from the study, including any connective tissue disease, vasculitis, vitamin deficiency, medication-induced aphthae, inflammatory bowel disease, and Behcet’s syndrome.

Blood sample collection

By aseptic venous puncture, two tubes containing 3 mL of peripheral blood were taken from each participant. Blood in the EDTA-containing tube was used for complete blood count (CBC) analysis by a Coulter LH 780 Analyzer (Beckman Coulter, USA) and MBL2 genotyping by PCR-restriction fragment length polymorphism (PCR-RFLP),...
Table 1. Comparing RAS patients and control groups in terms of their characteristics.

| Variable                        | RAS cases (n = 40) | Control (n = 40) | p-value |
|---------------------------------|--------------------|-----------------|---------|
| Age (years)                     | Median (IQR)       | 22.5 (16–55)    | 30.5 (18–51) | 0.069 |
| Sex, n (%)                      | Females            | 15 (37.5%)      | 15 (37.5%) | 1.000 |
|                                 | Males              | 25 (62.5%)      | 25 (62.5%) |       |
| Family history, n (%)           | RAS                | 15 (37.5%)      | 0 (0.0%)   | ≤0.001 |
|                                 | Autoimmune disease| 7 (17.5%)       | 0 (0.0%)   | 0.006  |
| CBC Median (IQR)                | TLC × 10^3/μl      | 6.65 (4.375–8.9) | 8.1 (7.025–9.3) | 0.006 |
|                                 | Neutrophils × 10^3/μl | 3.5 (2.66–5.6) | 4.35 (3.9–4.9) | 0.047  |
|                                 | Eosinophils × 10^3/μl | 0.21 (0.1–0.4) | 0.0 (0.0–0.1) | ≤0.001 |
| MBL genotype                    | AA                 | 16 (40.0%)      | 30 (75.0%) | 0.006  |
|                                 | AB                 | 14 (35.0%)      | 7 (17.5%)  |        |
|                                 | BB                 | 10 (25.0%)      | 3 (7.5%)   |        |
| MBL2 gene allele                | A                  | 46 (57.5%)      | 67 (83.8%) | ≤0.001 |
|                                 | B                  | 34 (42.5%)      | 13 (16.3%) |        |
| Serum MBL (ng/ml)               | Median (IQR)       | 975 (545–1320)  | 1760 (1254–2134) | ≤0.001 |

CBC: Complete blood count; IQR: Interquartile range; MBL: Mannose binding lectin; RAS: Recurrent aphthous stomatitis; TLC: Total leucocytic count. Statistical significance was set at 0.05.

whereas serum in the plain tube was used for MBL level detection by ELISA.

**Quantitation of MBL serum level**

The MBL serum levels of the study participants were determined using sandwich ELISA (Quantikine® ELISA, Human MBL immunoassay; R and D Systems, Minneapolis, USA; Catalog Number: DMBL00). The absorbance was measured using an ELISA Stat Fax® 303 Plus microplate reader (Awareness Technology, Inc., Palm City, USA) set at 450 nm with a 630 nm wavelength correction. MBL concentration in serum was expressed as ng/mL.

**Genotyping of the MBL2 (gly54asp - rs1800450)**

Amplification of genomic DNA from whole blood was done using a Phusion™ Blood Direct PCR kit (Thermo Scientific™, USA) with primers specific for the human exon 1 of the MBL2 gene (forward: 5’-TGGACAGAGGGCATGCTC-3; reverse: 5’-CAGGCCAGTTTCCCTCTGGAAGG-3). According to the manufacturer’s instructions, 5 μL of the whole blood was added to 25 μL of the Master Mix with 2.5 μL of each primer, and the final volume was then completed to 50 μL by nuclease free water. The PCR cycles were as follows: 40 cycles of 5 s denaturation at 98°C, 30 s annealing at 58°C and 30 s extension at 72°C, and then a final extension for 1 min. The 349-base pair (bp) product was then digested by restriction enzyme BanI (BshNI; Thermo Scientific™, USA) using 1 μL of the enzyme at 37°C overnight. Subsequently, the product was visualized by electrophoresis on a 2% agarose gel. The A allele was cleaved into two pieces (260 and 289 bp) by BanI, but the B allele remained undigested (349 bp).23

**Statistical analysis**

Analyses of data were done using the Statistical Package for Social Sciences version 21 (IBM Corporation, Armonk, NY, USA). Non-parametric quantitative variables were described in the form of medians and interquartile ranges. Qualitative data were expressed as numbers and percentages. For non-parametric quantitative data comparison, the Mann-Whitney U and the Kruskal-Wallis tests were used, while the Chi-squared test compared qualitative data. Spearman correlation coefficient was used to assess the association between not normally distributed variables. A p-value of 0.05 was considered significant.

**Results**

This case–control study included a total sample of 80 subjects stratified into two equal groups, the RAS group (n = 40) and the control group (n = 40). There were no significant differences between the two studied groups regarding age or sex. Among the included RAS patients, 37.5% had a family history of RAS, while 17.5% had a positive family history of other autoimmune diseases. There was no family history of RAS or other autoimmune disorders in any of the controls. Table 1

With respect to CBC parameters, decreased total leucocytic count (6.65×10^3/μl (4.375–8.9) vs. 8.1×10^3/μl (7.025–9.3); p = 0.006) and absolute neutrophil count (3.5×10^3/μl (2.66–5.6) vs. 4.35×10^3/μl (3.9–4.9); p = 0.047) and a higher absolute eosinophil count (0.21×10^3/μl (0.1–0.4) vs. 0.0×10^3/μl (0.0–0.1); p ≤ 0.001) in the RAS group than in the control group. Table 1

The median (IQR) serum MBL level was significantly lower in the RAS group compared to the control group.
(975 ng/mL (545–1320) vs. 1760 ng/mL (1254–2134); \( p \leq 0.001 \)) (Figure 1). The A allele was found in 57.5% of RAS patients and 83.8% of the controls, while the frequencies of B allele were 42.5% and 16.3%, respectively. Table 1 and Figure 2.

Serum levels of MBL varied significantly \((p = 0.002)\) among the three different genotypes in RAS patients being significantly lower in homozygous mutant BB genotype carriers with a median (IQR) of 525 ng/mL (442.5–725). In contrast, they were higher in homozygous wild type AA carriers with a median (IQR) of 1340 ng/mL (827.5–1932). The median (IQR) MBL level of the heterozygous AB genotype carriers was 990 ng/mL (695–1100). However, there was no statistically significant difference in MBL serum level between the AA and the AB genotypes \((p = 0.053)\). Table 2

Among the 40 RAS patients, three (7.5%) patients had herpetiform ulcers, eight (20.0%) patients had major ulcers, and 29 (72.5%) had minor ulcers. MBL serum levels \((p = 0.685)\) or

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**Figure 1.** Scatter diagram showing serum MBL levels in cases and controls. Control (gray circles), Cases (red circles)

**Figure 2.** Frequencies of MBL2 codon 54 genotypes (A) and alleles (B) in RAS cases and controls
the MBL2 codon 54 genotypes ($p = 0.382$) showed a non-significant association with the type of ulcer. Table 3

There was no significant correlation between the serum MBL level and age of RAS patients ($p = 0.479$), absolute neutrophil count ($p = 0.449$) and eosinophil count ($p = 0.205$). On the contrary, total leucocytic count showed a significant positive correlation ($p = 0.011$). Table 4

**Discussion**

RAS is one of the most prevalent oral disorders; however, its etiology remains undetermined. Several infectious, genetic, inflammatory, atopic, and autoimmune factors have been implicated in RAS pathogenesis. MBL deficiency may establish the pathogenesis of RAS by reducing apoptotic cell clearance, triggering adaptive proinflammatory pathways, and increasing local tissue destruction.

Serum MBL levels are significantly correlated with the MBL2 gene polymorphism and can be influenced by several factors like infections, drugs, and hormones. In this study, we aimed to investigate the association of MBL serum levels and MBL2 exon 1 codon 54 genotypes with RAS frequency in a group of Egyptian patients and healthy controls, bearing in mind that other genetic variations can be implicated in the etiology as well.

In our study, 37.5% of the RAS group had a positive RAS family history, and 17.5% had a family history of other autoimmune disorders. Consistent with this finding, in 1997, Rogers demonstrated that the chances of developing RAS elevated from 20% to 90% in the offspring of parents with aphthae. In other epidemiologic studies, a link between RAS and family history was detected in 24%–46% of RAS participants.

In our study, MBL serum levels were significantly lower in the RAS group than in the control group (975 ng/mL [545–1320] vs. 1760 ng/mL [1254–2134]; $p \leq 0.001$). Comparatively, Inanc and colleagues reported a wide range of serum MBL levels in both the RAS and control groups, with

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**Table 2.** Comparison of characteristics of different MBL2 codon 54 genotypes in RAS patients.

| Variable | AA ($n = 16$) | AB ($n = 14$) | BB ($n = 10$) | $p$-value |
|----------|---------------|---------------|---------------|-----------|
| Age (years) | Median (IQR) | 24 (17.25–28.5) | 21 (16–42) | 23 (15.5–30) | 0.681 |
| Sex n, (%) | | | | | |
| Females | 3 (18.8%) | 7 (50.0%) | 5 (50.0%) | 0.135 |
| Males | 13 (81.3%) | 7 (50.0%) | 5 (50.0%) | |
| Family history n, (%) | | | | |
| RAS | 6 (37.5%) | 3 (21.4%) | 6 (60.0%) | 0.157 |
| Autoimmune disease | 3 (18.8%) | 2 (14.3%) | 2 (20.0%) | 0.923 |
| CBC | | | | |
| TLC $\times 10^{3}/\mu l$ | Median (IQR) | 7.85 (4.15–9.925) | 6.65 (5.2–8.6) | 5.2 (3.8–6.975) | 0.162 |
| Neutrophils $\times 10^{3}/\mu l$ | Median (IQR) | 3.95 (2.6–5.6) | 3.66 (2.98–5.76) | 3.49 (1.59–3.82) | 0.300 |
| Eosinophils $\times 10^{3}/\mu l$ | Median (IQR) | 0.22 (0.07–0.59) | 0.3 (0.13–0.4) | 0.15 (0.07–0.6) | 0.898 |
| Serum MBL (ng/ml) | Median (IQR) | 1340 (827.5–1932) | 990 (695–1100) | 525 (442.5–725) | 0.002 |
| CBC: Complete blood count; IQR: Interquartile range; MBL: Mannose binding lectin; RAS: Recurrent aphthous stomatitis; TLC: Total leucocytic count. Statistical significance was set at 0.05.

**Table 3.** Comparison of MBL serum level and codon 54 genotypes according to the type of RAS ulcer.

| Variable | Herpetiform (3/40, 7.5%) | Major (8/40, 20.0%) | Minor (29/40, 72.5%) | $p$-value |
|----------|-------------------------|-------------------|-------------------|-----------|
| Serum MBL (ng/ml) | Median (IQR) | 1555 (1320–1790) | 980 (675–2038.25) | 780 (525–1100) | 0.685 |
| MBL genotype n, (%) | AA | 1 (6.3%) | 5 (31.3%) | 10 (62.5%) | 0.382 |
| | AB | 2 (14.3%) | 1 (7.1%) | 11 (78.6%) | |
| | BB | 0 (0.0%) | 2 (20.0%) | 8 (80.0%) | |

MBL: Mannose binding lectin. Statistical significance was set at 0.05.
Table 4. Correlation of MBL serum concentration with the other parameters in RAS patients.

| Variable    | Correlation coefficient (r) | p-value |
|-------------|-----------------------------|---------|
| Age         | 0.115                       | 0.479   |
| TLC         | 0.399                       | 0.011   |
| Neutrophils | 0.123                       | 0.449   |
| Eosinophils | 0.205                       | 0.205   |

TLC: Total leukocytic count. Statistical significance was set at 0.05.

no significant differences (2309 ng/mL (48–6152) vs. 3136 ng/mL (0–8320); p= 0.074). They also detected a non-significant difference between the studied groups regarding the percentage of patients with serum MBL levels <100 ng/mL (RAS: 14% vs. control: 12%; p > 0.05). In contrast, they found that serum MBL levels >3000 ng/mL were significantly more in the control group than in the RAS group (p < 0.05).26

On the other hand, Lewkowicz and colleagues reported higher, although not statistically significant (p = 0.08) MBL values in RAS patients compared to controls, in both the active and remission stages. They also reported that MBL levels remarkably increased during RAS remission.24

Depending on ethnicity, MBL deficiency induced by genetic abnormalities affects 5–30% of the population.29 In addition, genetic abnormalities can result in reduced blood concentration or dysfunctional MBL proteins.19 The most prevalent polymorphic variant in the coding region of the MBL2 gene was found to be at codon 54 (B allele).30 B allele was significantly correlated with the lowest serum MBL level, and the heterozygous polymorphisms reduced MBL levels 5-10-fold, while homozygous mutations resulted in almost undetectable MBL levels.31–33

Our results revealed that the homozygous BB variant was the least prevalent (25.0% in RAS patients and 7.5% in controls) and was associated with significantly lower levels of serum MBL in RAS patients with a median (IQR) of 525 ng/mL (442.5–725). In contrast, the homozygous wild AA genotype was the most prevalent among the study participants. It was associated with higher serum MBL levels in the RAS patients with a median (IQR) of 1340 ng/mL (827.5–1932).32

Suniya et al. in 1991 reported the B allele variant in children with low serum MBL levels and recurrent infections. MBL serum levels showed a tenfold decrease in individuals with a heterozygous genotype suggesting an autosomal dominant inheritance. It was surprisingly frequent in healthy Caucasians and rare in East Africa.17

The ‘B’ variant allele of the MBL2 gene in exon 54 has been linked to a variety of disorders, including resistant hepatitis and spontaneous bacterial peritonitis,34 acute respiratory tract infections,35 celiac disease,36 systemic lupus erythematosus,37 recurrent vulvovaginal candidiasis,38 and acute allograft rejection episodes in kidney transplant recipients.39

Our study revealed that the B allele was expressed in significantly higher percentages of RAS patients (42.5%) than controls (16.3%). In contrast, no significant association could be detected between the MBL2 codon 54 genotypes and severe chronic periodontitis, neonatal sepsis, and recurrent respiratory tract infection in children.40

Among the 40 RAS patients in our study, 29 (72.5%) had minor ulcers, 8 (20.0%) patients had major ulcers, and 3 (7.5%) patients had herpetiform ulcers. Similarly, Slebioda et al. reported that the most common type of RAS was minor aphthae (56 patients, 74.7%) and found major RAS in 14 (18.7%) as well as herpetiform RAS in 5 (6.7%) subjects.25

Our results showed no statistically significant relationship between MBL codon 54 genotype (p=0.382) or MBL serum levels (p=0.685) and the type of ulcer. This may be attributed to the low number of RAS subjects after subgrouping by ulcer type. More RAS patients should be studied to allow a more reliable statistical comparison between MBL2 genetic variants and the ulcer phenotype.

Regarding CBC parameters, in the RAS group compared to the control group, the total leukocytic count and the absolute neutrophil count were significantly lower (p=0.006 and 0.047, respectively), while the absolute eosinophil count was significantly higher (p ≤ 0.001). Moreover, the total leukocytic count showed a significant positive correlation (p=0.011) with the serum level of MBL.

In contrast, other studies reported a significant increase in the inflammatory markers, including total leukocytic count, absolute neutrophil count, and neutrophil-to-lymphocyte ratios in RAS patients compared to the control group.44,45 In addition, Liang and Neoh reported variable and non-specific changes in the inflammatory markers in RAS patients. They found that the total leukocytic count ranged from 5.7–15.7x10³/μL, with no neutropenic cases detected. They could not find any significant correlations between total leukocytic count and response to treatment and relapse rate.46 The significant increase in eosinophil counts observed in our included RAS patients compared to the controls could be attributed to the underlying hypersensitivity or atopic mechanism of RAS, which needs further investigation.

Many other mutations have been identified in the exon or promoter regions of the MBL2 gene and linked to MBL serum levels in various studies.15,41,47 Differences in results between prior studies and the current study could be explained by the variation in sample sizes, ages of participants, and ethnic groups included in each study. The major limitations in our research were the financial constraints that prevented us from studying other MBL2 gene variants and the relatively small sample size. Despite the high prevalence of the disease, only a few people seek medical assistance due to their lack of awareness and the transient nature of the disease, as most RAS cases are minor. To corroborate our findings and further detect the role of MBL in RAS pathogenesis, more studies on a larger
scale are needed; also, the evaluation of other significant MBL2 gene variants associated with MBL levels in RAS are highly recommended.

Conclusions

Our study showed that the B allele of the MBL2 codon 54 was significantly associated with the lowest serum MBL level and RAS susceptibility but not with RAS ulcer phenotype. Moreover, MBL may be considered as a future therapeutic target in RAS patients.

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