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Influence of Minimal Residual Disease at Day 15 of Induction Therapy on Survival of Children with Acute Lymphoblastic Leukemia

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Abstract

Objective. The aim of the study was to evaluate the impact of minimal residual disease (MRD) on day 15 of induction therapy (d15) on the treatment outcome in children with acute lymphoblastic leukemia (ALL). **Materials and Methods.** The study included 74 patients (1-18 years) with ALL, who were treated at the Pediatric Clinic of the University Clinical Center Banja Luka from January 2011 to May 2021. All patients were treated according to ALL IC-BFM 2009 protocol. MRD on bone marrow was assessed d15, using the multiparameter flow cytometry method (FCM). **Results.** Of all, 59.46% of patients had MRD d15 0.1−10%, MRD<0.1% had 18.92% of patients, and 21.62% had MRD >10%. Patients with the lowest MRD had the highest 5-year overall survival (OS) and event-free survival (89.5% and 91% respectively) and the lowest cumulative risk for relapse or death (9.7% and 8.1%), in contrast to patients with MRD>10% in whom OS was 80.0%, and the risk of recurrence is 20%. Predicted MRD d15 was significantly associated with prednisone response assessed in the peripheral blood on day 8 (P<0.001) and statistically significantly positive correlation (r=0.498; P<0.001) was found. **Conclusion.** MRD measurement d15 has a great prognostic significance for patients in the standard and high risk groups, but not for patients in the intermediate risk group. The introduction of additional testing is necessary for better identification of patients with an increased risk of disease recurrence.

Key Words: Minimal Residual Disease ▪ Acute Lymphoblastic Leukemia ▪ Children ▪ Outcome.

Introduction

Modern treatment of childhood acute lymphoblastic leukemia (ALL) is based on the individualization of therapy according to risk groups, which implies intensification of therapy for high-risk groups and optimization of therapy for low-risk groups, with the aim of achieving long-term remission while minimizing toxic complications of chemotherapy (1, 2). Thanks to risk-adapted therapy approach and better supportive care, the 5-year survival of children with ALL has increased significantly over the last decades, from 50% to 92% (3, 4). However, disease relapses are still the main cause of poor treatment outcome and occur in about 20% of children with ALL (5, 6).

Furthermore, it has been proven that the final outcome is influenced by many biological and clinical characteristics such as the age of the child, the number of leukocytes and infiltration of the central nervous system at diagnosis, cytogenetic characteristics of the disease as well as the initial response to therapy (1). Therefore, identifying the most sensitive prognostic factors for predicting relapse was of great importance in order to realize the concept of "risk-adjusted therapy" (6-10). It has been shown that an early treatment response is a significant prognostic indicator and a predictive factor for disease recurrence (9, 10). Patients with good prednisone on day 8 with a significant reduction in the number of blasts in the peripheral blood as well as a reduction in blasts in the bone marrow on day 15 have a significantly better prognosis (10). However, rapid development and modeling of therapy intensity have shown that their

association with relapse is not perfectly correlated. Some of the reasons are great morphological similarity of all blast cells with bone marrow lymphoid precursors (hematogones) and often with mature lymphocytes. Compared to standard bone marrow cytomorphology, minimal residual disease (MRD) analysis enables the detection of one blast cell per 10,000 to 100,000 normal cells, which represents a 100-fold higher sensitivity compared to standard cytomorphological analysis (11).

Today, two methods are most commonly used for the assessment of MRD, flow cytometry (FC) for the analysis of aberrant immunophenotypes and polymerase chain reaction (PCR) method with amplification of various fusion gene receptors for immunoglobulins or T-cell gene receptor rearrangements. Assessment of MRD during the treatment of various hematological malignancies has a high predictive value for disease recurrence, and therefore worse event free survival (ES) as well as overall survival (OS) (4, 11–13). Personalization of therapy based on MRD thus may improve the treatment outcome of children with ALL (4). Therefore, FC-MRD monitoring in different time points is used in current protocols and is the main criterion of stratification therapy with a more precise selection of intensity and duration (3, 14–18).

The aim of the study was to evaluate the impact of FC-MRD measured on day 15 of induction therapy (d15) on the treatment outcome in children with ALL.

Material and Methods

Patients and Treatment Protocol

The retrospective study included 74 patients aged 1 to 18 years, with newly diagnosed ALL, who were treated at the Pediatric Clinic of the University Clinical Center Banja Luka from January 2011 to May 2021. The Ethical Committee of University Clinical Centre Banja Luka approved the study (No. 01-19-360-2/23). All children were treated according to ALL IC-BFM 2009 protocol and were divided into three risk groups: standard, intermediate and high risk. Stratification of patients into risk groups was defined according to the treatment protocol, based on clinical, laboratory and genetic characteristics of the disease as well as initial response to therapy - prednisone response d8 and MRD d15 of therapy (19). The measurement of the absolute blast count (ABC) in the peripheral blood on d8 (after 7 days of prednisone and one intrathecal therapy) in the induction phase was done in all patients. Whereas, ABC lower than 1000/μL is considered as a good prednisone response (PGR), and ABC 1000/μL and higher as a poor prednisone response (PPR). In addition, MRD was assessed in all patients in a bone marrow sample obtained by bone marrow aspiration from the iliac crest after 14 days of corticosteroid therapy (prednisone), single doses of daunorubicin and vincristine and asparaginase, and two intrathecal therapies with methotrexate.

Sample Preparation and Multiparameter Flow Cytometry

In our study, MRD assessment was conducted by the eight-color FC (BD FACSCanto II) at the Department of Clinical Pathology and Immunophenotyping of the Institute for Health Care of Mothers and Children of Serbia "Dr. Vukan Čupić", Belgrade. The standardization process, including sample preparation, monoclonal antibody clone selection, staining-lysis procedure, and flow cytometric analysis, was performed according ALL IC-BFM 2009 protocol.

Briefly, bone marrow samples were transported immediately after aspiration in an ethylenediamine tetra acetic acid tube and analyzed by FC within 24 hours, using standard whole bone marrow lysis protocols (20, 21). Per sample, a standard acquisition consisted of 3×10^5 cell events. For FC analysis, samples were incubated with fluorochromes conjugated to antibodies specific for the proteins of interest. A stream of individual cells passes through multiple lasers that excite each fluorochrome and the emitted fluorescence intensity is captured and converted into digital signals that can be analyzed. Until 2019, a panel of four color combinations was used, and since then

an eight-color panel. For B-cell precursor ALL (B ALL) it was CD58/CD10/CD34/CD19/CD20/CD38/SYTO41/CD45, D10/CD11a/CD34/CD19/CD20/CD38/SYTO41/CD45 and for T-cell ALL (T ALL) CD99/CD56/ CD3/CD5/ iCD3/CD7/SYTO41/CD45 (antibodies ordered by channel sequence: fluorescein isothiocyanate, phycoerythrin, peridinin-chlorophyll-protein, allophycocyanin, phycoerythrin-cyanin 7, allophycocyanin-cyanine 7, violet 450, Violet 500. Data collection and analysis was performed using Diva software (BD Life Sciences, San Jose, CA USA).

Addionally, live cell permeant SYTO 16 or SYTO 41 nucleic acid fluorochrome staining (Invitrogen™, Thermo Fisher Scientific, Waltham, MA USA) was used to exclude residual anucleated erythroid cells, platelets or debris. This staining allowed unbiased proportional quantification of MRD among total NC (SYTO+).

Overall Survival and Event-Free Survival Definition

The OS was defined in months from disease diagnosis to death or last contact, and EFS from disease diagnosis to some event: relapse, death or follow-up contact. All data related to patient demographic characteristics (age, sex), immunophenotypic, cytogenetic, and molecular characteristics of leukemia, early response to prednisone, and FC-MRD assessment of were collected from the hospital's electronic database and patient medical history.

Statistical Analysis

The difference in the frequency of the observed characteristics according to patient groups was evaluated by Pearson's χ^2 contingency test. The OS and EFS survival were estimated by the Kaplan-Meier method, and the significance of differences between different risk groups in terms of OS and EFS survival was tested by the log rank test. The cumulative risk of recurrence or death is shown as a Hazard Ratio (HR). Spearman's non-parametric correlation was used to determine the degree of association (correlation) of the characteristics. The SPSS program for Windows (SPSS, Chicago, IL, USA) was also used for statistical analysis, whereas $P<0.05$ was considered significant.

Results

Thirty-nine boys (52.7%) and 35 girls (47.3%) aged 1 to 18 years, with an average age 6.88 years (median 4.0 years) were included in the study. The mean follow-up time was 78.5 months (from 36 to 120 months). Precursor B ALL was diagnosed in 59 patients (79.73%), and T ALL in 15 patients (20.27%). Considering the risk group, patients were stratified into three groups: the intermediate risk group, high risk group and standard risk group (Table 1).

Regarding FC-MRD d15, 44 patients (59.46%) had MRD 0.1–10%, 14 (18.92%) <0.1%, and 16 (21.62%) patients >10%. Sixty-four (86.49%) patients had GPR d8, 64 (86.49%). The main event

Table 1. Demographic and Clinical Characteristics of Patients

Characteristics	Value
Age: Median (IQR*)	4.0 (3.0–12.0 yrs)
Gender: Male (N; %)	39 (52.70)
Imunofenotype (N; %)	
T cell	59 (79.73)
B cell	15 (20.2)
MRD† (N; %)	
<0.1%	14 (18.92)
0.1–10%	44 (59.4)
>10%	16 (21.62)
RISK group (N; %)	
Standard risk	7 (9.40)
Intermediate risk	48 (64.90)
High risk	19 (2570)
Cytogentics (N; %)	
Normal	20 (27.03)
Hyperdiploidia	19 (25.68)
Hypodiploidia	4 (5.40)
No results	31 (41.89)

*Interquartile interval; †Minimal residual disease.

Table 2. Distribution of Events According to FC-MRD at 15 Day of Induction Therapy

Events	FC-MRD*			
	<0.1%	0.1-10%	>10%	Overall
	N (%)			
Overall	14 (18.92)	44 (59.46)	16 (21.62)	74 (100)
Relapse	1 (10.00)	8 (80.00)	1 (10.00)	10 (12.16)
Death	1 (10.00)	6 (60.00)	3 (30.00)	10 (12.16)
After relapse	1	6	-	7 (70.00)
In induction	-	-	3	3 (30.00)

*Flow Cytometry-Minimal Residual Disease.

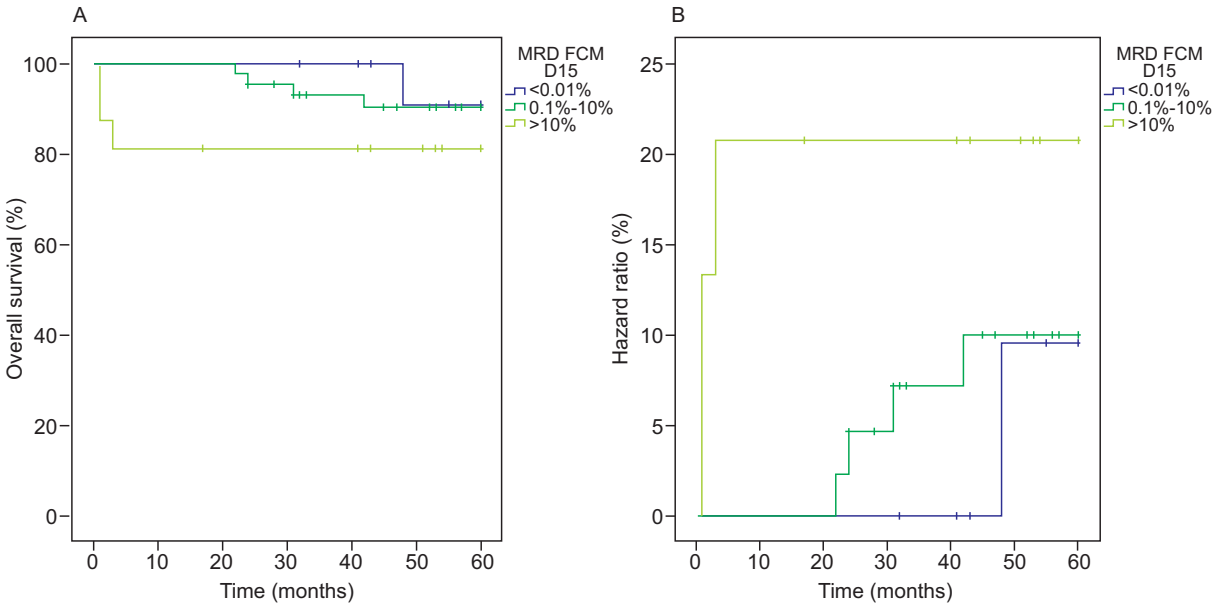

Figure 1. (A) Estimated overall survival according to FC-MRD at day 15 of induction therapy by flow cytometry and (B) cumulative hazard ratio for death according to MRD at day 15. D=Day; MRD=Minimal residual disease.

that affected the survival of our patients was disease recurrence: 10 (12.16%) patients had a recurrence. We found that the majority (8 patients, 80%) relapsed from group with MRD d15 0.1-10%, and 1 patient each with MRD d15 <0.1% and >10% (Table 2).

Ten (12.16%) patients died, of which disease recurrence was the cause of death in 7 (70%) patients. The other three patients died in induction phase and had high MRD d15 (above 10%), one patient died from an anaphylactic reaction to asparaginase, two from severe infection, and none of these three patients achieved remission d33.

The 5-year OS of patients with MRD d15 <0.1%, 0.1–10% and >10% was 89.5%, 88% and 80.0%, respectively (Figure 1A). The cumulative hazard ratio (HR) for death or relapse was 9.7%, 10.2% and 20%, respectively (Figure 1B) confirming that patients with FC-MRD d15 <0.1% had the highest OS with the lowest risk for relapse or death and that patients with MRD >10% had the lowest OS and the highest risk of recurrence or death. The log rang test determined that differences in OS according to MRD were not statistically significant (P=0.384).

Similar results were obtained in the analysis of the 5-year EFS. The highest EFS was in patients with MRD d15 <0.1% (91%), and the lowest EFS was in patients with MRD d15 >10% (72%) (Figure 2A). Also, the lowest risk for recurrence

Table 3. Distribution of FC-MRD at 15 Day of Induction Therapy according to Prednisone Response

FC-MRD*	Good prednisone response N (%)	Poor prednisone response N (%)	P-value†
< 0.1%	17 (26.56)	0 (0)	
0,1–10%	40 (62.50)	1 (10)	<0.001
>10%	7 (10.94)	9 (90)	
Total	64 (100)	10 (100)	-

*Flow Cytometry-Minimal Residual Disease: †Pearson ^{2} test.

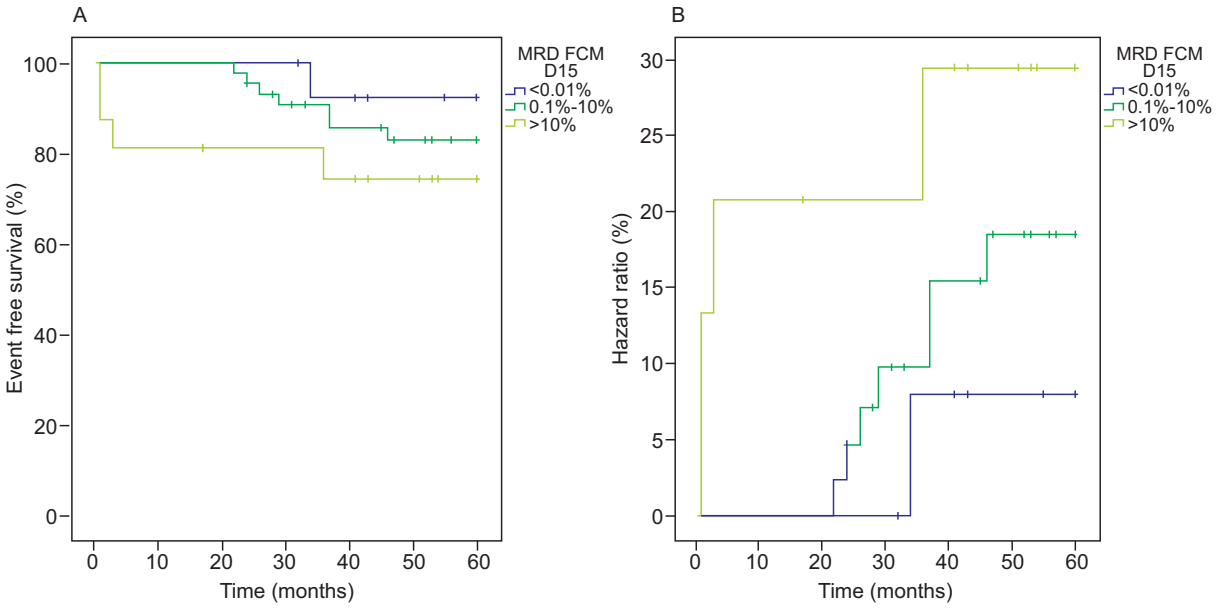

Figure 2. (A) Estimated event-free survival according to FC-MRD at day 15 of induction therapy by flow cytometry and (B) cumulative hazard ratio for relapse according to MRD at day15. D=Day; MRD=Minimal residual disease.

or death was in patients with MRD d15 <0.1% (8.1%), and the highest in patients with MRD d15 >10% (29.9%) (Figure 2B). The log rang test determined that differences in EFS according to MRD were not statistically significant (P=0.341). From patients with PPR, 90% had FC-MRD d15 >10%, and only one patient 0.1% to 10%. Whereas, no patient with MRD d15 <0.1% had PPR (P<0.001) (Table 3). Additionally, when bone marrow FC-MRD d15 and peripheral blood ABC d8 values were compared, a statistically significant positive correlation was found (r=0.498; P<0.001).

Discussion

Demographic and clinic characteristics of study patients are in agreement with the results of other studies (1, 22–25). By modulating the intensity of therapy based on the precise stratification of patients depending on the risk of disease recurrence, significant progress has been achieved in the treatment of pediatric ALL, with a five-year survival rate of around 80% in developed countries (2, 3). Assessment of early response to therapy plays a significant role in the treatment and cure of children with ALL (4, 13–16). Furthermore, long-term experience has shown that the minimization of leukemic cells in the peripheral blood on d8 of therapy, known as the prednisone response, and in the bone marrow (morphological and FC-MRD assessment) represents an important factor in assessing the success of therapy and predicting the disease recurrence, and therefore has become an integral part of the BFM protocol and criteria for stratification of patients into risk groups (13). Additionally, in the modern therapeutic protocols

such as ALL IC-BFM 2009, MRD assessment at different time points is used to assess therapeutic response and optimization of further treatment (18, 20, 26–28).

In the previous treatment protocol that we also used, ALL IC-BFM 2002, only bone marrow cytomorphology day 15 and 33 was used to assess the therapeutic response. When the results of morphological remission were compared with FC-MRD values in bone marrow day 15 and 33, a significant correlation was observed. However, it was determined that this positive correlation applies more to patients who are stratified into the high risk group, and less to the low risk group (20, 27). Since the main goal of the treatment strategy for children with ALL is the reduction of all toxicities, it is also important to identify low-risk group patients who require less intensive therapy (4). Nevertheless, the assessment of therapeutic response by morphological analysis of bone marrow is still of great importance and is used as the only assessment in countries with limited resources (20). By analyzing MRD d15, our results showed that patients with MRD <0.1% had a much better outcome (OS 89.5%, EFS 91%), compared to patients with MRD d15 >10% (80%, 72%). The same results apply to the overall cumulative risk of relapse or death, which is highest in patients with MRD d15 >10% (20%, 29.9%), and lowest in patients with MRD <0.01% (9.7%, 8.1%). Although our results were not statistically significant, they are consistent with the results of other similarly designed studies and confirm the importance of early MRD measurement in predicting prognosis (13, 29).

However, in contrast with AIEOP-BFM-2000 study, we cannot state that the MRD d15 is the most important predictive prognostic, which especially applies to patients in the intermediate risk group (18, 20). The main event that affected the survival of our patients was the recurrence of the disease, which occurred in 12.16% of patients, which was related predominantly to patients, with MRD d15 0.1–10%, (80% patients). Recurrence was also the most common cause of death in our patients (70%). Similar results were obtained by other authors with a significantly higher incidence of relapse and death in patients in the intermediate risk group, which confirms the fact that MRD d15 assessment cannot clearly define all patients with a high risk of disease recurrence (30, 31). In our study disease relapse was also recorded in one patient with the lowest MRD d15 values, suggesting that genotypic and phenotypic heterogeneity of leukemia, in addition to MRD, significantly influence the outcome of the disease (8). Also, despite the clear standardization of tests for MRD d15 assessment, other testing limitations, the possibility of diagnostic errors and the sensitivity of the tests should not be ignored (29). Early assessment of prednisone response as well as bone marrow morphological and FC-MRD d15 analysis are an integral part of all BFM protocols and stratifying of patients into risk groups. The results of our study showed that no patient with FC-MRD d15 <0.1% had PPR, and that 90% of patients with PPR had FC-MRD d15 >10% (P<0.001), which is consistent with the results of other studies (20, 26, 27). Our study also found a statistically significant positive correlation between prednisone response d8 and MRD d15 (r=0.498; P<0.001). Data from the literature have shown that prednisone response and morphologic criteria can identify most patients with MRD d15 >10%, but such a correlation does not apply to patients with a lower MRD values, who represent the target group for reducing the intensity of therapy and the absence of long-term complications (4, 27).

Limitations of the Study

Despite that our study has certain limitations such as retrospective nature of data analyses, relatively small number of patients which was influenced by fact that this was single center experience we summarized that our results are very important as this was first publication about children ALL in our country.

Conclusion

Our study is the first study in Bosnia and Herzegovina that showed the impact of bone

marrow FC-MRD d15 on the classification of patients into risk groups and the disease. The results confirmed the importance of MRD d15 for patients in the low and high-risk groups, but not for patients in the intermediate risk group. Given the large proportion of patients with a poor outcome from this risk group, in order to better identify patients with an increased risk of relapse, additional testing of MRD on day 33, is necessary in our center.

What Is Already Known on This Topic:

The identifying of most sensitive prognostic factors for predicting disease relapse was of great importance for the concept of "risk-adjusted therapy". It was shown that an early treatment response is a significant prognostic indicator and predictive factor for disease recurrence. MRD assessment during the treatment of various hematological malignancies has a high predictive value for disease recurrence, and therefore EFS and OS. Personalization of therapy based on MRD thus may improve the treatment outcome of children with ALL. Therefore, the monitoring of MRD levels in different time points is used in current protocols and is the main criterion of risk group assignment and risk-adjusted therapy.

What This Study Adds:

Our study is the first study in Bosnia and Herzegovina that showed the impact of bone marrow FC-MRD d15 on the stratification of patients into risk groups and the outcome of the disease. The results of our research confirmed the importance of MRD measurement d15 for patients in the low and high risk groups, but not for patients in the intermediate risk group. In order to better identify patients with an increased risk for relapse, it is necessary to implement additional MRD testing on day 33 of the therapy.

Author's Contributions: Conception and design: JSP; Acquisition, analysis and interpretation of data: BĐB and DMZ; Drafting the article: BĐB and DMZ; Revising it critically for important intellectual concept: JSP; Approved final version of the manuscript: JSP.

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Inherited Thrombophilia and Risk of Thrombosis in Children with Cancer: a Single-center Experience[a]

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Abstract

Objectives. Thrombosis is an increasingly recognized complication of childhood malignancy and its treatment. The incidence and etiology of pediatric cancer-related thrombosis is still not well understood. The aim of this study was to evaluate the prevalence of common prothrombotic genetic conditions in children with cancer, the frequency of thrombosis, and the role of inherited thrombophilia in the development of thrombosis in a pediatric oncology population. **Patients and Methods.** Forty-seven children (36 treated for hematological malignancies and 11 for solid tumors) with a median age of 8.8. years (range 0.4 – 19.3 years) were included in the study. Genetic polymorphisms of Factor V Leiden (G1691A), prothrombin G20210A, and methylenetetrahydrofolate reductase (MTHFR) C677T were determined by real-time polymerase chain reaction-based DNA analysis. **Results.** Four (8.5%) patients were heterozygous for Factor V Leiden, 3 (6.4%) were heterozygous for prothrombin G20210A mutation, and 3 (6.4%) were homozygous for MTHFR C677T mutation. All patients had implanted central venous catheters. Four (8.5%) children had documented thrombosis, three of which were in the upper venous system. Two of the four patients with thrombosis had Factor V Leiden heterozygosity. **Conclusions.** Thrombosis is an important complication of childhood cancer. The risk of thrombosis may be increased in patients with Factor V Leiden. In the absence of consensus guidelines, our results support the recommendation for thrombophilia screening in children with cancer.

Key Words: Inherited Thrombophilia ▪ Cancer ▪ Thrombosis ▪ Children,

Introduction

Thrombosis is a well-recognized complication of malignancy. It is estimated that up to 20% of all cancer patients develop thrombosis throughout the course of the disease, with an annual incidence rate of 0.5% compared to 0.1% in the general population (1, 2). There is substantially less knowledge about thrombosis in the pediatric cancer population, with reported rates varying from 2% to 16%, depending on the type of malignancy (3). Children with cancer and thrombosis have an increased risk of mortality, higher rates of recurrent thrombosis and thrombosis-related morbidity, and decreased quality of life (4, 5). The pathophysiology of pediatric cancer-related thrombosis is multifactorial, and may reflect prothrombotic genetic factors, and tumor-related and treatment-related factors (6). The role of inherited thrombophilia in the development of thrombosis in children with cancer is poorly investigated and still unclear.

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This study was undertaken to determine the prevalence of Factor V Leiden, prothrombin G20210A and methylenetetrahydrofolate reductase (MTHFR) C677T gene polymorphisms in children with hematological malignancies and malignant solid tumors, the frequency of cancer-associated thrombosis, and the role of inherited thrombophilic alterations in thrombotic events.

Patients and Methods

Patients

Forty-seven children (34 boys and 13 girls) with primary cancer consecutively admitted from January 1st, 2010 to December 31st, 2015 to the Division of Hematology and Oncology, Department of Pediatrics, Clinical Hospital Centre Rijeka, Croatia, were included in the study. The following data were collected from medical records: gender, age at diagnosis, the type of cancer, previous and family history of thrombosis, insertion/type of a central venous catheter (CVC), and the presence/developmental time/site of thrombosis. Ethical approval was obtained from the institutional ethics board. Informed written consent was obtained from the parents of all patients.

Methods

The samples were taken from the peripheral blood in tubes containing EDTA. The genomic DNA was prepared from the whole blood with a NucleoSpin Blood kit (Macherey-Nagel GmbH & Co. KG, Düren, Germany). Genetic polymorphisms of Factor V G1691A (Factor V Leiden), Factor II-Prothrombin G20210A and MTHFR C677T were screened by real-time polymerase chain reaction (RT-PCR) on a Light Cycler® 1.5 Instrument, Roche Diagnostics, Germany. Tests were performed by binding specific DNA probes marked with fluorescent colors during PCR and melting curve analysis of marked PCR products, according to the manufacturer's instructions. Plasma homocysteine levels were not routinely assessed.

Statistical Analyses

Descriptive statistics were used to summarize the data. The results were compared with the relative frequencies of heterozygous and homozygous variants of each polymorphism in the general population. Fisher's exact test was used to compare the prevalence of Factor II and Factor V Leiden polymorphism between boys and girls with cancer, between children with hematological malignancies and solid tumors, and between patients with and without thrombotic events. The Chi-squared test was used to describe MTHFR genotype distribution in boys and girls with cancer, and between patients with hematological malignancies and solid tumors. A P value of <0.05 was considered statistically significant.

Results

The median age of the patients was 8.8 years (range 0.4 – 19.3 years). Thirty-six patients had hematological malignancies (acute lymphoblastic leukemia [ALL] = 26, acute myeloid leukemia = 2, non-Hodgkin lymphoma = 7, Hodgkin lymphoma = 1) and 11 patients had solid tumors (malignant brain tumor = 3, soft tissue sarcoma = 3, osteosarcoma = 2, Ewing sarcoma = 1, neuroblastoma = 1, nasopharyngeal carcinoma = 1). All patients had implanted CVC: 33 patients had tunneled catheters (Broviac®) and 14 had implantable ports (Port-a-cath®).

Three (6.4%) patients (all boys) had heterozygous Factor II G20210A mutation, while no homozygosity was detected. Heterozygous Factor V Leiden was identified in 4 (8.5%) children (2 boys and 2 girls) with cancer, and no homozygous Factor V Leiden was found. MTHFR C677T heterozygosity was present in 21 (44.7%) patients, and homozygosity in 3 (6.4%). Six (46.2%) girls and 15 (44.1%) boys were heterozygous for MTHFR C677T, while 1 (7.7%) girl and 2 (5.9%) boys were homozygous. There was no statistical significance in the prevalence of FII G20210A mutation (Fisher's exact test, P=0.550), Factor V G1691A mutation (Fisher's exact test, P=0.304),

and MTHFR C677T mutation (Chi-squared test, P=0.928) between male and female patients.

Two (5.6%) patients with hematological malignancies and one (9.1%) with a solid tumor had heterozygosity for Factor II G20210A mutation. Factor V Leiden heterozygosity was identified in 3 (8.3%) children with hematological malignancies, and in 1 (9.1%) with a solid tumor. A heterozygous MTHFR C667T mutation was identified in 16 (44.4%) children with hematological malignancies and in 5 (45.5%) children with solid tumors, while 2 (5.6%) patients with hematological malignancies and 1 (9.1%) with a solid tumor had MTHFR C667T homozygosity. No statistical significance was found in the prevalence of Factor II G20210A mutation (Fisher's exact test, P=0.560), Factor V Leiden (Fisher's exact test, P=01.000) and MTHFR C667T mutation (Chi-squared test, P=0.936) between patients with hematological malignancies and solid tumors. The previous or family history of thrombosis was negative in all patients.

Four (8.5%) children (all boys) had a documented thrombotic event during treatment: right axillar and brachial vein thrombosis in a patient with non-Hodgkin lymphoma; right brachial vein thrombosis in a patient with neuroblastoma; right subclavian, axillary and brachial vein thrombosis in a patient with nasopharyngeal carcinoma, and right atrial thrombosis in a patient with osteosarcoma. No patient had any recurrent thrombosis. Two patients had heterozygous Factor V Leiden (both combined with heterozygous but no homozygous MTHFR C677T mutation), and one patient had a heterozygous MTHFR C677T mutation. Heterozygosity for Factor V Leiden was statistically more frequent among patients with thrombotic events than in patients without thrombosis (50% versus 5.3%, Fisher's exact test, P=0.039), while there was no statistical difference in the prevalence of MTHFR C677T mutation between cancer patients with and without thrombosis (Fisher's exact test, P=0.332). Homocysteine levels were normal in all patients. In one patient no thrombophilia gene alteration was detected.

The characteristics of patients with thrombosis are shown in Table 1.

Discussion

In our study, thrombosis was documented in 4 of 47 (8.5%) children with cancer, which is substantially higher than in the general pediatric population. The incidence of thrombosis ranges from 0.14 to 0.21 per 10,000 children per year, and 0.2 to 0.6% among hospitalized pediatric patients (7). The majority of affected children have at least one underlying condition or trigger for thrombosis, the most common being CVC, inherited thrombophilia, malignancy, congenital heart disease, chronic neuromuscular disease, surgery, major trauma, immobility, estrogen-containing contraceptives, obesity, and severe infection (8-11).

Table 1. The Characteristics of Patients with Thrombosis

Patient number	Sex/Age at diagnosis (years)	Type of malignancy	Site of thrombosis	Time of thrombosis	CVC* type	CVC* duration (days)	Inherited thrombophilic factors
1	Male / 16.5	Non-Hodgkin lymphoma	Right axillar and brachial vein	During therapy	Broviac	52	Factor V Leiden heterozygous (MTHFR† C677T heterozygous)
2	Male / 15.6	Osteosarcoma	Right atrium	During therapy	Port-a-Cath	96	Factor V Leiden heterozygous (MTHFR† C677T heterozygous)
3	Male / 17.2	Nasopharyngeal carcinoma	Right subclavian, axillary and brachial vein	During therapy	Port-a-Cath	209	(MTHFR† C677 heterozygous)
4	Male / 2.4	Neuroblastoma	Right brachial vein	During therapy	Port-a-Cath	491	None

*Central venous catheter; †Methylenetetrahydrofolate reductase.

The association between thrombosis and pediatric cancer is well established, and overall, 25% of children with thrombosis have an underlying diagnosis of cancer (12). The reported prevalence of thrombosis in children with cancer ranges from 2 to 16%, while the occurrence of asymptomatic events is approximately 40% (13-19). The risk is highest in children with ALL, followed by sarcoma and lymphoma, and the lowest risk is in children with brain tumors (4, 20, 21). The occurrence of thrombosis in the current study is in agreement with the published data, although thrombosis was more frequent in children with solid tumors (3/11) compared to hematological malignancies (1/36).

The etiology of thrombosis in children with cancer is multifactorial and includes patient-related (inherited thrombophilia), disease-related and treatment-related factors. Cancer may be considered a hypercoagulable state. Tumor cells express tissue factor, procoagulant proteins, metalloproteases, and molecules that can induce direct and indirect activation of coagulation. Several additional mechanisms, such as inflammatory, immune, and angiogenic responses, are involved (22, 23). Major risk factors for thrombosis in children with hematological malignancies include the presence of CVC, older age, prothrombotic genetic defects, non-O blood group, obesity, and medications (asparaginase, concomitant use of steroids, anthracyclines) (4, 24-26). Proposed prothrombotic risk factors in children with solid tumors include the presence of CVC, age > 10 years, certain tumor types and sites, metastatic disease, thrombophilia, obesity, and type of treatment (surgery, radiation, anthracyclines, and platinum) (4, 17, 27). CVC is the most important risk factor (28). Reported rates of symptomatic catheter-related thrombosis range from 2.6 to 36.7%, and rates of asymptomatic catheter-related thrombosis range from 5.9 to 43% (3, 29). The pathogenesis of catheter-related thrombosis is not well characterized, and it may involve endothelial damage and local activation of blood coagulation (30). The most common sites are the upper venous system, and the lower extremities for non-catheter-related thrombosis (28, 31). Central nervous system thrombosis is more common in children with ALL, with approximately half of patients having sinus venous thrombosis (19, 28). The incidence of cerebral sinus venous thrombosis in pediatric ALL patients varies from 1.4 to 10.5% (32-35). Right atrial thrombosis is reported in 2% of patients with symptomatic thrombosis (36).

In our study, all patients had CVC in place, and all thrombotic events occurred during chemotherapy. All four patients were male, and three were adolescents. Three patients had upper extremity thrombosis, and one had right atrial thrombosis. Two patients (50%) had heterozygous Factor Leiden (combined with MTHFR C677T heterozygosity).

The contribution of inherited thrombophilia to the occurrence of thrombosis in cancer patients has been documented. The two most common genetic causes of thrombophilia identified to date are Factor V Leiden and prothrombin G20210A mutation (37, 38). MTHFR C677T heterozygosity is a very frequent polymorphism, but it only increases the risk of thrombosis when it results in hyperhomocysteinemia (39). A meta-analysis of 17 prospective studies comprising 1752 pediatric patients with ALL reported the overall thrombotic risk of 5.2%. Prothrombotic genetic defects were studied in 557 children. Thirty-one thrombotic events were observed in 113 children affected by at least one genetic alteration, pointing to an approximately 8-fold increased thrombotic risk (relative risk [RR]:8.5; 95% CI: 4.4-17.4) in patients with inherited thrombophilia (26). Similar results were reported by Nowak-Göttle et al., who documented venous thrombosis in 46.5% (27/58) of children with ALL carrying a prothrombotic defect, compared to 2.2% (5/131) of children with no identified prothrombotic defect ($P<0.0001$; chi-square 137.0). Homozygous MTHFR mutation with hyperhomocysteinemia was diagnosed in 12.5% (4/32) children with thrombosis, and in a further 9.4% (3/32) patients combined with Factor V Leiden or increased lipoprotein A concentrations. In addition, an increased risk of thrombotic complications was clearly demonstrated in leukemia patients with combined prothrombotic risk factors, compared to patients with single

alterations (40). The study by Knöfler et al. included 77 children with malignancies and in 11 (14%) of them catheter-related thrombosis was detected. Prothrombotic genetic defects were found in 23% (17/77) patients, and in 7 of 11 (64%) patients had thrombosis. Three children had combined defects (heterozygous Factor V G1691A combined with heterozygous prothrombin G20210A mutation, protein S deficiency or hyperlipoproteinemia), and 4 had a single defect (heterozygous Factor V G1691A, heterozygous prothrombin G20210A mutation, hyperlipoproteinemia, and protein C deficiency type I) (41). Ünal et al. evaluated inherited and acquired prothrombotic risk factors in 37 children with malignancies and thrombosis. Congenital defects were detected in 15 (40%) patients: 8 had heterozygous Factor V G1691A, 1 had heterozygous prothrombin G20210A mutation, 4 had lipoprotein(a) elevation, 1 had decreased protein S level, and 1 had decreased protein C level. The risk of thrombosis increased when accompanied by additional prothrombotic risk factors (42). A large population-based study in Israel of 1191 children with ALL reported venous thromboembolism in 89 (7.5%) children. Thrombophilia screening was performed in 584 children, and findings were positive in 84 (14.4%). Patients with thrombophilia had significantly more thrombotic events compared to children without thrombophilia ($p < 0.001$) (43). Other studies failed to show any impact of thrombophilic gene mutations on thrombosis risk in patients with cancer (28, 44-47). Thus, the impact of inherited thrombophilic markers on the development of thrombosis in pediatric oncology patients has not been completely clarified. Our study confirms the higher occurrence of symptomatic thrombosis in children with cancer. Two out of 4 children with thrombosis had heterozygosity for Factor V Leiden as an inherited prothrombotic risk factor.

Limitation of Study

Our study has several limitations, including retrospective design, the small number of patients, heterogenous underlying malignancies, and the limited panel of genetic prothrombotic traits tested. Moreover, no investigations for asymptomatic vessel occlusion were performed. This could result in underestimation of thrombotic events, which in turn leads to an overestimation of the role of inherited prothrombotic risk factors. Larger multicenter prospective studies, development of guidelines for thrombophilia screening, identification of high-risk groups, individualized reevaluation of additional prothrombotic risk factors and appropriate measures might help in the prevention and early intervention of thrombotic events.

Conclusion

Children with cancer are at increased risk for developing thrombosis secondary to disease- and treatment-related factors, and other poorly characterized conditions. The prevalence of inherited thrombophilia in our patients was within the prevalence in the healthy population, but fact that two out of four patients with thrombosis had documented congenital prothrombotic risk factors should not be overlooked. There is still much to be learned regarding the risk factors, prevention, and treatment of thrombosis in children with cancer. In the absence of consensus guidelines, our results support a recommendation for thrombophilia screening in this population.

What Is Already Known on This Topic:

Children with cancer constitute the largest subset of patients who experience thrombosis. The pathophysiology of pediatric cancer-associated thrombosis is multifactorial, and the role that inherited thrombophilia plays in the pathogenesis is largely unknown. Thrombosis is a serious condition that can lead to significant long-term morbidity, as well as early mortality. With over 80% cure rates of childhood cancer, strategies for prevention, early diagnosis, and optimal intervention of cancer-related thrombosis in pediatric patients are of great importance.

What This Study Adds:

This is the first study in the Republic of Croatia to investigate the frequency of thrombosis and the prevalence of common prothrombotic genetic defects in children with cancer, as well as to evaluate the role of inherited thrombophilia in the development of pediatric cancer-related thrombosis. Our results confirm that children with cancer experience increased risk of thrombosis. To identify patients at increased risk for thrombosis better, we suggest thrombophilia screening in the routine clinical care of children with cancer.

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Conflict of Interest: The authors declare that they have no conflict of interest.

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Investigation of Correlation between Resistance to Diazepam and Expression of Inflammatory Markers in The Peripheral Blood of Patients with Status Epilepticus

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Abstract

Objective. This study investigated several inflammatory markers' gene and protein expression in status epilepticus (SE) and their correlation with diazepam resistance. **Materials and Methods.** Peripheral blood samples were collected from 18 adult patients with SE in Cipto Mangunkusumo Central Hospital, consisting of 12 diazepam-responsive and six diazepam-resistant samples, within 72 hours of the onset of the seizure. We collected baseline demographic and clinical data from each subject. Peripheral blood mononuclear cells (PBMCs) were isolated, cultured, stimulated with lipopolysaccharide (LPS) 1 mg/ml, and harvested for RNA isolation. The RNA was used to determine the expression of Human Mobility Group Box 1 (HMGB1), Interleukin-6 (IL-6), IL-10, Toll-like Receptor 4 (TLR4), and Glial fibrillary acidic protein (GFAP). In addition, we performed serum protein assay of HMGB1, IL-6, IL-10, TLR4, and GFAP to compare with gene expression. **Results.** We found a significant difference between the responsive and resistant groups for serum HMGB1 and IL-6 concentration. The mRNA expression of HMGB1 and IL-6 was significantly higher in LPS-stimulated samples in the responsive but not in the resistant groups. The ratio of IL-6 to IL-10 showed a significant difference between LPS and control in the responsive group. Diazepam response was significantly correlated with seizure duration and serum protein concentration of HMGB1. **Conclusion.** HMGB1 was highly expressed in the resistant group and strongly correlated with diazepam response, and there was a significant increase in HMGB1 mRNA expression in response to LPS stimulation. These findings suggest that targeting HMGB1 may be a promising therapeutic strategy and that HMGB1 levels could be a valuable biomarker for predicting diazepam resistance in SE.

Key Words: Diazepam Resistance ▪ HMGB1 ▪ GFAP ▪ Interleukin ▪ Status Epilepticus ▪ TLR4.

Introduction

Status epilepticus (SE) is a seizure that lasts more than 5 minutes or a series of seizures without recovery to baseline (1). SE is a life-threatening neurological and medical emergency that can lead to long-term consequences such as neuronal injury, epilepsy, drug resistance, and death (1-3). Worldwide, the incidence of SE ranges from 8 to 41 per 100,000 people per year (3-5), with mortality reaching up to 30% in adults and 39% in refractory SE (6). Current treatment protocols for SE suggest a timely progression of treatment, with the conceptual definition of SE by the ILAE task force of 5 minutes that indicates the time to start the SE emergency treatment with benzodiazepines (BZD) (e.g., diazepam) as the preferred initial treatment (7, 8). Studies have shown that 30-40% of cases of status epilepticus are not effectively treated by benzodiazepines (9). Escalation of pharmacoresistance, particularly to benzodiazepines, with prolonged seizure duration has been demonstrated in several studies (10). Time-dependent pharmacoresistance

is a major issue in SE. The anticonvulsant efficacy of BZD may decrease up to 20-fold after 30 minutes of seizure activity (11-13). In addition, 30 minutes of convulsive SE may also indicate irreversible brain damage, as shown experimentally (14).

Recent studies show that inflammatory reactions can cause seizures, and pro-inflammatory pathways may contribute to the development of SE. Hence, it might be feasible for researchers to regulate epileptic seizures by altering inflammatory signals in the brain (1). Higher levels of High-mobility group box 1 (HMGB1) protein and specific pro-inflammatory cytokines have been identified in the serum of children with febrile convulsions and cerebrospinal fluid of patients with refractory SE, in comparison to patients affected by other inflammatory diseases (15-19). Several inflammatory biomarkers have been associated with SE, including HMGB1 (2, 20-22), GFAP (23, 24) and other pro-inflammatory cytokines such as IL-6 and IL-1β (22, 25, 26).

HMGB1, a non-histone DNA binding protein, has been identified as a significant cytokine when released into the extracellular environment. The pro-inflammatory properties of HMGB1 are activated by binding to receptors such as toll-like receptor 4 (TLR 4), thereby mediating nuclear factor-κB (NF-κB) or other pathways (27). In both animal models and human patients, HMGB1 is actively expressed in epileptic tissues (2, 28). TLR4 belongs to the TLR family and is expressed in microglia, oligodendrocytes, and astrocytes within the central nervous system (29). Dysregulation of TLR 4 is implicated in the pathogenesis of several neurological diseases (29–33), including status epilepticus(7, 25). Additionally, there are also unattached forms of TLR4 identified as soluble TLR4 (sTLR4) (34, 35). These unattached protein complexes are considered to have the same structural characteristics as their membrane-bound counterparts. However, they do not participate in the TLR pathway. Rather, they mitigate inflammatory responses by competing with TLRs for ligands (35). HMGB1 and TLR4 can quickly trigger the activation of pro-inflammatory signaling pathways, disrupt the blood-brain barrier (BBB), and increase the severity of seizures (7, 21, 28, 36). Multiple studies in animal models of epilepsy have shown that activating the HMGB1/TLR4 signaling pathway leads to a marked increase in the frequency of seizures (7, 27, 37, 38). The impairment of the blood-brain barrier could result in the release of serum proteins into the brain, resulting in inflammation and glial activation (39-41). In cases of SE, the elevated expression of GFAP, a marker of glial cells have been observed. Glial fibrillary acidic protein (GFAP) is an intermediate filament found only in white matter astrocytes of the CNS. In the CNS, GFAP is crucial for the structural organization of astrocytes. The communication between astrocytes and certain types of neurons, such as Purkinje cells, is imperative in upholding the integrity of the BBB. GFAP could be a promising blood biomarker since there is no evidence of extracerebral GFAP production (39).

In response to infection, tissue injury, trauma, as well as seizures the innate immune system is rapidly activated and triggers an inflammatory response. Lipopolysaccharide (LPS), known to bind to and stimulate cell surface receptors known as TLR4, are a well-studied example of this signal (42–44). LPS-induced inflammatory response in peripheral blood mononuclear cells (PBMC) cultures can serve as a model to investigate immune response in SE patients, which can provide insights into the role of inflammation in the development and progression of the condition (45). In this study, the researchers examined the expression of several genes, including HMGB1, TLR4, IL-6, IL-10, and GFAP, after stimulating with LPS, using qPCR analysis on mRNA isolated from PBMC of patients with SE. The aim of this study was to identify the expression and the correlation between the gene expression and protein concentration of several inflammatory markers with resistance to diazepam. No evidence currently supports a direct link between inflammation and resistance to diazepam, hence the need for this study.

Methods

Patient Characteristics and Clinical Data

Peripheral blood samples were collected from 18 adult patients with Status Epilepticus in Cipto Mangunkusumo Central Hospital Jakarta,

consisting of 12 diazepam-responsive (DRV) and 6 diazepam-resistant (DRT) subjects. We took the samples within 72 hours of the onset of the seizure. The clinical data collected were age, gender, previous history of epilepsy, etiology of seizure, and seizure duration and frequency.

PBMC Isolation, Storage, Thawing, and Culture

PBMCs were isolated from freshly heparinized peripheral blood of patients with SE using the density gradient separation medium Ficoll Paque™ Premium (Cytiva) and centrifugation. After cell counting, the PBMCs were stored at -80 °C for 24 h and then transferred to liquid nitrogen until used for culture. After the thawing protocol at 37 °C, PBMCs were cultured in a complete medium composed of RPMI-1640 with L-glutamine, FBS, 100 U/ml Penicillin + Streptomycin, and Gentamycin, at a concentration of 10^6 cells per well. The cell culture was then incubated in a 5% CO2 humidified incubator for 4 hours at 37°C. After this incubation, LPS from Pseudomonas aeruginosa (L7018, Sigma) was added in a final concentration of 1 µg/ml. There was an unstimulated well (control) in the same cell culture plate for each stimulated well. The cells were harvested at 24 hours after stimulation. The cell culture supernatant was taken from each well, and the harvested cells were kept at –80 °C until use.

RNA Extraction and cDNA Synthesis

The Quick-RNA™ Miniprep Plus kit (R1057, Zymo Research) was used to extract total RNA from PBMC according to the manufacturer's protocol. The Nano spectrophotometer (BioDrop) was used to measure the RNA's quantity and quality. The purified RNA was stored at –80 °C for subsequent cDNA synthesis. The isolated RNA was used to synthesize cDNA according to the manufacturer's protocol by utilizing the ReverTra Ace™ qPCR RT Master Mix (FSQ-301, Toyobo), and continued with the PCR reaction.

Determination of HMGB1, IL-6, IL-10, TLR4, and GFAP expression levels in PBMC by Quantitative real-time PCR (qRT-PCR)

Primers for IL-6, IL-10, HMGB1, TLR4, and GFAP were designed using Primer3 software. The details of the primers are shown in Table 1. Primers and cDNA samples were diluted with nuclease-free water (NFW). Each primer was used along with the reference gene primer in a PCR reaction using Thunderbird™ Next SYBR® qPCR Mix (QPX-201, Toyobo) as described by the manufacturer and analyzed with Applied Biosystem® 7500 StepOnePlus™. The $2^{\Delta\Delta Ct}$ method was used to assess the expression level using β-actin (ACTB) as the reference gene.

Table 1. Primers Used in the PCR Reaction

Gene name	Genbank accession number	Primer sequence (5'-3')		Amplicon length (bp)
ß-actin	NM_001101.5	F	GCT GGA AGG TGG ACA GCG A	613
		R	GGC ATC GTG ATG GAC TCC G	
IL-6	NM_00600.5	F	CACTCACCTCTTCAGAACGAAT	107
		R	GCTGCTTTCACACATGTTACTC	
IL-10	NM_000572.3	F	GCTGGAGGACTTTAAGGGTTAC	106
		R	GATGTCTGGGTCTTGGTTCTC	
GFAP	NM_002055.5	F	GATCAACTCACCGCCAACA	107
		R	AGCCTCAGGTTGGTTTCATC	
HMGB1	NM_001313893.1	F	GGCCCGTTATGAAAGAGAAATG	119
		R	CAGAGCAGAAGAGGAAGAAGG	
TLR4	NM_003266.4	F	TTTCAGCTCTGCCTTCACTAC	107
		R	GACACCACAACAATCACCTTTC	

Detection of HMGB1, IL-6, IL-10, TLR4 and GFAP levels in serum samples by ELISA

The concentration of HMGB1, IL-6, IL-10, TLR4, and GFAP in the serum was measured using ELISA kits that are commercially available, as follows: HMGB1 (Cusabio, catalog number: CSB-E08223h), GFAP (Cusabio, catalog number: CSB-E08601h), TLR4 (Cusabio, catalog number: CSB-E12954h), IL-6 (Quantikine (R&D), catalog number: S6050), and IL-10 (Quantikine (R&D), catalog number: S1000B). The assays were carried out according to the manufacturer's instructions. The concentrations of cytokines were expressed as pg/ml.

Ethical Considerations

This study was approved by the Ethics Committee of the University of Indonesia and Cipto Mangunkusumo National Hospital (under permit number: KET-220/UN2.F1/ETIK/PPM.00.02/2021) and had informed consent from all patients.

Statistical Analysis

The statistical analyses were conducted using IBM SPSS statistics software for Windows, version 26.0 (SPSS Inc., USA). Categorical variables are analyzed with chi-square (or the alternatives: fisher's exact test, and Kolmogorov-Smirnov test for variables >2). Continuous data were validated for normality with Saphiro-Wilk test, presented as mean ± standard error of the mean (SEM), and were analyzed using unpaired t-tests or Mann-Whitney U tests for unpaired data and paired t-tests or Wilcoxon tests for paired data. The calculation of rank correlation coefficients according to Spearman. Values of $P<0.05$ were indicative of statistically significant differences.

Results

Characteristic of Subjects

Eighteen adult subjects with SE consisting of 12 DRV and 6 DRT were included in this study. The mean age of the subjects was 47.17±18.26. There were significant differences in age distribution between the DRV and DRT groups, with the DRT group having a younger age profile. Acute symptomatic etiology was present in most subjects. Overall, the majority of subjects were female and had intracranial etiology, with a seizure duration of under 30 minutes. The clinical characteristics of the subjects are shown in Table 2.

Table 2. Characteristics of Subjects

Characteristics of the subjects		DRV (N=12)	DRT (N=6)	P value
		Age (year), median (min-max)		
		56.5 (25–75)	22.5 (20–63)	0.013*
		PBMC count ($\times 10^6$), median (min-max)		
		8.53 (5.76–20.8)	7.59 (6.84–21.12)	0.851*
Gender N (%)	Male	2 (16.7)	0 (0)	0.529†
	Female	10 (83.3)	6 (100)	
History of epilepsy N (%)	Yes	2 (16.7)	2 (33.3)	0.569†
	No	10 (83.3)	4 (66.7)	
Etiology N (%)	Intracranial	8 (66.7)	2 (33.3)	0.766‡
	Metabolic	2 (16.7)	2 (33.3)	
	Mixed	2 (16.7)	2 (33.3)	
Seizure duration N (%)	<30 min	12 (100)	3 (50)	0.025†
	≥30 min	0 (0)	3 (50)	

DRV=Diazepam-responsive; DRT=Diazepam-resistant. *Mann-Whitney test; †Fisher's Exact test; ‡Kolmogorov-Smirnov test.

Concentration of Serum HMGB1, TLR4, IL-6, IL-10, and GFAP Protein Expression

A significant difference in serum HMGB1 and IL-6 concentration was found between the two groups (P=0.005 and P=0.013, respectively), whereas TLR4, IL-10, and GFAP and the ratio of IL-6 to IL-10 did not differ significantly between the two groups (Figure 1).

HMGB1 and Associated-genes Expression in PBMC in Response to LPS Stimulation

HMGB1 gene expression was found in all samples tested. There was a significant difference in HMGB1 mRNA expression between control and LPS samples in the DRV group (P=0.019), whereas it was not significantly increased in the DRT group (P=0.297) (Figure 2A). The highest expression, 40.162, was found in the DRV group stimulated with LPS. No difference was found between the LPS samples in both groups (P=0.51).

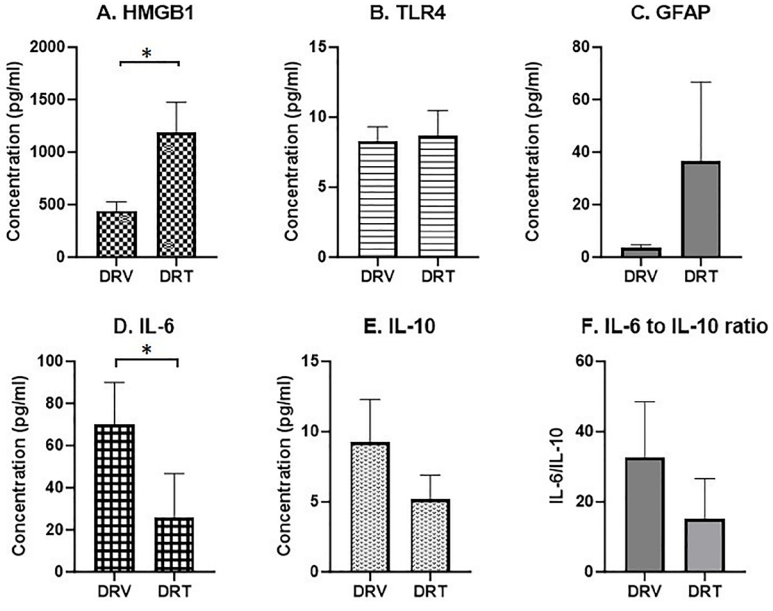
The mean expression of TLR4 did not significantly differ between control and LPS samples in both the DRV (P=0.844) and DRT groups (P=0.875) (Figure 2B). Additionally, there were no differences in TLR4 expression between LPS samples in both groups (P=0.7).

The expression of the IL-6 gene was low in the control-DRV group, and it was significantly different from the LPS-DRV group (P=0.001). However, there was no difference between the control and LPS samples in the DRT group (P=0.096), nor were there any differences in the LPS samples for either group (P=0.240) (Figure 2C).

There was no significant difference in the expression of the IL-10 gene in the control and LPS samples, either in the DRV (P=0.100) or in the DRT group (P=0.220). No difference was found in the LPS samples for either group (P=0.888) (Figure 2D). GFAP gene expression was found in only one subject (for each control and LPS-stimulated samples) in the DRV group, while none were found in the DRT group.

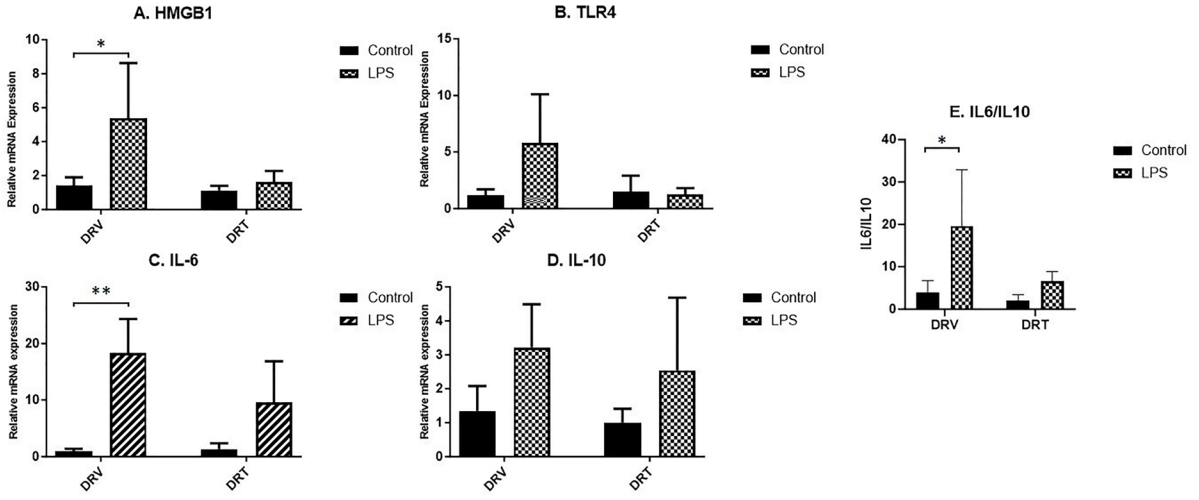
In the DRV group, the IL-6 to IL-10 ratio was 3.891±2.821 and 19.68±13.20 for the control and LPS, respectively (P=0.005), while in the DRT group the ratio was not different significantly between control and LPS samples (P=0.142). There was no discernible difference in the IL-6/IL-10 ratio between the LPS-stimulated samples of the DRV and DRT groups (P=0.981) (Figure 2E).

In terms of fold change, there was no difference in the fold change of any of the genes between the DRV and DRT groups (Figure 3).



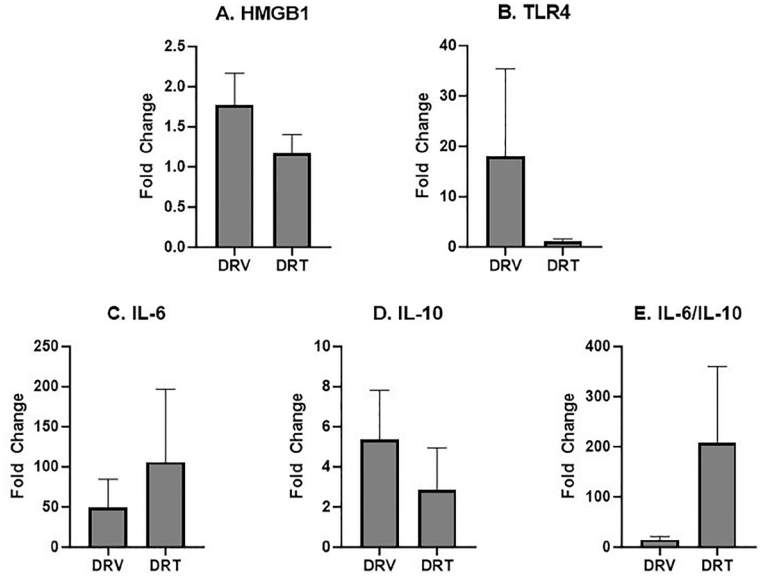
DRV=Diazepam-responsive; DRT=Diazepam-resistant; PBMC=Peripheral blood mononuclear cells; HMGB1= High-Mobility Group Box 1; TLR4=Toll-like receptor 4; GFAP=Glial fibrillary acidic protein; IL=Interleukin; Statistical analyses were performed using Mann-Whitney test. Data represent mean ± SEM. *P<0.05

Figure 1. Serum protein concentration of HMGB1, TLR4, IL-6, IL-10, GFAP and IL-6 to IL-10 Ratio (using ELISA method; DRV, N=18; DRT, N=6).



DRV=Diazepam-responsive; DRT= Diazepam-resistant; PBMC=Peripheral blood mononuclear cells; LPS= Lipopolysaccharide; HMGB1=High-Mobility Group Box 1; TLR4=Toll-like receptor 4; IL=Interleukin; Statistical analyses were performed using Wilcoxon test or paired t-test (paired data; compared control and LPS samples in each group) and Mann-Whitney test (unpaired data; compared LPS samples between DRV and DRT group). Data represent mean ± SEM. *P<0.05; **P<0.01

Figure 2. Gene expression of inflammatory markers from PBMC culture of SE patients. (using qPCR; LPS, N=18; Control, N=18, for each group).



DRV=Diazepam-responsive; DRT=Diazepam-resistant; PBMC=Peripheral blood mononuclear cells; LPS=Lipopolysaccharide; HMGB1=High-Mobility Group Box 1; TLR4=Toll-like receptor 4; IL=Interleukin. Statistical analyses were performed using Mann-Whitney test. Data represent mean ± SEM.

Figure 3. Fold Change between DRV and DRT groups. Fold change of LPS-stimulated and control samples, from PBMC culture of SE patients.

Correlation of Diazepam Response, Seizure Duration, Serum Protein Concentration and Fold Change of mRNA Expression

There were significant correlation between diazepam response with seizure duration (r_s=0.63; P=0.005), serum protein HMGB1 concentration (r_s=0.57; P=0.014) and IL-6 (r_s=–0.59; P=0.010); while seizure duration showed significant corellation with serum protein HMGB1 concentration (r_s=0.56; P=0.015) (Table 3). In addition, the serum IL-10 concentration was negatively correlated with the serum GFAP concentration (r_s=–0.51; P=0.031), and had moderate correlation with serum IL-6 concentration (r_s=0.42; P=0.077).

Table 3. Correlation of Diazepam Response, Seizure Duration, Fold Change of Mrna Expression, and Serum Protein Concetration

	Variables		DZP Response	Seizure Duration
Spearman's rho	DZP Response	r_s	1	0.632*
		p (2-tailed)	0	0.005
	Seizure Duration	r_s	0.632†	1
		p (2-tailed)	0.005	0
	FC HMGB1	r_s	-0.158	0.193
		p (2-tailed)	0.575	0.491
	FC TLR4	r_s	-0.087	0
		p (2-tailed)	0.825	1
	FC IL-6	r_s	0.267	0.171
		p (2-tailed)	0.377	0.577
	FC IL-10	r_s	-0.254	0
		p (2-tailed)	0.403	1
	Elisa HMGB1	r_s	0.568†	0.56†
		p (2-tailed)	0.014	0.015
	Elisa TLR4	r_s	0.091	-0.043
		p (2-tailed)	0.72	0.865
	Elisa IL6	r_s	-0.59†	-0.101
		p (2-tailed)	0.01	0.691
	Elisa IL-10	r_s	-0.091	-0.101
		p (2-tailed)	0.719	0.69
	Elisa GFAP	r_s	0.296	-0.072
		p (2-tailed)	0.233	0.776

DZP=Diazepam; FC=Fold Change of mRNA expression; *Correlation is significant at the 0.01 level (2-tailed); †Correlation is significant at the 0.05 level (2-tailed).

Discussion

Our study was done to investigate the correlation between diazepam response and several inflammatory biomarkers. To the best of our knowledge, this study represents the initial demonstration of the utility of mRNA and protein expression in HMGB1, TLR4, IL-6, IL-10, and GFAP from peripheral blood of adults SE patients in correlation with diazepam response. Our findings demonstrated that serum protein concentrations of HMGB1 were significantly higher in SE patients with diazepam resistance. Diazepam resistance in SE exhibits a robust correlation with seizure duration, as shown in the positive correlation in this study. As seizures become more prolonged, the exacerbation of inflammation occurs (46, 47), which can partially explain the significantly higher HMGB1 level in the DRT group. Neuroinflammation plays a significant role in various forms of epilepsy, including SE. According to reports, HMGB1 binds to TLR4 or RAGE, rapidly initiating pro-inflammatory signaling pathways, which disrupt the BBB and worsen seizure severity (7, 48). HMGB1 has the potential to intersect oxidative stress and inflammation as it enhances the production of reactive oxygen species (ROS), exacerbating the inflammatory process (1, 2).

Conversely, IL-6 was higher in responsive patients. This may be due to other factors such as the pro- and anti-inflammatory properties of IL-6 in certain condition such as in acute phase response (49), and other comorbidities including sepsis and hypoxia in DRV group, which may confound the results. IL-6 exhibits pro-inflammatory effects in acute inflammation whilst also displaying immunosuppressive and anti-inflammatory properties when expressed at lower concentrations.

Additionally, IL-6 may exhibit opposing functions in varying cell types. It is evident that IL-6 has a complex and multifaceted influence on inflammation and immune responses (49). IL-6 also helps regulate the production of anti-inflammatory protein such as IL-10 (49, 50), which explains the moderate correlation found between protein expression of IL-6 and IL-10, as well as the similar pattern of IL-6 and IL-10 protein expression, as well as the IL-6/IL-10 ratio that were found in this study.

There was no difference in serum TLR4 protein concentration between DRT and DRV groups in this study. The Heterogeneity of SE cases, and individual variation, in addition to timing of measurements, concurrent medications and comorbidities, as well as the presence of systemic inflammation, might affect the results, obscuring the association between sTLR4 and diazepam responsiveness (51–53).

We observed that the mRNA expression of HMGB1 in diazepam-responsive SE patients after LPS stimulation was about 3.5 times greater than in the control group. Conversely, there were subtle changes in the relative expression in diazepam-resistant subjects. Similar findings were noted with regards to IL-6 expression. These findings are consistent with the study results conducted by several researchers, which documented increased cytokine concentrations following LPS stimulation (54–56). LPS, the major component of Gram-negative bacterial walls can provoke acute inflammatory responses by stimulating the release of numerous inflammatory cytokines in various cell types, particularly monocytes/macrophages (57, 58). Resistant SE, which tended to be of longer duration than responsive SE, may have had more severe changes, including inflammation and BBB breakdown, as a result of the ongoing underlying pathology and co-morbidities in the patients (46). The mechanisms underlying diazepam-resistant SE are multifactorial and can involve changes in neuronal excitability, receptor function, and network properties (11, 46). These mechanisms along with the co-medication and other severe pathology in these conditions could potentially override the effects of LPS-inducing inflammation in the DRT group.

The IL-10 mRNA expression level increased after LPS stimulation in the DRV and DRT groups, but they did not differ significantly. This finding suggests that IL-10 expression may be a common response to this kind of stimulation (59, 60). The lack of significant difference between groups suggests that although both groups show this response, the level of IL-10 expression may not differentiate diazepam response or resistance in the context of SE.

There was no significant difference in TLR4 mRNA expression between control and LPS samples in either group, or between LPS samples in the DRV and DRT groups. This can be caused by the downregulation of TLR4. In response to prolonged inflammation, the body may downregulate the expression of TLR4 as a regulatory mechanism to avoid excessive immune activation and tissue damage. TLR4 is a key receptor involved in the recognition of LPS and chronic activation of TLR4 can lead to a state of immune tolerance or desensitisation, where cells become less responsive to the stimulus (e.g. LPS or bacterial products). This downregulation of TLR4 can result in lower mRNA levels (61, 62). Furthermore, TLR4 gene expression might be influenced by several things such as age, stimulus, environtment or patient's condition and cell part/type (63). The TLR4-dependent NF-–B signaling pathway is the primary pathway through which LPS induces an inflammatory response (44, 64). TLR4-independent host response to LPS has also been identified (65).

In this study, GFAP was only detected in one subject. GFAP is known to be expressed predominantly, but not exclusively, in astrocytes of the central nervous system (CNS), and to a lesser extent in other cells, including PBMCs (66).

Fold change indicates whether a gene is upregulated or downregulated between LPS-stimulated and control. In this study, except for TLR4 in the DRT group, there was an upregulated fold change for all other genes tested in both the DRV and DRT groups, but there was no significant difference between the groups. Some possible reasons for these findings were the heterogeneity within SE groups (6, 67), the small sample size in this

study, and the timing of measurements that might change overtime (68–71). Nevertheless, there were some trends in the results.

Serum IL-10 concentration was negatively correlated with the serum GFAP concentration. GFAP was exclusively expressed in astrocytes in the CNS white matter. Findings in the serum could potentially suggest BBB leakage in the neuroinflammation and gliosis that occur in the pathophysiology of SE (39). IL-10 is an anti-inflammatory cytokine that can modulate immune responses and reduce inflammation. Its role in epilepsy and SE is a subject of many ongoing researches, as the immune system and neuroinflammation play complex roles in these conditions (72). As the seizure activity is controlled, and the brain attempts to resolve the inflammatory response, the levels of IL-10 may increase as part of the body's anti-inflammatory response. This could potentially lead to a negative correlation between IL-10 and GFAP, as IL-10's anti-inflammatory effects might be associated with a reduction in astrocyte activation (GFAP expression) (66, 72, 73). On the contrary, serum IL-10 concentration had positive moderate correlation with the serum IL-6 concentration. IL-10 is a double-edged sword for the immune system: while it is a potent anti-inflammatory and immunosuppressive cytokine, it can also have immunostimulatory properties. To activate multiple signaling pathways that either inhibit or activate immune cells, it is crucial to consider the different sources of IL-10, the target cells on which it acts, as well as the timing and site of its secretion. Each of these features contributes to different functions (72).

In terms of clinical characteristics, the DRT group was found to have a younger age profile than the DRV groups. This may be attributed to several factors. Younger individuals may exhibit distinc underlying causes for SE (14, 74), or have different physiological responses to diazepam compared to older ones (75, 76). Additionally, age-related discrepancies may reveal diverse epilepsy types or other neurological ailments that are more prevalent in particular age categories (3, 5, 14). Further research is indispensable to investigate the particular mechanisms and factors that contribute to this observed age disparity in drug responsiveness among SE patients.

Limitation of Study

The small sample size is a constraint of this study. The limited resources and funding available at the time caused a small number of samples to be examined. To address this, further investigation is necessary with a bigger sample size in the future. Further research is required to study diazepam resistance in SE. This may include an investigation into GABA receptor and gene mutations, as well as other associated factors.

Conclusion

HMGB1 was highly expressed in the diazepam-resistant group and strongly correlated with response to diazepam treatment, and there was a significant increase in HMGB1 mRNA expression in response to LPS stimulation. These findings suggest that targeting HMGB1 may be a promising therapeutic strategy and that HMGB1 levels could serve as a valuable biomarker for predicting diazepam resistance in SE.

What Is Already Known on This Topic:
Time-dependent pharmacoresistance is still an issue in SE, and all the researchers are trying to look for alternative approaches to control the seizures. Recent studies have shown widespread brain inflammation in SE, suggesting that inflammation plays an essential role in the onset and development of SE. SE can cause damage to the BBB and activate various immune cells, releasing large amounts of pro-inflammatory mediators that can induce neuroinflammation through a variety of signaling pathways.

What This Study Adds:
This study provides new insights into the trends of various inflammatory biomarkers, particularly HMGB1, in relation to diazepam response in adult patients with status epilepticus, suggesting that it may be possible to regulate epileptic seizures by altering inflammatory signals in the brain.

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The Effects of *Helichrysum italicum* Extract on the Extracellular Matrix of the Skin

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Abstract

Objective. An in-vitro study was performed to investigate the molecular basis of the wound healing and skin protective features of *Helichrysum italicum* (*HI*), a medicinal plant from the Mediterranean basin. **Materials and Methods.** A dermal fibroblast cell line culture was treated with *HI* hydro-alcoholic extract to detect the gene expression levels of three selected primers: *FGF-2*, *HAS-2* and *MMP-9*. Cell proliferation assay was performed using a XTT reagent. RNA isolations were carried out from both the extract treated study cell group and the control cell group using a TRI reagent. *GAPDH* was used as the reference gene. Gene expressions were determined by real time RT-qPCR. The results were represented as 'Target/*GAPDH* Fold Change'. Statistical evaluation was performed by Student's t test. **Results.** *HI* extract caused statistically significant upregulation of *FGF-2* (P=0.0473) and *HAS-2* (P=0.0335) gene expressions compared to the untreated control cells. The treatment ended with 1.74 and 3.10 fold changes for *FGF-2* and *HAS-2*, respectively. **Conclusion.** In general, it may be considered that *HI* has certain anabolic effects on the extracellular matrix of the skin because of the significant increases it causes in *FGF-2* and *HAS-2*. Therefore, it may have a promising future in anti-aging studies and cosmetic dermatology. The results obtained in this study may also partially explain the molecular basis of the health benefits of *HI* on skin, including improvement in wound healing, and protection against the detrimental effects of ultraviolet exposure.

Key Words: *Helichrysum italicum* ▪ FGF-2 ▪ HAS-2 ▪ MMP-9.

Introduction

Medicinal plants are important sources of novel drug discoveries (1). Plants contain miscellaneous molecules with significant pharmacological actions. The genus *Helichrysum* (family *Asteraceae*) includes more than one thousand species and subspecies, most of which grow in the Mediterranean basin (2). There are numerous reports on the traditional uses of *Helichrysum italicum* (*HI*) or the "everlasting plant" in Northern Mediterranean countries. Data from various ethnopharmacological surveys show that the most frequently reported traditional uses of *HI* are related to respiratory diseases, digestive disorders, wound healing and inflammatory skin conditions. Wound healing and skin protective properties seem to be the best documented therapeutic effects of *HI*, as shown by in vivo studies performed with topical application of its extracts (3). A large variety of extracts of *HI* can be prepared, including the volatile oil, and the resulting products differ in their chemical compositions (4-6). Therefore *HI* bioactivity may show differences, depending on the chemical composition of its different extracts, from which most of the main active compounds have already been isolated (5). Extraction with organic solvents, such as ethanol, methanol and acetone, is most frequently used to attain non-volatile *HI* extracts, whereas hydrodistillation and steam distillation are favored for the isolation of volatile essential oils (7). *HI* extracts and essential oils have a wide variety of chemical classes, among which flavonoids, α-pyrones, phenolic acids, acetophenones, tremetones, pholoroglucinol derivatives

monoterpenes, sesquiterpenes, and triterpenes dominate (3, 7). Flavonoids, acetophenones, and pholoroglucinol derivatives have been shown to have inhibitory activity in different pathways of arachidonic acid metabolism and other pro-inflammatory mediators (3). It was reported that the flavonoid fraction may protect skin from ultraviolet (UV)-induced damage by a combination of UV-absorbing, DNA-protective, anti-oxidant, and anti-inflammatory properties (8). Arzanol, a heterodimeric phloroglucinol identified as the major anti-oxidant, anti-inflammatory and anti-viral constituent of *HI* (4, 9), potently inhibits the biosynthesis of pro-inflammatory lipid mediators, such as prostaglandin E_2 (PGE_2), both in vitro and in vivo. It also showed potent antibacterial action against multidrug-resistant *Staphylococcus aureus* isolates (9). Phytochemical investigations of *HI* essential oil revealed some sesquiterpenes contents, such as γ-curcumene and β-selinene, as well as monoterpenes, such as α-pinene and neryl acetate. These terpenes, as the most characteristic constituents, might be effective as potential wound healing agents (10). Neryl acetate was also demonstrated to strengthen the skin barrier function by increasing lipid and ceramide content in the stratum corneum, through increasing the expressions of ceramide synthesis-related enzymes (11). These observations validate the topical use of *HI* extracts to prevent wound infections in the traditional medicine of the Mediterranean area (12).

In this study, we investigated the effects of *HI* extract on a fibroblast cell line with selected factors which are all highly effective in the metabolism of the cutaneous extracellular matrix (ECM). These were *Fibroblast Growth Factor-2* (*FGF-2*), a potent mitogen for the cells of mesenchimal and neuroectodermal origin (13); *Hyaluronan Synthase-2* (*HAS-2*), the major enzyme synthesizing hyaluronic acid (HA) (14); and *Matrix Metalloproteinase-9* (*MMP-9*), an enzyme with essential roles in basement membrane remodeling through its proteolitic activity (15).

Methods

Plant Material and Preparation of the Extract

Only the flower part of the plant was used. Five grams of dried yellow flowers were extracted with 500 mL of distilled water-ethanol mixture (70:30 v/v) using soxhlet apparatus for two cycles. The extract was filtered through 0.45 μm filter paper and kept in a refrigerator at between 4-8°C until further analysis. The extract was evaporated in a rotavapor until 5% dissolved solids content remained. The 5 brix extract was used for the cell culture experiments. The solvent alcohol was eliminated during evaporation under vacuum. The final solution was a concentrated aqueous extract, and the dissolved botanical content of the extract was 50 mg/mL.

Cell Culture

Human skin fibroblast cells (HSF 1184) were cultured in Dulbecco's Modified Eagles Medium with high glucose, supplemented with 15% heat-inactivated fetal bovine serum and 1% gentamicin. The cells were maintained at 37°C in a humidified atmosphere at 5% CO_2 in a Newbrunswick incubator. All supplements and media were purchased from Sigma Aldrich.

Cell Proliferation Assay and Cytotoxicity Analysis

The cellular toxicity of *HI* extract was investigated using 2,3-bis (2-methoxy-4-nitro-5-sulfophenyl)-S-|(phenylamino)carbonyl|-2//-tetrazolium hydroxide (XTT) cell proliferation assay (Roche Diagnostics) according to scientific principles (16) and manufacturers' instructions. The cells were seeded into 96-well plates (10^4cells/well) and incubated for 24 h at 37°C, in a humidified atmosphere at 5% CO_2. On the second day, new medium was added, after aspiration of the previous one, subjected to different concentrations (100%, 10%, 5%, 0%) of the extract and incubated in the same conditions for 72 h. XTT reagent was added to the plates after the incubation period to obtain

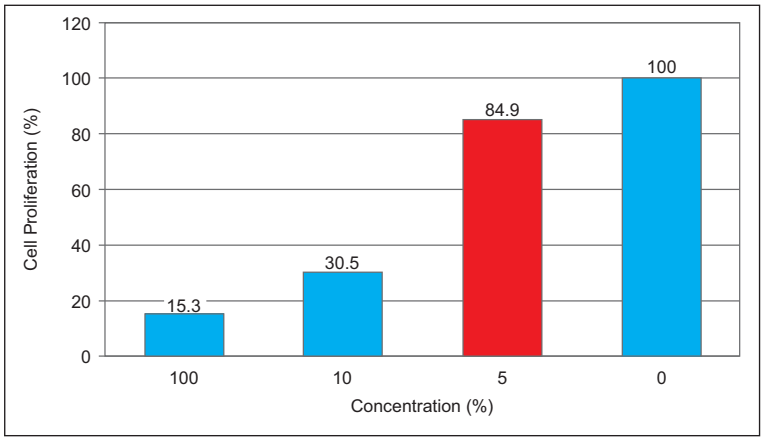

Figure 1. Cytotoxicity results of *HI* extract. The red bar represents the concentration used for incubation.

a concentration of 0.3 mg/mL. Then the cells were incubated at 37°C for 4 h in order to reduce the XTT reagent to an orange formazan compound. The optical density of the soluble formazan compound was measured at 450 nm, with 650 nm reference level by microplate reader (Bio-Rad). On the basis of the cell proliferation ratios of the treated cells with respect to the control cells, the cytotoxicity levels of the extract were determined. Higher concentrations were found to be cytotoxic for fibroblast cells. For the subsequent analysis, the possible highest concentration was determined as 5%, having optimum cell viability of approximately 80%, and the fibroblast cells were incubated with a 5% concentration of extract solution before total RNA isolation (Figure 1).

Reverse Transcription

Total RNA was extracted from the cells treated with *HI* extract solution and from untreated cells, using the TRI reagent (Sigma Aldrich) according to the scientific principles (17, 18) and the manufacturer's instructions. The concentration and purity of the isolated RNA samples were determined by measuring optical densities at 260 nm and 280 nm using BioSpec-nano. A Transcriptor First Strand cDNA Synthesis Kit (Roche Diagnostics) was used for cDNA synthesis. 500 ng total RNA and 10 mM gene specific primers of *FGF-2*, *HAS-2*, *MMP-9* as study material, and *glyceraldehyde-3-phosphate dehydrogenase* (*GAPDH*) as the reference gene (Integrated DNA Technologies) were added to RNAse free test tubes and the final volume was reached at 13 µL for each, by adding distilled water. After incubation for 10 min at 65°C in a Thermal Cycler, the tubes were transferred over ice. Later they were incubated for 30 min at 55°C and 5 min at 85°C in a Thermal Cycler, after adding 4 µL of Reverse Transcription Buffer (5X), 2 µL of dNTP mix (10 mM), 0.5 µL of Protector RNAse Inhibitor and 0.5 µL of Reverse Transcriptase. The primer sequences are given in Table 1.

Gene Expression Analysis

A Fast Start DNA Green Master Kit (Roche Diagnostics) was used for the real-time reverse transcription quantitative polymerase chain reactions (RT-qPCR). The analysis was performed according to the scientific principles (17, 18) and the manufacturers' instructions. Briefly, the total volume of reaction mix was 20 µL, containing 10 µL Master Mix, 10 mM of reverse and forward

Table 1. Primers (5'–3') of the Genes Studied

Primers	Forward primer	Reverse primer
FGF-2	CCTCAAGGTTCTCAAGGCCG	AGCACGTATATTCCCCAGCG
HAS-2	GCCTGGGCTATGCAACAAAA	GTAGGACTTGCTCCAACGGG
MMP-9	GTACTCGACCTGTACCG	AGAAGCCCCACTTCTTGTCG
GAPDH	ATGGGTGTGAACCATGAGAA	GTGCTAAGCAGTTGGTGGTG

FGF-2=Fibroblast growth factor-2; HAS-2=Hyaluronan synthase-2; MMP-9=Matrix metalloproteinase-9; GAPDH=Glyceraldehyde-3-phosphate dehydrogenase.

primers, 25 ng template cDNA and the appropriate amount of RNAse free distilled water. All samples were run as triplicates in each run, including a non-template control and four standards (1:1, 1:10, 1:100, 1:1000). The real-time RT-qPCR parameters were determined separately for each target according to the melting and annealing temperatures of the primers. Each parameter included a pre-incubation step for 10 min at 95°C, followed by 45 cycles of three amplification and melting steps. Melting curve analysis was performed to verify specificity. Absolute quantification analysis was performed using a Light Cycler 96 (Roche Diagnostics). For quantitation of real-time RT-qPCR results, the $\Delta\Delta C_t$ method was used. The gene expression results were represented as 'Target/*GAPDH* Fold Change'.

Statistical Analysis

All data were representative of the three experiments and expressed as mean ± standard deviation, together with 95% confidence interval (CI). Statistical evaluation was performed by Student's t test (Graph Pad Prism 6), and statistical significance was defined as $P<0.05$.

Results

HI hydro-alcoholic extract caused statistically significant upregulation in human skin fibroblast cells for *FGF-2* (P=0.0473) and *HAS-2* (P=0.0335) gene expressions. The treatment resulted in 1.74±0.26 (95% CI: 1.74±0.21) and 3.10±0.76 (95% CI: 3.10±0.61) fold changes for *FGF-2* and *HAS-2*, respectively. Also, as a positive outcome, the treatment resulted in a 0.72±0.19 (95% CI: 0.72±0.16) fold change for *MMP-9* gene expression, however, the result was not statistically significant. The fold changes and the P values of the gene expression analyses are given in Figure 2.

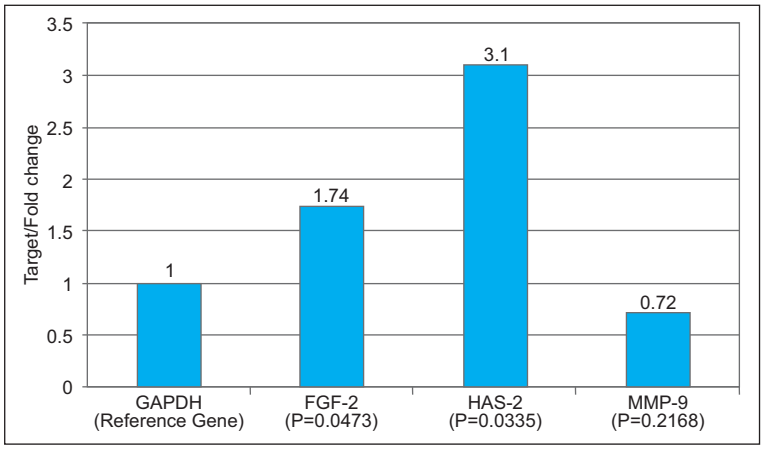

Figure 2. Gene expression levels and P values of *FGF-2*, *HAS-2* and *MMP-9* after treatment with *HI* extract, compared to untreated control cells.

Discussion

In our results, there were statistically significant increases in *FGF-2*, and *HAS-2*, and some decrease was recorded in *MMP-9* which was not statistically significant. Long term exposure to environmental or internal disturbances cause tissue damage through the formation of reactive oxygen species and the decline of cell functions. These inflammatory reactions increase the synthesis of dermal enzymes which brings on the degradation of ECM (19). The *FGFs 1* and *2*, also known as acidic and basic *FGF,* respectively, are produced by inflammatory cells, vascular endothelial cells, fibroblasts and keratinocytes. They are expressed upon dermal injury and have important functions in re-epithelization, angiogenesis, and granulation tissue formation (20). *FGF-2* increases the synthesis of matrix macromolecules, and notably that of HA, by stimulating the expression of *HAS* genes (21). HA is an essential component of the skin, responsible for captivating water and giving the dermis its volume (22). The concentration of HA in various tissues is in correlation with the transcription of *HAS* genes, especially with *HAS-2* (14). It was demonstrated that *HAS-2* protects skin fibroblasts against apoptosis, induced by environmental stress, mainly UV-B (23). The synthesis of HA, regulated by *HAS-2*, conducts keratinocyte migration, which is crucial for the reconstruction of squamous epithelia after wounding

(24). *MMPs* are secreted by keratinocytes and dermal fibroblasts in reaction to various stimuli, such as oxidative stress, UV and cytokines (25). *MMP-9* is thought to have critical functions in the remodeling of the basement membrane zone because several ECM proteins in this region have been determined as substrates of this proteinase (15).

Considering the significant increases in *FGF-2* and *HAS-2* gene expressions in our study, it may be suggested that *HI* has some anabolic effects on the ECM of the skin, mainly due to the angiogenesis inducing and granulation tissue enhancing effects of *FGF-2* (20), and the fibroblast protecting activities of *HAS-2* against UV-B mediated stress (23). The results obtained in this study may also partially explain the molecular basis of the health benefits of *HI* on skin, including improvement in wound healing, and protection against the detrimental effects of UV exposure. It is quite possible that these results are largely related to the plant's strong anti-inflammatory and anti-oxidant activities (3, 5, 8, 9, 12). Suppression of the degradation and increasing the synthesis of the ECM components of the skin are also the well known targets of anti-aging studies. Therefore, regarding the anabolic effects of *HI* in dermal ECM, it may have a promising future in cosmetic dermatology.

Limitations of the Study

Although the strong anti-oxidant and anti-inflammatory activities of *HI*, related to its phenolic and flavonoid constituents, have been revealed in recent studies performed by other researchers, the lack of phytochemical analyses in the particular plant of our study, prevents us from establishing a solid connection between the current results and the chemical features of the plant.

Conclusion

In general we may consider that *HI* has some anabolic effecst on the ECM of the skin because of the significant increases it induced in *FGF-2* and *HAS-2*. The results obtained by this study may also partially explain the molecular basis of the health benefits of *HI* on skin, including improvement in wound healing, and protection against the detrimental effects of UV exposure.

What Is Already Known on This Topic:

There are numerous reports on the traditional uses of HI in the Northern Mediterranean countries. Although data from various surveys show that the most frequently reported uses of HI are related to respiratory diseases, wound healing, digestive disorders and inflammatory skin conditions, its wound healing and skin protective properties seem to be the best documented therapeutic effects of this plant. HI's bioactivity depends on the chemical composition of its different extracts, from which most of the main active compounds have been isolated. These compounds are mainly flavonoids, acetophenones, pholoroglucinol derivatives and terpenes, which have been demonstrated to have anti-inflammatory, anti-oxidant, anti-microbial and wound healing features. Despite these scientific data, the molecular basis of the suggested activities is still lacking. Therefore, we performed an in vitro study to reveal the activities of HI on skin fibroblast cells, to see whether it has an effect on FGF-2, HAS-2 and MMP-9, the three functional proteins of skin.

What This Study Adds:

Considering the significant increases in FGF-2 and HAS-2 gene expressions in our study, it may be suggested that HI has some anabolic effects on the extracellular matrix of the skin, mainly due to the angiogenesis inducing and granulation tissue enhancing effects of FGF-2, and the fibroblast protecting activities of HAS-2 against UV-B mediated stress. The results obtained in this study may also partially explain the molecular basis of the health benefits of HI on skin, including improvement in wound healing, and protection against the detrimental effects of UV exposure.

Authors' Contributions: Conception and design: EP and MT; Acquisition, analysis and interpretation of data: EP and MT; Drafting the article: EP and MT; Revising it critically for important intellectual content: EP and MT; Approved final version of the manuscript: EP and MT.

Conflict of Interest: The authors declare that they have no conflict of interest.

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Original Research Study
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Animal Model of Cisplatin-Induced Oral Mucositis: Dose Optimization

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Abstract

Objective. The present study aimed to develop and validate an animal model of chemotherapy-induced oral mucositis due to cisplatin administration. **Materials and Methods.** Oral mucositis was induced in Wistar rats by cisplatin. Twenty healthy male Wistar rats were divided into four groups: a control group, and cisplatin 3 mg/kgBW (D1), cisplatin 5 mg/kgBW (D2), and cisplatin 6 mg/kgBW groups (D3). The D1, D2, and D3 groups received the cisplatin intraperitoneally on days 1, 3, and 5, whereas the control group did not receive anything. On day 7 and day 14 the entire experiment was terminated in all groups and the changes in body weight, oral mucositis grades, and histopathological scores were evaluated. **Results.** Cisplatin administration created a strong oral mucositis effect on groups D2 and D3. All the cisplatin doses decreased the rats' body weight by day 14. The worst oral mucositis grades and histopathological scores resulted from the administration of cisplatin at a dose of 5 mg/kgBW. **Conclusions.** In conclusion the cisplatin 5 mg/kgBW administered on days 1, 3, and 5 by intraperitoneal administration was the optimum dose to induce oral mucositis.

Key Words: Cisplatin ▪ Oral mucositis ▪ Rats ▪ Histopathology.

Introduction

The specific first-line therapy for cancer can vary widely depending on the type and stage of the disease. It may involve a combination of treatments, such as surgery, chemotherapy, radiation therapy, targeted therapy, or immunotherapy (1). Chemotherapy is the one of the first line therapy to treat cancer, however chemotherapy induces several side effects, including tissue damage reactions along the epithelium of the mouth and gastrointestinal tract (GIT) (1, 2). Chemotherapy-induced oral mucositis (CIOM) is a serious side effect of cytotoxic drugs (3). Patients with head and neck cancer are most affected by CIOM, with a risk of roughly 40% cases (4). Combination cancer treatment with radiation increases the chance of CIOM up to 100%. Cisplatin is the first-line chemotherapy with reported incidences of induced CIOM (5–7). Animal models enable highly regulated experimental circumstances, precise insights into the oral organs, standardized, clinically appropriate treatment regimens, and the development of new biomarkers to aid our understanding of the progression of CIOM, and how to avoid or treat it (8). The adverse effects of CIOM are not well managed, due to the lack of an understanding of the mechanism of its formation, so appropriate therapy cannot be provided (9). Therefore, in this study we developed and validated an animal model of chemotherapy-induced oral mucositis.

A previous study reported that cisplatin induced reactive oxygen species (ROS) and immune depression lead to erythema, edema, and CIOM

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(10–12). Furthermore, high levels of ROS cause apoptosis due to induction of DNA damage, and lead to increasing numbers of CIOM cases (13). The deterioration of CIOM results in forced disruption of treatment, leading to a loss of consciousness (3). These symptoms reduce the patient's quality of life. Therefore, preventing CIOM or treating it quickly brings improvement to the patient's quality of life, and reduces the need to interrupt treatment. Currently, various treatments are used, including antiinflammation drugs, however they are not adequate and have little preventive effect (14, 15). A novel drug for CIOM is needed. To develop new drugs, development of animal models is important.

A previous study reported on a CIOM mouse model induced by acetic acid injection into the oral mucosa (16). However, the study did not specifically measure the pain associated with oral mucositis, as it can induce pain in various body regions. A previous study that used acetic acid injection also did not assess the macroscopic picture of tissue damage in the oral mucosa, but only described changes in body weight and the area where CIOM formed. In addition, the induction used in the study did not describe the CIOM formed due to chemotherapy. In this study, we tried to prepare a CIOM animal model using rats in which CIOM was induced by cisplatin chemotherapy. Most animal models of CIOM induced by chemotherapy have reported CIOM along with intestine or gastric ulcers (17, 18). Surprisingly, despite considerable research, no CIOM model has led to the development of appreciable ulcers in the oral cavity.

Therefore, in this study we developed an animal model for chemotherapy-induced oral mucositis to determine the optimal dose of chemotherapy by measurement of the ulcerated area, histopathological epithelial specimens, and the CIOM grade.

Methods

Material and Study Design

This post-test only control group study design was conducted at the Stem Cell and Cancer Research (SCCR) Laboratory, Semarang, Indonesia, from January - March 2023.

Chemical and Drug Preparation

The cisplatin 0.5 mg/ml injections were obtained from PT. DANKOS FARMA (A Kalbe Company) Jakarta, Indonesia.

CIOM Induction

Twenty male Wistar rats (250 g ± 20 g) were used in this study. The rats were maintained at a controlled room temperature (21 °C ± 2), humidity at approximately 55% ± 10, light and dark cycles every 12 h, and no restriction of food and drink. After a week of acclimation, the rats were randomly divided into the following four groups: control/untreated, and D1 (Cisplatin 3 mg/kgBW), D2 (Cisplatin 5 mg/kgBW), and D3 (Cisplatin 6 mg/kgBW) groups. Cisplatin was administered intraperitoneally (i.p.) on days 1, 3, and 5 (19-22). The control group did not receive anything. The rats were sacrificed on day 14 by guillotine decapitation.

Epithelial Specimen Analysis

The buccal oral mucosa tissue was taken out and fixed with 10% formalin for 24 hours to create a paraffin block preparation. Tissue preparations 5 µm thick were removed and stained with hematoxylin-eosin.

Mucositis Grade Analysis

A modified version of the National Cancer Institute Common Terminology Criteria for Adverse Events was used to grade CIOM in the rats on days 7 and 14, as follows: Grade 0, normal mucosa; Grade 1, redness of the mucosa with punctate ulcers or a pseudo membrane; Grade 2, confluent ulceration or a pseudo membrane with no bleeding following slight stimulation; Grade 3, confluent ulceration or a pseudo membrane with bleeding following a slight stimulation; and Grade 4, tissue necrosis or spontaneous bleeding (23).

Mucositis Score Analysis

Buccal samples were collected from rats for histopathological analysis on days 7 and 14 after cisplatin administration. Specimens were fixed in the 10% neutral-buffered formalin, dehydrated, and embedded in paraffin. Five-micrometer-thick sections were obtained for hematoxylin and eosin staining, and examined under a light microscope (×100). Histological parameters were assessed in a single-blind manner and graded as follows (24): Score 0, normal epithelium and connective tissue who no vasodilatation, cellular infiltration, hemorrhagic areas, ulceration, or abscesses; Score 1, scattered vasodilatation, areas of reepithelization, diffuse cell infiltration with multiple mononuclear leukocytes, and absence of bleeding, edema, ulcers and abscesses; Score 2, moderate vasodilatation, epithelial hydropic degeneration (vacuolization), moderate cell infiltration dominated by polymorph nuclear leukocytes, the presence of hemorrhagic areas, edema and rarely small ulcers but absence of abscesses; Score 3, marked vasodilation, cell infiltration with multiple polymorph nuclear leukocytes, the presence of hemorrhagic sites, the presence of edema and ulceration, and the absence of abscess; Score 4, severe vasodilatation and inflammatory infiltration, characterized by neutrophils, abscesses and diffuse ulcers (21).

Ethical Considerations

The study was approved by the Medical/Health Research Bioethics Commission, Faculty of Medicine, Sultan Agung Islamic University (N0. 399/X/2022).

Statistical Analysis

The data are presented as mean ± standard deviation (SD). Normal distribution was assessed using the Shapiro-Wilk test, and homogeneity was examined via the Levene's test. Furthermore, data analysis used one-way ANOVA and continued with the Least Significant Difference (LSD) test with $P<0.05$ under SPSS version 23.

Result

Body weight decreased after the development of oral mucositis in all the rats in the three groups (Figure 1). Significant differences were observed in body weight on day 14 between groups D1, D2, and D3 compared to the control group ($P<0.05$). Significant differences were also noted between groups D1 and D3, D2 and D3, and D2 and D3.

Mucositis grades were highest on day 14 in the D3 group. The grades worsened over 14 days in all treated groups compared to the control group (Figure 2). The mucositis grades of groups D1, D2, and D3 were 1.4, 2.2, and 3.2 on day 7, respectively.

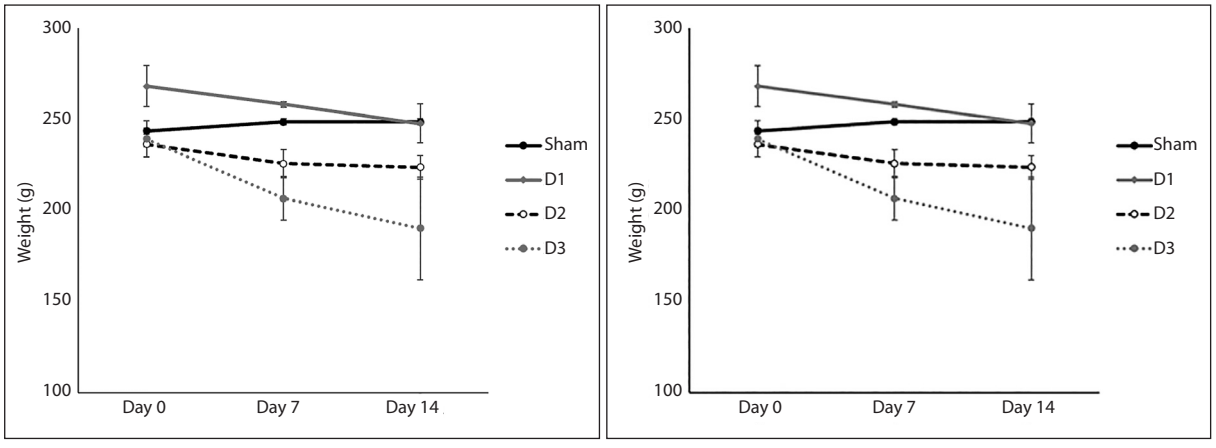

Figure 1. Evaluation of the rat cisplatin-induced oral mucositis model. Changes in body weight of the rats in the four groups.

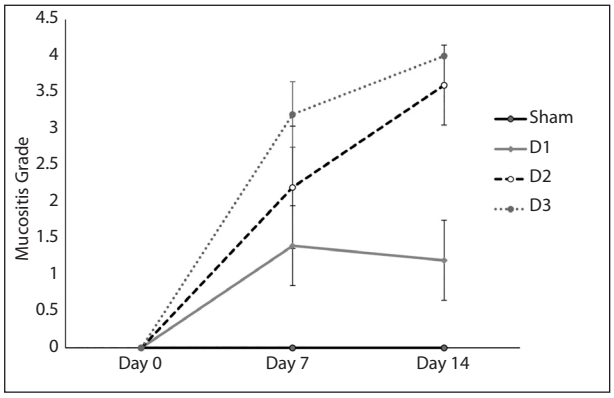

Figure 2. Evaluation of the rat cisplatin-induced oral mucositis model. Mucositis grades in the four groups of rats.

Interestingly, the mucositis score worsened on day 14 in groups D2 and D3. The mucositis grades in D1, D2, and D3 were 1.2, 3.6, and 4, respectively. In group D1 the mucositis grades improved on day 14. This phenomenon indicates that the administration of cisplatin at doses of D2 and D3 successfully induced mucositis consistently.

The histopathological scores were highest on day 14 in groups D2 and D3. In group D1 the histopathological scores were improved on day 14 (Figure 3). Intragroup comparisons revealed no significant differences between D2 and D3 groups on day 14. The findings from the histopathological

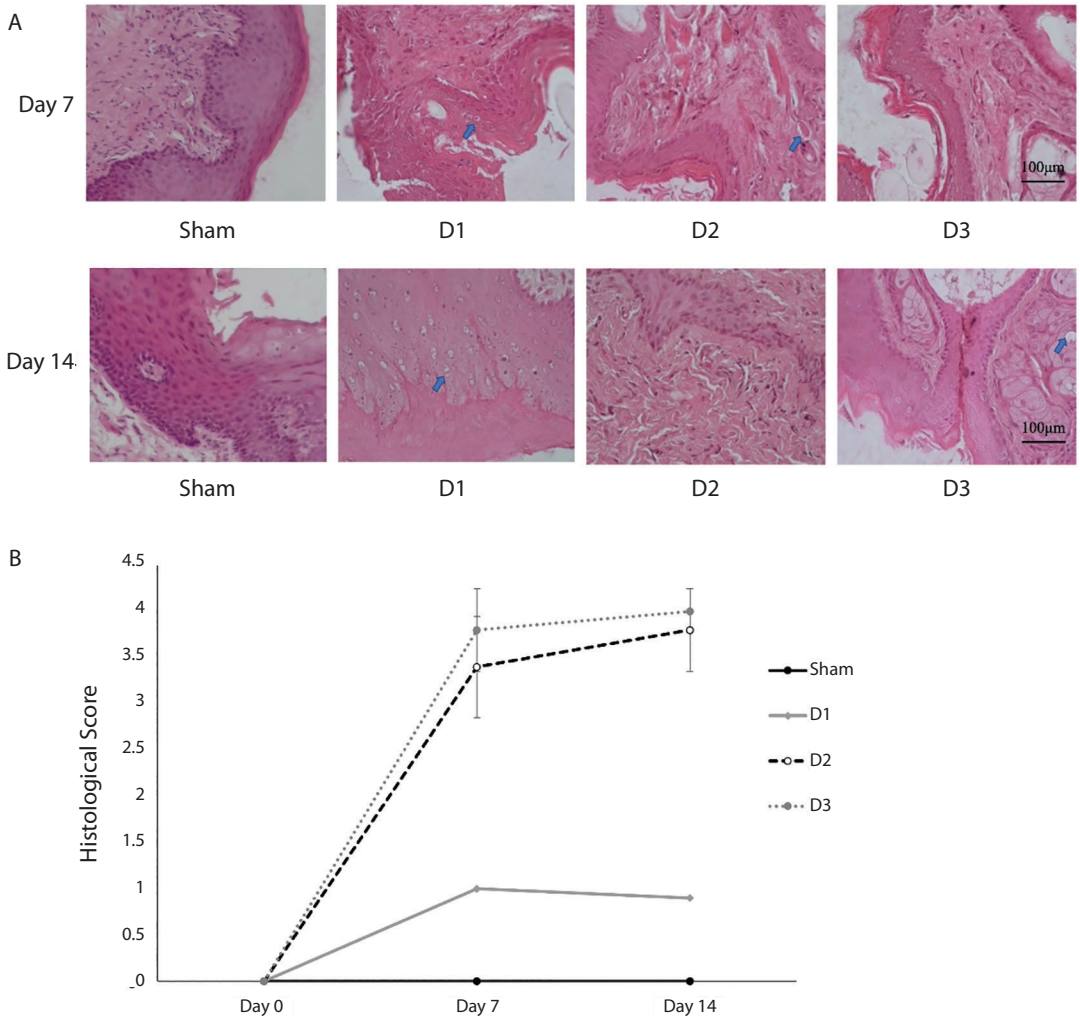

Figure 3. Evaluation of the rat cisplatin-induced oral mucositis model. (A) Histological morphology of buccal on days 7 and 14 after cisplatin administration. (B) Changes in the mucositis scores of the four groups of rats. D1 (Cisplatin 3 mg/kgBW), D2 (Cisplatin 5 mg/kgBW), and D3 (Cisplatin 6 mg/kgBW) groups.

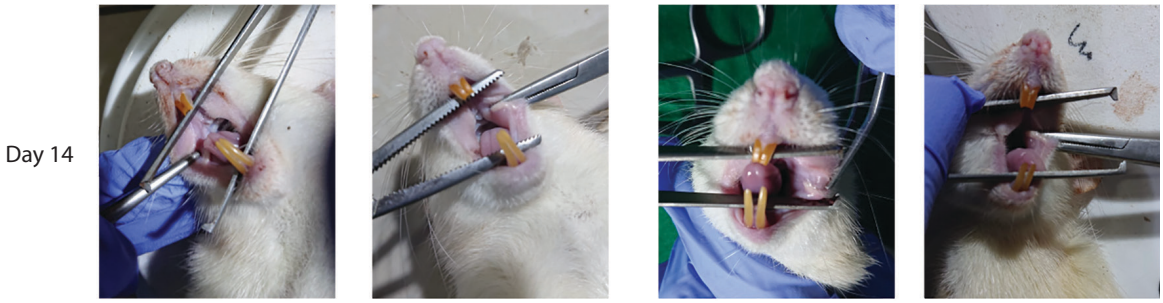

Figure 4. Macroscopic changes in the oral cavity of the rat cisplatin-induced oral mucositis model. D1 (Cisplatin 3 mg/kgBW), D2 (Cisplatin 5 mg/kgBW), and D3 (Cisplatin 6mg/kgBW) groups.

examination align with the macroscopic observations of the oral cavity, indicating that the alterations in the oral cavity of both groups D2 and D3 did not demonstrate significant disparities. This was substantiated by the presence of a whitish hue in the cheek region of the mice, particularly notable in groups D2 and D3 (Figure 4).

Discussion

The present results revealed markedly lower body weights in groups D1, D2, and D3, suggesting that cisplatin inhibits appetite. Decreased food intake was noted after the onset of oral mucositis, along with a corresponding reduction in body weight, and these changes were attributed to the pain associated with oral mucositis. This result supported a previous study showing that the body weight of a oral mucositis animal model had significantly decreased on days 9 and 11 after 5-FU administration (25).

The inhibition of the growth of granulation tissues is an important step in oral mucositis (4). Inhibition of fibroblast also plays the most important role in the formation of un-granulation tissues (15). The administration of cisplatin in D2 and D3 significantly increased confluent ulceration or the formation of a pseudo membrane, with bleeding. Interestingly, on day 14 in group D3 the oral mucosa of the animals was necrotic with spontaneous bleeding. However, in group D1 the mucositis grades had improved on day 14. Furthermore, macroscopic, and histopathological findings indicated more rapid tissue damage in groups D2 and D3 than in D1 and the control group. The tissue damage of the oral mucosa was exacerbated by the inhibition of the cell migration-promoting effect of cisplatin. These results indicate that the doses in D2 and D3 did not show any significant differences. Therefore, the D2 dose was sufficient to induce oral mucositis caused by cisplatin chemotherapy. In this condition, it was also confirmed that the D2 dose was able to maintain the condition of oral mucositis until day 14, while in D1 there was improvement without treatment. A previous study also reported that the administration of 5-FU induced vasodilatation and inflammatory infiltration on days 9 and 11 (25). On the basis of these results, we concluded that the cisplatin 5 mg/kgBW administrated on days 1, 3, and 5 by intraperitoneal administration was the best dose to induce oral mucositis. However, future studies are needed to investigate the molecular mechanism of cisplatin-induced oral mucositis in more detail.

Taken together, this finding holds significant implications for research pertaining to oral mucositis and related therapies. By establishing the optimal dosage and a reliable induction method, subsequent research can be more directed towards intervention studies and the development of therapies to address this condition. This research may serve as a foundation for testing pharmaceuticals or medical procedures aimed at preventing or reducing oral mucositis in a rat population, expediting progress in this field and potentially yielding benefits for the treatment of oral mucositis in humans.

Conclusion

Cisplatin administered intraperitoneally at a dose of 5 mg/kgBW produced histopathological oral mucositis without death, and was validated and shown to be optimal.

What Is Already Known on This Topic:
Chemotherapy-induces oral mucositis is a serious side effect of cytotoxic drugs. However, the adverse effects of chemotherapy-induced oral mucositis are not well managed, due to a lack of understanding of its mechanism of formation. At present, there is no established method for creating an animal model of oral mucositis induced by chemotherapy. Many studies have primarily focused on gastric mucositis, and no standardized approach has been developed for oral mucositis induced by chemotherapy in animal models.

What This Study Adds:
In this study, we developed an animal model of chemotherapy-induced oral mucositis. Additionally, we validated the conditions of oral mucositis using several inflammation parameters. Through the findings of this research, valuable information was obtained regarding the establishment and validation of a method for inducing oral mucositis through chemotherapy induction.

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Authors' Contributions: Conception and design: OK and DGT; Acquisition, analysis, and interpretation of data: BW and OK; Drafting the article: OK; Revising it critically for important intellectual content: DGT and MS; Approved final version of the manuscript: DGT.

Conflict of Interest: The authors declare that they have no conflict of interest.

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Original Research Study
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A Comparison of Cathelicidin Levels in the Skin of Leprosy Patients and Their Household Contacts

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Abstract

Objective. This study aimed to compare cathelicidin levels in the skin of leprae patients and leprae contacts. **Patients and Methods.** This research is an analytic observational study with a cross-sectional approach. Fifty-four research subjects participated in this study. They consisted of leprae patients, household contacts, and healthy individuals. Cathelicidin levels were measured using the ELISA method. Data analysis was carried out with the help of SPSS software, and univariate and bivariate analysis was conducted. **Results.** Cathelicidin levels in the leprae group (256.8±22.9 pg/ml) were higher than in the contact group (25.9±2.7 pg/ml). Likewise, the contact group had higher cathelicidin levels than healthy controls (1.4±0.1 pg/ml). Statistically, there were differences in cathelicidin levels between groups, P<0.050. **Conclusion.** Cathelicidin levels in leprae patients were higher than those in household contacts.

Key Words: Antimicrobial Peptide ▪ Cathelicidin ▪ Enzyme-Linked Immunoabsorbent Assay ▪ Leprosy ▪ Neutrophils.

Introduction

Leprosy is a chronic infection caused by *Mycobacterium leprae* and is still a serious health problem in many countries (1). Data from WHO state that in 2022 there were 174,087 new cases of leprosy recorded, with 9554 of them accompanied by grade 2 disabilities (G2D) (2). New cases of leprosy are always present in endemic areas, and in some endemic areas, it continues to increase (3, 4). Indonesia is the third-highest country globally in terms of leprosy cases, following India and Brazil. In 2022, there were 15,052 registered cases of leprosy and 12,095 new cases, resulting in a new case detection rate of 45.16 per 1,000,000 population (5, 6). The clinical manifestations of leprosy are varied, and the mechanism of this infection is closely related to the innate immune response (7–9).

The innate response is a natural response that exists in the body for the body's defense against infection (9). Antimicrobial peptide (AMP) is part of the innate immune response (10, 11). Defensin and cathelicidin are part of the antimicrobial peptide (12). Cathelicidin is an antimicrobial protein found in neutrophils and keratinocytes (13, 14). Several studies have shown that cathelicidin deficiency is related to the severity of the infection experienced (15, 16). One study showed that cathelicidin deficiency in salivary neutrophils was associated with more severe oral infections (16). Cathelicidin in skin neutrophils is also believed to play a role in the severity of leprosy (12, 14, 15, 17). A study on *Mycobacterium tuberculosis* infection showed that AMP from neutrophils could potentially prevent the severity of pulmonary

[*] The study was conducted at the Faculty of Medicine, Universitas Sriwijaya, Palembang, Indonesia.

tuberculosis (18). The similarity of the causative genus between leprosy and tuberculosis infections suggests that the potential of cathelicidin to reduce the severity of tuberculosis could have a similar effect on leprosy cases. Cathelicidin is believed to be able to act as a marker of the presence and severity of *Mycobacterium leprae* infection (17).

As human beings, of course, leprosy patients cannot live alone. Leprosy patients often come into contact with many people, such as family members at home, friends, and neighbors (household contacts) (2). Their condition means that those who have close contact with leprosy patients have the potential to experience leprosy infection (19). However, these household contacts (HCs) do not necessarily become directly infected with leprosy. Many HCs are clinically healthy, which is believed to result from the immune response in household contacts. HCs are believed to have innate and adaptive immune responses, which are more optimal than leprosy patients (20). Ideally, leprosy patients will have lower cathelicidin levels than HCs. However, another study on the severity of tuberculosis patients and HCs using cathelicidin markers presented a different picture (21). The study stated that tuberculosis HCs have lower cathelicidin levels than tuberculosis patients.

This study compared cathelicidin levels in leprosy patients' skin and that of household contacts.

Methods

Study Design and Participants

This study is an analytic observational study with a cross-sectional approach. The study was conducted between September and December 2022. This study used primary data, where the research subjects were PB and MB type leprae patients and their families who live at home and always accompany patients for treatment at the Dermatology Polyclinic of Dr. Mohammad Hoesin General Hospital, Palembang, Indonesia. Fifty-four research subjects participated in this study, including leprosy patients, leprae household contacts (HCs), and healthy individuals. The inclusion criteria for leprosy patients were: patients diagnosed with leprosy (WHO classification (PB and MB types) (2) by a dermatologist at Dr. Mohammad Hoesin General Hospital, aged over 18 years, who agreed to participate in this study. Exclusion criteria for leprosy patients and HCs were those suffering from skin diseases other than leprosy, and those taking antibiotics or corticosteroids. HC inclusion criteria were subjects living at home with leprosy patients for at least six months, aged over 18 years, and who agreed to participate in this study. Detailed sociodemographic data of the patients, consisting of gender, age, and daily activities were recorded. The daily activities of patients were investigated to determine their interactions with their household contacts.

Skin Scraping Method

The research participants underwent procedures for specimen collection, including skin scraping from both ear lobes and two different skin lesions (for patients), and both upper arms (for household contacts and healthy participants). The scalpel used for skin scraping was put into a tube containing 70% alcohol. Using the non-sharp edge of a scalpel, skin scrapings are collected from the skin lesions (macules) and unaffected skin of individuals with leprosy, their household contacts, and healthy individuals, in the brachii area. We conducted the dermal scraping process on leprosy patients, obtaining two samples measuring 5 cm × 5 cm from the afflicted lesion area (macules) and healthy skin located 7 cm apart from the lesion site. One hand selected and stretched the skin area, while the other hand held the incision in a manner that ensured the cut remained parallel to the skin's surface. Subsequently, the scraping procedure was performed approximately 10–20 times in a single direction, followed by three repetitions on each dull edge of the scalpel while exerting substantial force.

Cathelicidin Level Evaluation

Examination of cathelicidin levels was carried out using the enzyme-linked immunosorbent assay (ELISA) technique. The skin scraping samples of

the research subjects were homogenized and centrifuged at 5000 rpm for 10 minutes at 4°C (22). The supernatant was taken and used for ELISA examination. The ELISA procedure was carried out according to the ELISA kit cathelicidin manual (Cloud Clone*, Hangzhou, PRC).

Ethical Approval

This study received ethical approval from the Ethics Committee of the Faculty of Medicine, Universitas Sriwijaya (Ref. No. 155/FKUNSRI/XI/2022), and informed consent was provided by each volunteer participating.

Statistical Analysis

Data were analyzed using SPSS 25.0 (SPSS Inc., Armonk, NY, United States). Univariate analysis was performed to present the data distribution for each test variable. Bivariate analysis was performed to compare cathelicidin levels between test groups, with $P<0.05$.

Results

A total of 54 research subjects participated in this study, including leprosy patients, leprae household contacts (HCs), and healthy individuals.

Table 1 shows the baseline characteristics of the research subjects. Most of the leprosy group were male, aged 21–40 years, and performed activities outside the home. Most HC groups were aged 41–60 years old and worked outside their home. The healthy group was predominantly male, aged 21–40, and most worked at home.

Table 2 shows a comparison of cathelicidin levels between groups. Cathelicidin levels in the leprosy group were higher than in the HC group. Likewise, the HC group had higher cathelicidin levels than the healthy controls. Statistically, there were significant differences in cathelicidin levels between groups (Table 3).

Table 1. Baseline Characteristics of Participants

Variables	Groups		
	Leprosy patients N (%)	Household contacts N (%)	Healthy N (%)
Gender			
Male	10 (55.6)	10 (55.6)	10 (55.6)
Female	8 (44.4)	8 (44.4)	8 (44.4)
Age (years old)			
<40	11 (61.1)	7 (38.9)	13 (72.2)
≥40	7 (38.9)	11 (61.1)	5 (27.8)
Daily activities			
Inside home	11 (61.1)	7 (38.9)	11 (61.1)
Outside home	7 (38.9)	11 (61.1)	7 (38.9)
Type of leprosy			
Paucibacillary	10 (55.6)	-	-
Multibacillary	8 (44.4)	-	-

Table 2. Comparison of Cathelicidin Levels between Groups

Variable	Groups			P-value*
	Leprosy patients	Household contacts	Healthy	
Cathelicidin levels	256.8±22.9	25.9±2.7	1.4±0.1*	0.0001

*One-way ANOVA; †pg/ml±SD.

Table 3. Pos-hoc Analysis between Groups

Groups	Leprosy patients	Household contacts	Healthy
Leprosy	--	0.0001*	0.0001*
Household contacts	0.0001*	-	0.0001*
Healthy	0.0001*	0.0001*	-

*Pos-hoc Bonferroni.

Discussion

This study showed that cathelicidin levels in leprosy patients were higher than in household contacts (HCs). Cathelicidin is part of the innate immune system, where this protein is an antimicrobial protein (AMP) produced by neutrophils to treat various infections. The higher the cathelicidin level, the more moderate the severity of *Mycobacterium* infection (11, 12). Another study showed that keratinocytes and skin cells, such as eccrine gland cells, produce and secrete AMPs, including cathelicidin (14). In our study, cathelicidin was evaluated in skin scrapings because it is synthesized by epithelial cells and provided by infiltrating immune cells, such as neutrophils and macrophages (23). The infiltrating immune cells transport cathelicidins to infected or injured skin (23).

The results of this study are inconsistent with several studies that state that cathelicidin deficiency causes *Mycobacterium tuberculosis* infection to become more severe compared to the HCs group (24, 25). There are several theories and other studies that can explain the findings of this study. Previous studies have measured cathelicidin levels in *M. tuberculosis* infection, where the primary infection site is in the lungs so that the cathelicidin levels that represent the immune system are in the serum (21, 24). In leprosy patients, the primary site of infection is in the skin, so the level of cathelicidin in infected skin scrapings represents the patient's infection status (26). Other studies state that cathelicidin levels are identical to bacterial load or how many microorganisms there are in the body (27, 28). The more bacteria or microorganisms in the body, the higher the production of cathelicidin (28, 29). This can explain why cathelicidin levels in the HC group are lower compared to leprosy patients. HCs have a lower bacterial load than leprosy patients.

The immune system is a simultaneous process triggered by antigenic stimuli that aim to destroy the stimulus triggers (30). The body's defense mechanism has three levels: the physical barrier of the skin and mucosal surfaces, the innate immune system, and the adaptive immune system (30). The physical skin barrier is essential because it protects against contact with the outside world. The skin surface is also inhabited by various microbes, viruses, and fungi, known as the skin microbiome, to strengthen the skin barrier (31). The innate immune system cooperates with the physical defenses of the skin and mucosa, enzymes, macrophages, polymorphonuclear, eosinophils, and natural-killer cells, to deal with non-specific foreign bodies or organisms.

Vitamin D and downstream receptor signaling are essential in enhancing the capabilities of macrophages and other immune cells (32). Increasing the immune cells' ability will encourage the human body's antimicrobial defense (33). Several AMPs are induced by vitamin D signaling, including cathelicidins, defensins, hepcidins, and neutrophil peptides acting as major intrinsic antibiotics. Previous studies have also suggested that vitamin D signaling is related to the transcriptional activation of AMPs, including cathelicidins and defensins (32, 33).

Cathelicidin activation-induced vitamin D, as a component of immunity in the skin, is affected by sun exposure. As a tropical country, Indonesia has sufficient sun exposure to activate vitamin D (34). Cathelicidin levels in the skin show the immunity system activity against *M. leprae* infection. Cathelicidin levels on the surface of the skin are an accumulation of the results of the synthesis of

skin epithelial cells and neutrophils that infiltrate the skin that is being infected (13). Cathelicidin has the potential to be developed into a marker to assess the bacterial load of leprosy infection. The limitation of our study is the number of samples that were only taken from one region in Indonesia. In future studies, multicentre sampling should be carried out so that the results obtained are more representative of leprosy patients and their household contacts.

Conclusion

Cathelicidin levels in leprosy patients were higher than those in household contacts and healthy individuals.

What Is Already Known on This Topic:
Cathelicidin is an antimicrobial protein found in neutrophils and keratinocytes. Cathelicidin in skin neutrophils is also believed to play a role in the severity of leprosy. Household contacts or people who live together with leprosy patients are believed to have more innate and adaptive immune responses than the patients.

What This Study Adds:
This is the first study to explore and compare cathelicidin levels in skin scrapings of leprae patients and their household contacts. Cathelicidin is believed to be able to act as a marker of the presence and severity of Mycobacterium leprae infection. In this study, we found that cathelicidin levels in leprosy patients were higher than those in household contacts.

Authors' Contributions: Conception and design: FA and OS; Acquisition, analysis and interpretation of data: FA and HG; Drafting the article: FA, HG and AB; Revising it critically for important intellectual content: HG, AB; Approved final version of the manuscript: FA, OS, HG and AB.

Conflict of Interest: The authors declare that they have no conflict of interest.

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The Relative Role of Family Affluence and Social Support on Depression and Self-Esteem among Adolescents in Nigeria: a Cross-Sectional Study

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Abstract

Objectives. To assess the relative importance of social support and family affluence in depression and self-esteem among adolescents in Calabar, Nigeria. **Methods.** This cross-sectional study was conducted among adolescent students in Calabar, Nigeria. Using stratified random sampling, 332 students were selected for participation. The Family Affluence Scale (FAS), Oslo Social Support Scale (OSS), Becks Depression Inventory (BDI) and Rosenberg Self-Esteem Inventory (RSES) were administered. To facilitate comparisons, the sample was divided into four groups: those with low OSS and low FAS scores, those with low OSS and high FAS scores, those with high OSS and low FAS scores, and those with high scores in both OSS and FAS. Groups were compared using the Kruskal-Wallis Test. Linear regression analysis was conducted to determine the predictors of depression and self-esteem. All analyses were performed using IBM SPSS version 21. **Results.** Respondents with low levels of social support, irrespective of family affluence, had significantly higher depression scores and significantly lower self-esteem scores ($P< 0.05$). In linear regression analyses, social support (95%CI [-1.35,-0.58]) and female gender (95%CI [1.49,5.29]) emerged as predictors of depression, while social support (95%CI [0.25,0.64]) and age (95%CI [-0.79,-0.11]) emerged as predictors of self-esteem. Affluence did not predict depression or self-esteem. **Conclusions.** Social support is of greater relative importance in depression and self-esteem in our study. In developing nations with lean resources, enhanced social support might buffer against the effect of low socio-economic status on mental health.

Key Words: Depression ▪ Social Support ▪ Socio-economic Factors ▪ Adolescent.

Introduction

Mental disorders are a significant global health concern, comprising 7.4% of the global disease burden and affecting over one billion people worldwide (1, 2). They are also the leading cause of years of life lived with disability, accounting for up to 4% of gross domestic product loss in sub-Saharan Africa (3, 4). In Nigeria, it is estimated that 40 to 60 million individuals suffer from mental disorders (5). Furthermore, the associated disability in sub-Saharan Africa is projected to increase by 130% over the next 40 years (6).

Adolescence represents a sensitive phase in human development, characterised by heightened neuroplasticity that enables the brain to adapt to various physical, emotional, cognitive, and environmental challenges (7). This developmental period involves significant brain rewiring, associated with substantial learning and laying the foundation for adulthood (8). However, it is also a vulnerable phase, with nearly 50% of all mental disorders beginning around the age of 18, peaking at 14.5 years (8, 9). Unfortunately, the majority of affected adolescents do not receive any form of treatment until several years after the onset of their conditions, and this situation is likely exacerbated in sub-Saharan Africa, where over 90% of mental disorders remain undiagnosed (10).

Adolescents with untreated mental health issues face increased risks, including substance abuse, violence, academic struggles, unemployment, poor social functioning, and compromised physical health (11, 12). Depression and anxiety are among the most common disabling problems, with suicide ranking as the third leading cause of death in this age group (13, 14). Given the current and future disease burden and its consequences, it is imperative to understand the determinants of mental well-being among adolescents.

Numerous factors influence mental well-being, spanning biological, psychological, and social dimensions (15). The theoretical framework for this study aligns with the Social Determinants of Health (SDOH) Model developed by the World Health Organization (16). This model posits that conditions related to where individuals are "born, live, grow, work, and age," particularly those related to social and economic disadvantages, can result in health inequalities. Social determinants of health encompass early childhood experiences, educational opportunities, socio-economic backgrounds, food security, neighbourhood conditions, access to clean air and water, gender inequity, social support, employment opportunities, and exposure to crime (17). Research consistently demonstrates that these social determinants of health affect physical and mental well-being. They are causally linked to mental health, interacting with polygenic risk factors to shape psychological well-being (18). Social determinants of health also significantly influence positive mental health and mental disorder among adolescents (19).

Socio-economic status segregates the population, creating health disparities through its impact on an individual's occupation, health-seeking behaviour and risk exposure (20). These social inequalities become evident when examining the direct effects of socio-economic factors on mental well-being (13). Socio-economic status has been associated with mental health problems in adolescents, with more pronounced effects observed in younger children (21). Its impact is especially significant in countries like Nigeria, where over 60% of the population lives in extreme poverty (22). According to the social equalisation theory, health disparities in childhood might diminish during adolescence due to the influence of youth culture pressures (23). However, research has shown that these inequalities can persist during adolescence despite the equalisation effect, influencing health behaviours and mental wellness (24, 25).

Social support also plays a critical role in mental well-being, particularly during adolescence, a phase characterized by heightened stress (13, 26). It is defined as everyday behaviours that communicate directly or indirectly to an individual that they are valued and cared for by others (27). Effective communication with parents and peers, adequate family time, and engaging in recreational activities with friends improve stress coping, enhancing social skills, self-esteem, and a sense of security and belonging (13, 20). Various dimensions of social support, including emotional, instrumental, financial, and informational support, can be derived from family, peers, and teachers (20, 26, 28).

While some studies have explored how social support and socio-economic status might be associated with depression or self-esteem, most were conducted in high-income countries and different socio-cultural settings (29-32). Few studies have aimed to determine the relative importance of these factors as predictors of depression or self-esteem in adolescents.

This study aims to fill this gap by assessing the comparative significance of social support and socio-economic status as predictors of depression and self-esteem among adolescents in a low-middle-income country in sub-Saharan Africa. Depression and self-esteem were selected as outcome variables to evaluate the two essential dimensions of mental health: negative and positive aspects, respectively.

Methodology

Study Setting and Population

Participants for this cross-sectional study were recruited from secondary schools in Calabar, a city located in the southern senatorial district of

Cross River State, Nigeria. Calabar encompasses two local government areas, Calabar South and Calabar Municipality, and shares boundaries with Akpabuyo, Odukpani, and Akamkpa local government areas. Calabar is a tropical city renowned for its serenity, cuisine, and rich pre-colonial history. During the colonial era, while exporting local resources and enslaved people, the colonialists introduced Christianity, healthcare, and education to the region. Education remains one of the enduring legacies of this exchange, beginning with the establishment of the famous Hope Waddell Training Institute, one of Nigeria's first secondary schools, in 1895. According to the State Ministry of Education, Calabar currently boasts 80 secondary schools, with 56 (70%) being privately owned.

Eligibility Criteria: Students from the most senior class in private and public schools in Calabar were eligible for inclusion.

Sample Size Estimation

The sample size was determined using the formula for estimating sample size when the prevalence is known ($Z\alpha^2pq/d^2$) (33). Using an assumed prevalence of 50%, the desired precision level of 0.05 and a 95% confidence interval, we had an estimate of 384 (34).

Sampling Procedure

The study employed a stratified sampling technique. Out of the 80 secondary schools in Calabar, 73 were coeducational, and the remaining seven, which were single-gender schools, were excluded to maintain uniformity. Sampling was conducted in the 73 coeducational secondary schools. These secondary schools were further stratified into privately and government-run institutions. Using simple random sampling, two schools were selected from each group, resulting in four schools (two private and two government-run).

Participants were randomly selected from the senior class, SS3 (typically divided into sub-classes, e.g., SS3A, SS3B, SS3C), in each school. The data collection period lasted six weeks, from January to February 2020.

Study Instruments

1. Sociodemographic questionnaire: This collected data on the respondent's age, gender, and school type.
2. The Family Affluence Scale (FAS-II): This is a four-item self-report measure of family affluence first designed and used in the WHO-Health Behavior in School-aged Children (HBSC) survey (35). It was designed to assess family socio-economic status using ownership of material possessions as a response to the observation that many adolescents cannot accurately report other indicators of family affluence, such as parental income, occupation, education, etc. A composite score for the scale can be computed as follows: low affluence (0-2), medium affluence (3-5), and high affluence (6-9) (35). In this study, affluence was graded as high or low based on the median, giving a dichotomous variable for simplicity of presentation. It has been found to have cross-cultural validity and has been used in several countries worldwide, including Nigeria (35, 36). In a multi-country validation study, it had good rank order correlations of .87 with country GDP, suggesting good criterion validity (35). In this study, the Cronbach's alpha was 0.57.
3. Oslo Social Support Scale (OSS-3): This is a 3-item self-report scale which measures the level of social support an individual receives(37). It inquires about the number of people the respondent feels close to, the ease of accessing help when needed and the level of interest others show in the respondent's person or life. Its brevity is considered an advantage, and it has been found to be reliable and valid in many countries worldwide, including Nigeria (37). The overall score spans from 3 (minimum) to 14 (maximum), delineating social support into categories: inadequate (3-8), moderate (9-11), and strong (12-14). In this study, the social support was graded into high and low based on the

median, giving a dichotomous variable for simplicity of analysis and presentation. It has an internal consistency of 0.67, which is acceptable given the scale's brevity (38). In this study, the value was 0.52.

4. Beck Depression Inventory (BDI-II): This is a 21-item self-report measure of depressive symptoms that was originally designed to assess the intensity of depression. It is one of the most popular screening tools for depression and has been used across several countries worldwide (39). The scale assesses depressive symptoms such as sadness, loss of interest, insomnia, tiredness, feelings of guilt and suicidality (39). Each question has a possible score of 0 to 3, with 0 indicating the absence of symptoms and 3 indicating severe symptoms. The 21 items are summed to get a composite score, which is graded as follows: 0-13: Minimal depression, 14-19: Mild depression, 20-28: Moderate depression, and 29 to 63: Severe depression (40). It has also been validated and widely used in Nigeria with a sensitivity of 0.91 and a specificity of 0.97 (40). In this study, the scale was used as a continuous variable to indicate the overall presence of depressive symptoms as defined by the BDI. The Cronbach alpha for this sample was 0.86.
5. Rosenberg Self-Esteem Scale (RSES): This is a 10-item self-report measure of self-esteem, originally designed for adolescents (41). Some examples of scale items are as follows: "*I feel that I am a person of worth, at least on an equal plane with others*; I take a positive attitude toward myself; I certainly feel useless at times, etc". Each question is graded using a Likert scale ranging from 1 (strongly agree) to 4 (strongly disagree). A score between 21 and 30 on the RSES indicates high self-esteem, scores between 11 and 20 signify average self-esteem, and scores ranging from 0 to 10 suggest low self-esteem. In this study, the scale was used as a continuous variable to indicate the overall level of self-esteem. It has been used in several contexts globally and is known for its good psychometric properties (42). It has also been validated and used in Nigerian studies with a Cronbach alpha of 0.87 (43). In this sample, the Cronbach alpha was 0.66.

Ethical Consideration

This study adhered to the Declaration of Helsinki. Ethical approval (Ref no: FNPH/HREC/01/05) was obtained from the ethics board of the researcher's institution, the Federal Neuropsychiatric Hospital Calabar. Additionally, permission for the study was granted by the State Ministry of Education and the administrators of the selected schools. For students under the age of 18, informed parental consent was sought, and the students also provided their assent to participate. Students aged 18 or older provided informed consent themselves.

Statistical Analysis

Analysis was done using the IBM SPSS version 21 software. Frequencies and percentages for sociodemographic variables were computed. The dependent variables (depression and self-esteem) were not normally distributed. Therefore, the four groups were compared using Kruskal-Wallis (KW) non-parametric tests with post-hoc analyses. A possible association between affluence and social support was explored using Spearman rank correlation, as both variables were also non-normal. Results were considered significant if $P<0.05$. Linear regression was also done to determine the predictors of both depression and self-esteem scores.

Results

The final sample comprised 332 adolescents from four secondary schools in Calabar, with slightly more than half being male. Ages ranged from 11 to 20 years, and 182 (54.8%) attended private schools, while the rest attended government-owned schools. Sociodemographic data are presented in Table 1.

Table 1. Characteristics of Respondents

Variable	Frequency (N; %)
Age (yrs)*	
11-14	125 (38.3)
15-20	205 (61.7)
Gender	
Male	181 (54.5
Female	151 (45.5)
School type	
Public	150 (45.2)
Private	182 (54.8)

*Mean±SD (14.99±1.40).

Four comparison groups were generated based on median scores of 5 on the Affluence Scale (FAS) and 11 on the Oslo Social Support Scale (OSS). First, using the median score, the social support (OSS) and family affluence (FAS) variables were categorized into high and low levels. Thus, for FAS, low affluence was defined by scores of 0-4, while scores of 5 or above were considered high. Also, for OSS, scores of 3-10 were designated as low social support, while scores of 11 or above were deemed high affluence. Then, based on binary combinations, four groups were created as follows: Group One comprised of respondents with low OSS and FAS scores; Group Two consisted of respondents with low OSS and high FAS scores; Group Three was composed of respondents with high OSS and low FAS; and Group Four comprising respondents with high scores in both measures. These four groups were compared to determine how different levels of social support and affluence in binary combinations might be associated with depression and self-esteem.

Using the normal P-P plot, data was approximately normally distributed for both regression analyses. Also, a scatter plot of regression standardized residuals against regression standardized predicted values showed that the data was homoscedastic. All VIF values were less than 10, with the Durbin-Watson statistic as 1.95 for the depression regression and 1.87 for the self-esteem, suggesting low multicollinearity.

A weak positive correlation was observed between OSS and FAS ($r=0.19$; $P<0.05$). Table 2 presents a comparison of depression levels across the four groups. Depression scores (as measured by the BDI) were similar for two groups with low social support and the other two with high social support, irrespective of affluence level. Overall, the differences in depression scores across the four groups were statistically significant ($H=25.37$; $P<0.05$). Post hoc tests revealed that the depression score for Group One (low OSS + low FAS) was significantly higher than that of Group Three (high OSS + low FAS) and Group Four (high OSS + high FAS) ($P<0.05$). Similarly, the depression score for Group Two (low OSS + high FAS) was significantly higher than that of Group Three (high OSS + low FAS) and Group Four (high OSS + high FAS) ($P<0.05$). The two groups with low OSS (one and two) were not significantly different from each

Table 2. Comparison of Depression Scores across Groups

Group	N (%)	BDI Mean± SD	Kruskal-Wallis test P-value	Post Hoc (Tukey HSD) Comparisons			
				Low OSS and Low FAS P-value	Low OSS and Low FAS P-value	Low OSS and Low FAS P-value	High OSS and High FAS P-value
Low OSS and Low FAS	79 (23.8)	14.74±9.17	<0.001	-	-	-	-
Low OSS and High FAS	79 (23.8)	14.16±9.25		1.00	-	-	-
High OSS and Low FAS	65 (19.6)	9.60±6.98		0.006	0.03	-	-
High OSS and High FAS	109 (32.8)	9.99±9.14		<0.001	0.002	1.00	

BDI= Becks Depression Inventory; OSS=Oslo Social Support Scale; FAS=Family Affluence Scale.

Table 3. Comparison of Self-Esteem Scores across Groups

Group	N (%)	RSES Mean±SD	Kruskal-Wallis Test P-value	Post Hoc (Tukey HSD) Comparisons			
				Low OSS and Low FAS P-value	Low OSS and High FAS P-value	High OSS and Low FAS P-value	High OSS and High FAS P-value
Low OSS and Low FAS	79 (23.8)	19.96±4.00	<0.001	-	-	-	-
Low OSS and High FAS	79 (23.8)	19.60±4.85		1.00	-	-	-
High OSS and Low FAS	65 (19.6)	21.16±3.93		0.006	0.009	-	-
High OSS and High FAS	109 (32.8)	20.89±4.20		0.01	0.02	1.00	-

RSES=Rosenberg Self-Esteem Inventory; OSS=Oslo Social Support Scale; FAS=Family Affluence Scale.

other in terms of depression score, irrespective of affluence score (P>0.05). Also, the two groups with high OSS (three and four) did not differ significantly with regard to their depression score, regardless of their affluence level (P>0.05).

Table 3 is a comparison of the levels of self-esteem in the four groups. The self-esteem scores (as measured by the RSES) were similar for two groups with low social support and the other two with high social support, irrespective of affluence level. Overall, the differences in self-esteem scores across the four groups were statistically significant (H=18.83; P<0.05). The post hoc test revealed that the self-esteem score for Group One (low OSS + low FAS) was significantly lower than that of Group Three (high OSS + low FAS) and Group Four (high OSS + high FAS) (P<0.05). Also, the self-esteem score in Group Two (low OSS + high FAS) was significantly lower than that of Group Three (high OSS + low FAS) and Group Four (high OSS + high FAS) group (P<0.05). The two groups with low OSS (one and two) were not significantly different from each other in terms of self-esteem score, irrespective of affluence score (P>0.05). The same trend was present in the comparison of groups (three and four) with high OSS scores (P>0.05).

Table 4 shows predictors of depression scores based on OSS score, FAS score, age, and gender. A significant regression equation was found (F=10.17, P<0.05), with an R^2 of 0.11. Both OSS (95%CI [-1.35,-0.58]) and female gender (95%CI [1.49,5.29]) were significant predictors of BDI scores. Depression scores decreased by 0.97 for each unit increase in OSS score and increased by 3.39 for females compared to males (reference category).

In Table 4, OSS score, FAS score, age, and gender were assessed as predictors of self-esteem scores.

Table 4 Linear Regression Showing Effect of Family Affluence and Social Support on Depression and Self-Esteem Scores

Variables	Standardized β Coefficient	95% CI
Predictors of depression		
OSS	-0.27	-1.35, -0.58
FAS	0.01	-0.32, 0.41
Age	0.09	-0.068, 1.29
Gender	0.18	1.49, 5.29
Constant	-	-0.39, 22.78
R=0.230		
R^2=0.053		
Predictors of self-esteem		
OSS	0.25	0.25, 0.64
FAS	-0.06	-0.29, 0.08
Age	-0.14	-0.79, -0.11
Gender	-0.01	-1.12, 0.78
Constant	-	17.92, 29.51
R=0.29		
R^2=0.08		

CI=Confidence Interval; OSS=Oslo Social Support Scale; FAS=Family Affluence Scale.

A significant regression equation was found ($F=7.58$, $P<0.05$), with an R^2 of 0.08. Both OSS (95%CI [0.25,0.64]) and age (95%CI [-0.79,-0.11]) were significant predictors of RSES scores. Self-esteem increased by 0.45 for each unit increase in OSS score and decreased by 0.45 for each unit increase in age.

Based on these results, we conclude that there is sufficient evidence to reject the null hypothesis in favour of the alternative, that family affluence and social support have differential roles in depression and self-esteem among adolescents in Calabar, Nigeria.

Discussion

This study aimed to determine the relative roles of social support and family affluence in depression and self-esteem. We found that social support and affluence had a weak positive correlation. Respondents with low social support consistently had poorer outcomes, regardless of their family affluence level. They exhibited significantly higher depression scores and significantly lower self-esteem scores. In regression analysis, social support and gender emerged as predictors of depression, while self-esteem was predicted by age and social support. Family affluence did not emerge as a predictor of either depression or self-esteem.

Previous research has indicated a positive correlation between social support and affluence (44, 45). Our study also found such a relationship; however, it was weak, which minimized multicollinearity in regression analysis. Another survey among Chinese adolescents reported an even weaker correlation, though they used different measures of social support and family affluence (46). This might suggest that while there is some relationship, they are largely independent phenomena with potentially convergent effects.

Although a high socio-economic level and family affluence are ideal in adolescence, there is a wide variation in these factors among individuals. We assessed four possible combinations and found that a low level of social support was associated with poorer outcomes in terms of depression and self-esteem, regardless of affluence level. In other words, those with strong social support generally fared better. This could imply that, at least in our study context, good social support leads to better mental health outcomes, regardless of the adolescent's socio-economic background.

This finding appears to trivialize the role of socio-economic status in mental health, which contradicts common knowledge in the field of psychological research (21). A systematic review of 55 studies, primarily conducted in North America, Europe, and Australia, reported an inverse relationship between socio-economic status and various mental health indicators (including depression and self-esteem) in children and adolescents (21). The review noted a wide disparity in the approach to measuring socio-economic status among included studies, which limited comparability. The majority used single variables such as parental occupation, income, or educational level as indicators of SES, while only a few utilized a composite index like the FAS. It is noteworthy that two out of the three studies that did not report an association used the FAS. The differences in measuring tools might explain the variance in our findings compared to previous work. Also, the social equalization theory which posits that the impact of social disadvantage in early childhood diminishes in adolescence, might explain the lesser role of family affluence on mental health in this population (23).

A cross-national pilot study conducted in Serbia, India, Nigeria, Turkey, and Indonesia, employing the FAS, revealed a significant relationship with mental well-being (36). However, their study included children from both rural and urban areas, likely capturing the poorest rural families. Our study was conducted among adolescents who are secondary school students in a metropolitan city. Therefore, our findings might be more applicable to adolescents in urban areas who are likely to be well-off in terms of family affluence compared to those from rural areas.

Another study in South Africa demonstrated that SES did not significantly predict variance in mental well-being (47). Additionally, they identified hope as a significant predictor and determined

that its presence mitigated the impact of low socioeconomic status. It is also possible that in a highly religious, collectivistic society like Nigeria, sociocultural factors, including social support, religiosity, and hope, could act as buffers against the effects of low SES on mental health risk. Another review also considered this possibility, suggesting that buffers, such as religiosity driven by cultural mechanisms, could explain the lack of association in some contexts (48). Taken together, these findings indicate that the link between SES and mental health is intricate and subject to moderation by various psychosocial or culturally dependent variables.

Our study underscores the importance of social support in adolescents' psychological well-being, aligning with previous reports (11, 29, 49). According to the *stress-buffering effect model*, social support exerts its effect by diminishing the impact of stress and helping the individual to cope better (50). Low SES, on the other hand, exacerbates health problems by elevating stress risk, thus exerting its effect in the opposite direction (51). Social support is more accessible in collectivistic societies –a phenomenon believed to serve an "anti-psychopathology" function (52). In resource-lean collectivistic communities like Nigeria, the relative ease of accessing social support may mitigate the impact of low SES.

Limitation of Study

Our study has some limitations. First, we relied solely on self-report questionnaires, which could introduce social desirability bias. Second, our study did not include adolescents from rural areas and may have predominantly excluded those from economically disadvantaged families. This may explain the lack of significance regarding family affluence and could limit the generalizability of our findings. It would be beneficial to reevaluate the validity and socio-metric capability of the FAS among Nigerians and conduct further research to determine the threshold at which SES begins to affect mental health adversely. Additionally, our regression models accounted for a low variance in the outcome variables, suggesting that while social support was a significant predictor, its overall impact may be relatively modest. Alternatively, the low variance might mean the relationship between study variables is non-linear. Lastly, causality cannot be inferred since this was a cross-sectional study.

Recommendation

Considering the importance of social support for mental well-being, interventions designed to strengthen social support systems for adolescents should be prioritized, especially in contexts with socioeconomic inequity. Such interventions could be school-based, as the structured educational environment might be more amenable to integrating such interventions. Parents should be educated and involved in such interventions to make them aware of the relative importance of social support in the well-being of adolescents. Further research is needed to elucidate the relative importance of social support and affluence. Also, it would be beneficial to reevaluate the validity and socio-metric capability of the FAS among Nigerians and conduct further research to determine the threshold at which SES begins to affect mental health adversely.

Conclusion

Based on our findings, we conclude that social support predicts depression and self-esteem and appears to hold greater relative importance for the mental well-being of adolescents in our sample compared to family affluence. Our study may be interpreted as highlighting the compensatory effect of factors like social support on mental well-being in developing countries where economic resources are scarce and the majority live below the poverty line.

What Is Already Known on This Topic:
It is known that social support and socio-economic factors influence the mental well-being of adolescents.

What This Study Adds:
This study examines the relative importance of socio-economic status and social support in depression and self-esteem among adolescents. Our findings suggest that social support influences adolescents' emotional health more than family affluence.

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Conflict of Interest: The authors declare that they have no conflict of interest.

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Vitamin D-Binding Protein and the Role of its Gene Polymorphisms in the Mortality of Sepsis Patients

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Abstract

Objective. This study aimed to determine the role of vitamin D-binding protein (VDBP) gene polymorphisms (especially at locus rs7041), vitamin D-binding protein levels, and vitamin D levels in mortality in sepsis patients. **Patients and Methods.** We performed the analytic observational study with a case-control approach. A total of 80 patients were included in this study, 40 patients were grouped as the case group and 40 patients were grouped as the control group. The patients were diagnosed with sepsis and treated in the Intensive Care Unit (ICU), M. Djamil Hospital, Indonesia. The VDBP rs7041 gene polymorphism was analyzed using the polymerase chain reaction procedure. VDBP and vitamin D levels were examined using the enzyme-linked immunosorbent assay (ELISA) method. **Results.** The case group showed lower mean vitamin D and VDBP levels than the control group ($P<0.05$). There were more variations in the rs7041 gene VDBP (mutant) locus in the case group than in the control group, and this difference was considered statistically significant, $P<0.05$. The results of this study indicate that the occurrence of polymorphism or variations at locus rs7401 (mutant) causes a decrease in VDBP and vitamin D levels. A decrease in vitamin D levels correlates with the incidence of mortality in sepsis patients. **Conclusion.** Polymorphism gene VDBP at locus rs7041 causes a decrease in the production of VDBP, a vitamin D carrier protein.

Key Words: Genetic Polymorphisms ▪ Intensive Care Units ▪ Sepsis ▪ Vitamin D ▪ Vitamin D-Binding Protein.

Introduction

Sepsis is a severe medical condition when the body responds to infection by releasing an excessive inflammatory reaction (1). This situation can cause organ damage, organ failure, and even mortality in patients (2). Sepsis is a significant global health problem, with high mortality rates worldwide (3, 4). Although many factors can influence the development of sepsis and patient outcomes, recent studies have shown that genetic polymorphisms in the immune system and vitamin D metabolism can contribute to the variability of patient response to sepsis (5, 6).

Vitamin D plays a role in regulating the immune system (7). Adequate levels of vitamin D help maintain a balance between inflammatory and anti-inflammatory responses. In septic patients, an exaggerated inflammatory response can lead to organ damage (8). Vitamin D is essential in protecting these organs and minimizing the damage caused by sepsis (9). Vitamin D deficiency or changes in vitamin D metabolism can affect the body's ability to fight infections associated with sepsis.

Vitamin D-binding protein (VDBP) is a protein produced by the liver and serves as the primary "vehicle" for transporting vitamin D in blood

circulation. Most of the vitamin D produced by the skin in response to sun exposure (vitamin D3) or taken from food (vitamin D2 or D3) must be bound to VDBP to circulate in the blood (7). This is a critical step in delivering vitamin D throughout the body. VDBP also has a role in protecting vitamin D from the degradation that can occur in the blood. This helps ensure that vitamin D remains active and ready for use by various cells in the body. Once bound to VDBP, vitamin D can easily be distributed to various cells and tissues in the body.

One gene that has caught the attention of researchers is the gene that encodes the vitamin D binding protein (VDBP). The genetic polymorphism at the rs7041 locus of the VDBP gene has been the subject of intense research because it is associated with variations in VDBP levels and blood levels of vitamin D (10, 11). VDBP, also known as Gc-globulin, plays a role in the transport of vitamin D in the blood, and can also have critical immunomodulatory effects (12). Vitamin D levels, which can also be affected by the rs7041 polymorphism, are essential in regulating the immune system and response to infection (11). Genetic polymorphisms are variations in the DNA sequence that can affect how specific genes function (13). Locus rs7041 is a VDBP gene locus with genetic variations that can affect VDBP expression and function (13). VDBP is a protein in the blood that binds to circulating vitamin D. Variations in the rs7041 locus can affect blood levels of VDBP. Some variants of rs7041 are associated with lower or higher blood levels of VDBP (14). VDBP levels strongly influence vitamin D levels in the blood (15). VDBP helps transport vitamin D from the skin and intestines throughout the body (12). Variations in VDBP levels can affect how efficiently vitamin D is distributed to the cells (16). Recent studies have shown that differences in the rs7041 polymorphism, VDBP levels, and vitamin D levels can affect an individual's susceptibility to sepsis, and the outcome (17, 18). These factors can affect the inflammatory response, the body's ability to overcome infection, and potential complications that may occur during sepsis.

This study aims to determine the role of the vitamin D binding protein polymorphism gene, vitamin D binding protein (VDBP) levels, and vitamin D levels in relation to mortality in sepsis patients.

Methods

Study Design and Participants

This study is an analytic observational study with a case-control approach. A total of 80 patients were included in this study, where the research subjects consisted of 40 patients grouped into the case group and 40 patients grouped into the control group. The patients were diagnosed as having sepsis and treated in the Intensive Care Unit (ICU), M. Djamil Hospital, Padang, Indonesia, from July to September 2022. The sampling process was carried out using consecutive sampling until the number of samples for the case and control groups was fulfilled. The inclusion criteria for the case group were sepsis patients aged 18-60 years who died in ICU care within 30 days of hospitalization, and who had complete medical record data, while the criteria for the control group were sepsis patients caused by bacterial infection, aged 18-60 years who did not die in ICU care and had complete medical record data. Sepsis diagnosis was based on Acute Physiology Age Chronic Health Evaluation (APACHE)-II and Sequential Organ Failure Assessment (SOFA) scores. Age and gender were matched in this study. Patients who received vitamin D supplementation and had viral, parasite, or fungal infections were excluded from this study. This study included observations on sociodemographic data as well as the clinical and laboratory test results of the patients, including routine blood laboratory tests and blood chemistry.

Gene Polymorphism Analysis

The VDBP rs7041 gene polymorphism was analyzed using the following procedure: genomic DNA was isolated from peripheral blood taken from

patients using a Vivantis Technologies, GF-1, and Blood DNA Extraction Kit, Malaysia, as instructed by the manufacturer. The amplification of 482 bp PCR rs7041 at the VDBP gene was accomplished using the following pairs of primers: F. Primer (5'AAATAATGAGCAAATGAAAGAAGAC3') R. Primer (5'CAATAACAGGAAAGAAATGAGTAG A3'). PCR reactions contained 10 µM of each primer, 12.5 µl of OnePCR™ Mix (2X) (GeneDirex Inc., Seoul, South Korea), 6.5 µl of nuclease-free water, and 3 µl of genomic DNA. A nuclease-free water test was run with each PCR experiment for contamination detection (negative control). PCR reactions were done using the Eppendorf Master Cycler. The amplified products were electrophoresed in 2% agarose gel containing ethidium bromide, visualized, and photographed using a UV transilluminator (Olympus, Tokyo, Japan). Each 482 bp PCR fragment was digested twice for all cases and controls using HaeIII restriction enzyme (Thermo Scientific, 2000 U) to determine genotypes of the c.1296 T > G variant (rs7041) according to the manufacturer's instructions. Fragments were analyzed by 2% agarose gel electrophoresis using a 100 bp DNA Ladder (Thermo Scientific, GeneRuler).

VDBP and Vitamin D Level Evaluation

VDBP and vitamin D levels were examined using the ELISA (Enzyme-Linked Immunosorbent Assay) method, using an ELISA kit (CloudClone®, Hangzhou, China) according to the manufacturer's instructions. According to the manufacturer's protocols (CloudClone®, Hangzhou, China), 50 µl of standard diluent or serum samples were added to the well and incubated at 37°C for 30 minutes. After the plates were washed, 100 µl of the biotinylated antibody solution was added and set for 30 minutes at 37°C. After washing three times, 50 ul avidin-peroxidase complex solution was added and incubated for 15 minutes at 37°C. After washing, 50 µl of tetramethylbenzidine color solution was added and set in the dark for 15 minutes at 37°C. Finally, a 50 ul stop solution was added to stop the reaction. The absorbance was measured at 450 nm using an ELISA reader (Epoch, Biotek, Winooski, VT, United States).

Ethical Approval

This study received approval from the medical and health research ethics committee of M. Djamil Hospital, Padang, Indonesia (LB.02.02/5/7/385/2022). In addition, participants, or their legal guardians, were informed about the study's objectives, and they provided informed consent to participate.

Statistical Analysis

Univariate and bivariate data analysis was performed using SPSS software version 25 (IBM, Jakarta, Indonesia). Univariate analysis was performed to present the distribution of data frequencies for each test variable. Meanwhile, bivariate analysis was carried out to determine the relationship between the test variables, where $P<0.05$.

Results

The basic clinical characteristics of research subject are summarized in Table 1. There was no statistically significant difference between the case and control groups in the variables age, gender, and body mass index (BMI), $P>0.05$. There was no difference between the case and control groups in the variables of past medical history (diabetes mellitus, chronic kidney injury, cardiovascular, and chronic obstructive pulmonary disease (COPD)), $P>0.05$. The study results showed no differences in the Acute Physiology Age Chronic Health Evaluation (APACHE)-II and Sequential Organ Failure Assessment (SOFA) scores between the case and control groups, $P>0.05$. Table 1 shows no difference between the case and control groups in the laboratory test results, $P>0.05$. The absence of statistically significant differences between the case and control groups in relation to demographic variables, medical history, and laboratory evaluation indicated that patients in the case and control groups were in equal and matching conditions.

Table 1. Basic Clinical Characteristics of Patients

Characteristics	Group		P-value
	Case, N (%)	Control, N (%)	
Age (years), Mean±SD	52.6±4.9	52.1±4.6	0.132*
Gender			
Male	15 (37.5)	15 (37.5)	1.000†
Female	25 (62.5)	25 (62.5)	
Body mass index			
Underweight	4 (10)	3 (7.5)	0.544†
Normoweight	14 (35)	15 (37.5)	
Overweight	22 (55)	22 (55)	
Diabetes mellitus	5 (12.5)	4 (10)	0.575†
Chronic kidney injury	4 (10)	5 (12.5)	0.537†
Cardiovascular disease	5 (12.5)	4 (10)	0.524†
COPD	3 (7.5)	3 (7.5)	1.000†
APACHE II Score, Mean±SD	21.3±1.4	21.8±1.6	0.622*
SOFA score, Mean±SD	7.6±0.7	7.1±0.8	0.562*
Hemoglobin, g/dL	10.24±1.4	10.64±1.6	0.122*
Leukocytes, $x10^3/mm^3$	14.11±1.9	13.96±1.8	0.132*
Platelets, $x10^3/mm^3$	208.9±16.2	208.2±17.7	0.089*
Procalcitonin, ng/mL	15.88±2.7	13.43±3.3	0.082*
Lactate, mmol/L	2.8±0.7	2.4±1.6	0.113*
Albumin, g/dL	2.69 ± 0.84	2.72±0.81	0.138*
Vitamin D, ng/mL	16.12±1.5	24.68±,97	0.002*
VDBP, ug/mL	161.29±14.12	224.36±17.98	0.003*
Variant locus rs7041			
Mutant (G/G)	28 (70)	14 (35)	0.011†
Wild type (T/T or T/G)	12 (30)	26 (65)	

*Independent t test; †Chi square test; APACHE II=Acute physiology age chronic health evaluation II; COPD=Chronic obstructive pulmonary disease; SD=Standard deviation; SOFA=Sequential organ failure assessment; VDBP=Vitamin D binding protein.

Table 1 also shows vitamin D levels, vitamin D binding protein (VDBP) levels, and variant locus rs7041. The case group showed lower mean vitamin D and VDBP levels than the control group, and they differed significantly statistically, P<0.05. The case group indicated a condition of vitamin D deficiency (16.12+1.5 ng/ml), whereas the control group exhibited a state of vitamin D insufficiency (24.68+1.97 ng/ml), when compared to the standard reference value for normal vitamin D levels in blood (30 ng/ml).There were more variations in the rs7041 gene VDBP (mutant) locus in the case group than in the control group, and this difference was also considered statistically significant, P<0.05. The study showed that in patients who died, there was polymorphism or variation at locus rs7401, and they had lower VDBP and vitamin D levels compared to patients who did not die.

A correlation test was performed to explore the correlation between the test variables, and this is presented in Table 2. Table 2 shows the very strong and statistically significant correlation between vitamin D and mortality. Strong correlations were also found between the mortality variables and VDBP levels. Moderate correlations were found between the mortality variables

Table 2. Correlation between Test Variables

Variables		Mortality	Vitamin D	VDBP	Variant rs7041
Mortality	r	-	-0.921	-0.911	0.490
	p value	-	0.000	0.000	0.001
Vitamin D	r	-0.921	-	0.971	-0.653
	p value	0.000	-	0.000	0.000
VDBP	r	-0.911	0.971	-	-0.891
	p value	0,000	0.000	-	0.000
Variant rs7041	r	0.490	-0.653	-0.891	-
	p value	0.001	0.000	0.000	-

*Pearson correlation-test; VDBP=Vitamin D binding protein.

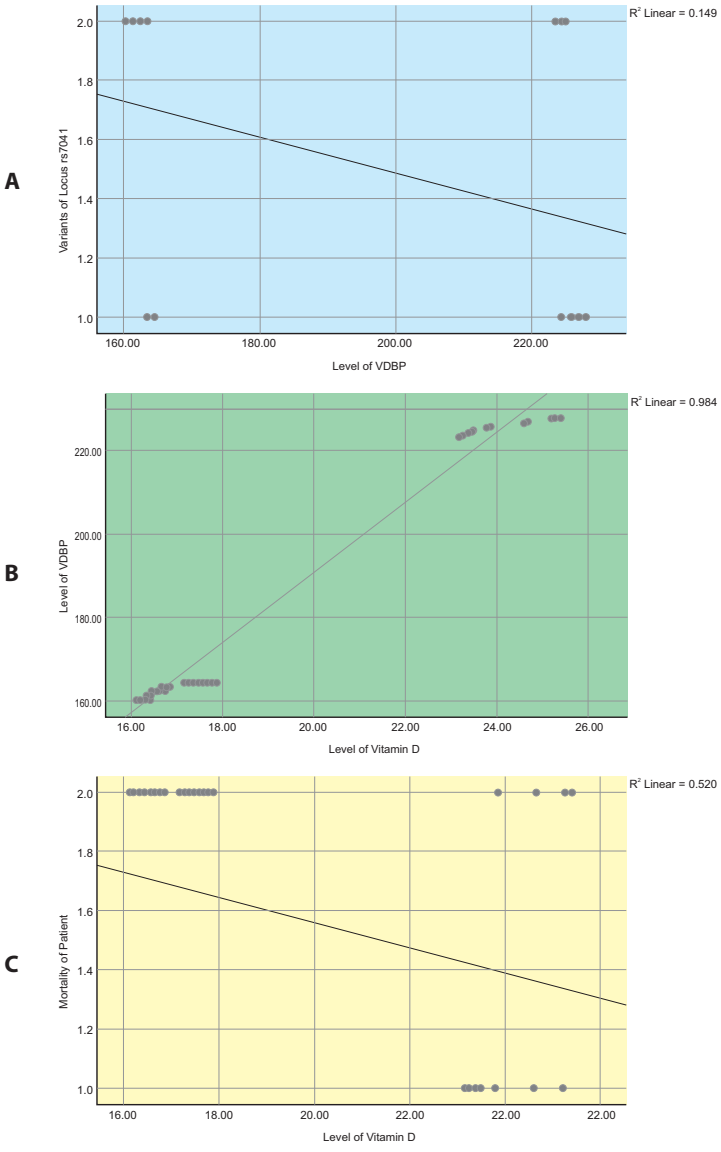

Figure 1. The graphs of correlation among the variable tests; (A) Graph of correlation between variant locus rs7041 and VDBP level; (B) Graph of correlation between VDBP level and vitamin D level; (C) Graph of correlation between vitamin D level and mortality.

and polymorphism or variations at locus rs7401. Polymorphism or variations at locus rs7401 had a strong and statistically significant correlation with VDBP levels. VDBP levels correlated strongly and statistically significantly with vitamin D levels. The correlation between polymorphism or variations at locus rs7401, VDBP levels, and vitamin D levels can be seen in Figure 1. The results of this study indicate that the occurrence of polymorphism or variations at locus rs7401 (mutant- G/G) causes a decrease in VDBP levels. In contrast, a decrease in VDBP protein also caused a decrease in vitamin D levels. A decrease in vitamin D levels correlated with the incidence of mortality in sepsis patients.

Discussion

This study shows that in sepsis patients who died, polymorphism or variation at the locus rs7401 of the VDBP gene was more dominant than in septic patients who did not die. VDBP protein levels and vitamin D levels also showed a decrease in septic patients who died compared to septic patients who did not die. Further studies were carried out to reconstruct the role of polymorphism or variations at the rs7401 locus of the VDBP gene, VDBP protein, and vitamin D in the incidence of mortality in sepsis patients. The correlation test showed that polymorphism or variations in the rs7401 gene VDBP locus caused a decrease in VDBP protein levels. Furthermore, decreased VDBP levels led to decreased vitamin D levels, which correlated with mortality in septic patients.

The rs7041 genetic polymorphism is a genetic variation in the human vitamin D binding protein (VDBP) gene (13). This gene encodes a protein essential for transporting vitamin D in the blood. There are two main variants of rs7041, namely T and G. Several studies have shown that individuals with the rs7041 G/G variant tend to have lower levels of vitamin D in the blood compared to individuals with the T/T or T/G variants (14, 19). The G/G variant is associated with the reduced capacity of VDBP to bind to vitamin D, so vitamin D may not be transported efficiently in the blood. Epidemiological studies have shown that individuals with the rs7041 G/G variant have a higher risk of developing vitamin D deficiency (20, 21). Vitamin D deficiency can contribute to several health problems, including an increased risk of bone diseases such as osteoporosis. Studies have also shown that individuals with different rs7041 polymorphisms may respond differently to vitamin D supplements (22, 23). G/G variants may require higher doses of vitamin D supplements to achieve the same levels as individuals with T/T or T/G variants (24).

A previous study stated that vitamin D can influence the activity of T cells, which are essential components of the adaptive immune system (8). In particular, vitamin D can help regulate the balance between regulatory T cells (Tregs) that inhibit inflammation, and cytotoxic T cells that trigger inflammatory responses (25, 26). It plays a role in controlling excessive inflammation, as occurs in sepsis. In particular, vitamin D has been associated with an increased number and activity of Tregs (27). This can result in a reduction in inflammation as Tregs inhibit the over activity of cytotoxic T cells (28).

One of the main mechanisms by which vitamin D reduces inflammation is by inhibiting the production of pro-inflammatory cytokines, such as interleukin-6 (IL-6) and tumor necrosis factor-α (TNF-α) (29, 30). It is a critical component in controlling the inflammatory response in the body. IL-6 is one of the main cytokines that trigger inflammation in the body. High levels of IL-6 may be associated with various inflammatory diseases, such as rheumatoid arthritis, heart disease, and autoimmune diseases (29). Vitamin D can inhibit the production of IL-6 by immune system cells. TNF-alpha is a cytokine that also plays a role in inflammation and can contribute to various chronic inflammatory diseases, such as Crohn's disease and psoriasis. Vitamin D has been shown to reduce TNF-alpha production in inflammatory responses. Macrophages are phagocytic cells that play an essential role in inflammation by producing large amounts of pro-inflammatory cytokines. Vitamin D can regulate the activity of macrophage cells and reduce the production of

pro-inflammatory cytokines by these cells (31). By inhibiting the production of pro-inflammatory cytokines, vitamin D helps maintain the proper balance between the inflammation that is needed to fight infection and excess inflammation that can damage body tissues (32). It is a crucial element in keeping the body healthy and preventing the development of chronic inflammatory diseases (32).

Prior studies supported the findings of our investigation. Shojaei et al. found a significant correlation between decreased blood vitamin D levels and mortality in individuals with sepsis (33). Multiple studies have also indicated that administering vitamin D supplements to individuals with sepsis might diminish the severity and enhance the prognosis of sepsis cases. A retrospective study by Guan et al., showed that vitamin D supplementation resulted in a lower risk of sepsis and a lower risk of mechanical ventilation requirement (34). Other studies by Bayat et al., and Rech et al., found an association between vitamin D and sepsis severity (35, 36). Furthermore, Rech et al., reported an increased risk of 30-day mortality for sepsis patients with vitamin D deficiency (36).

This study provides a pathway for connecting the rs7041 gene polymorphism, VDBP levels, vitamin D levels, and their correlation with mortality in sepsis patients. The limitation of this study is that it was conducted in a single center. In order to obtain results that may be applied to a wider population, it is necessary to conduct research involving multiple centers and greater sample sizes.

Conclusion

Polymorphism of the VDBP gene at locus rs7041 causes a decrease in the production of VDBP protein, a vitamin D carrier protein. This causes a decrease in vitamin D levels and plays a role in the incidence of mortality in sepsis patients. In future, more studies with larger samples and multiple centres in this field of expertise could yield useful data.

What Is Already Known on This Topic:

Vitamin D-binding protein is a protein produced by the liver essential for transporting vitamin D in the blood circulation. Vitamin D levels, which can also be affected by rs7041 polymorphism, are essential in regulating the immune system and response to infection.

What This Study Adds:

Our study reveals the vital role of vitamin D binding protein related gene polymorphisms (locus rs7041) in the mortality of sepsis patients. The correlation test emphasizes a pathway in the pathophysiology of patient death due to sepsis, namely a gene polymorphism at locus rs7041 that causes a reduction in VDBP; low VDBP will reduce vitamin D levels. Furthermore, a decrease in vitamin D will affect the immune system, which is necessary for the survival and recovery of sepsis patients.

Authors' Contributions: Conception and design: LAK, YY and TM; Acquisition, analysis and interpretation of data: LAK and JJ; Drafting the article: KA and WMS; Revising it critically for important intellectual content: LAK, YY and JJ; Approved final version of the manuscript: LAK, YY, JJ, TM, KA and WMS.

Conflict of Interest: The authors declare that they have no conflict of interest.

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The Hyoid Bone - the Anatomy of a Small Bone of the Neck in Hellenic and Greco-Roman Medical Literature

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Abstract

Objectives. To understand the nomenclature of the hyoid bone. **Materials and Methods.** Hyoid, a small bone of the neck, is a bony part that is rather difficult to unearth and discover among skeletal remains. **Results.** The named was coined by the ancient Greeks, along with its anatomic description. Galen (2nd – 3rd c. AD) and Theophilus Protospatharius (7th century AD), facing religious and social barriers, succeeded in presenting its anatomy and suggesting its probable function in speech and swallowing, regarding the bone as a muscle pillar of the neck area. **Conclusion.** Authorities of Hellenic antiquity surprise us with their accuracy and the resilience of their anatomical descriptions.

Key Words: Hyoid Bone ▪ Galen ▪ Theophilus Protospatharius.

Introduction

The hyoid bone is found in the superior anterior region of the neck (Figures 1 and 2). It is characteristic of this bone that it is one of the few that have no contact with any other bone, but is held in position by muscles alone. It has a role as a supportive structure for the tongue and the various muscles around it. It may have various shapes, but it is mainly U-shaped or receives a similar one. In very rare cases it is not present or missing one of the greater or lesser of its horns. Sometime it is situated higher or lower than its usual anatomical area (1). During the 19th century it was regarded as the border distinguishing the upper and lower parts of the neck area (2). This small bone has been situated above the larynx since the era of the Neanderthals, but specimens are very hard to find in human skeleton remains (3). Since the Hellenic antiquity the hyoid bone has been well known as a point used to produce severe pain in opponents in the martial arts (4). However, it was scarely described in the literature on human anatomy, as the

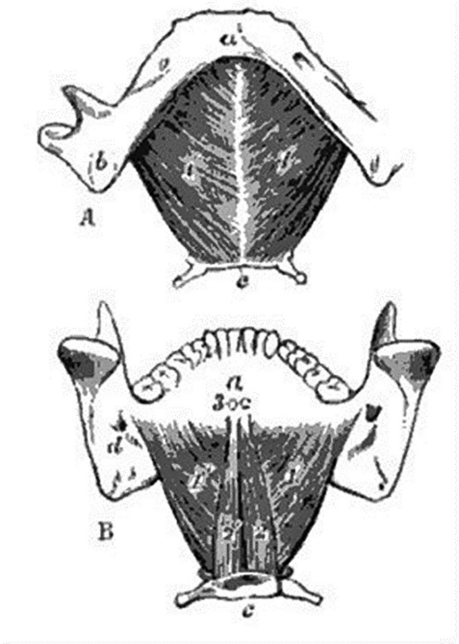

Figure 1. A. The lower jaw and hyoid bone (c) from below, with the mylo-hyoid muscles attached; B. The same with the genio-hyoid muscles attached. Figure by Allen Thomson in Jones Quain's Elements of Anatomy, Longmans, Green, and Company, London, 1894.

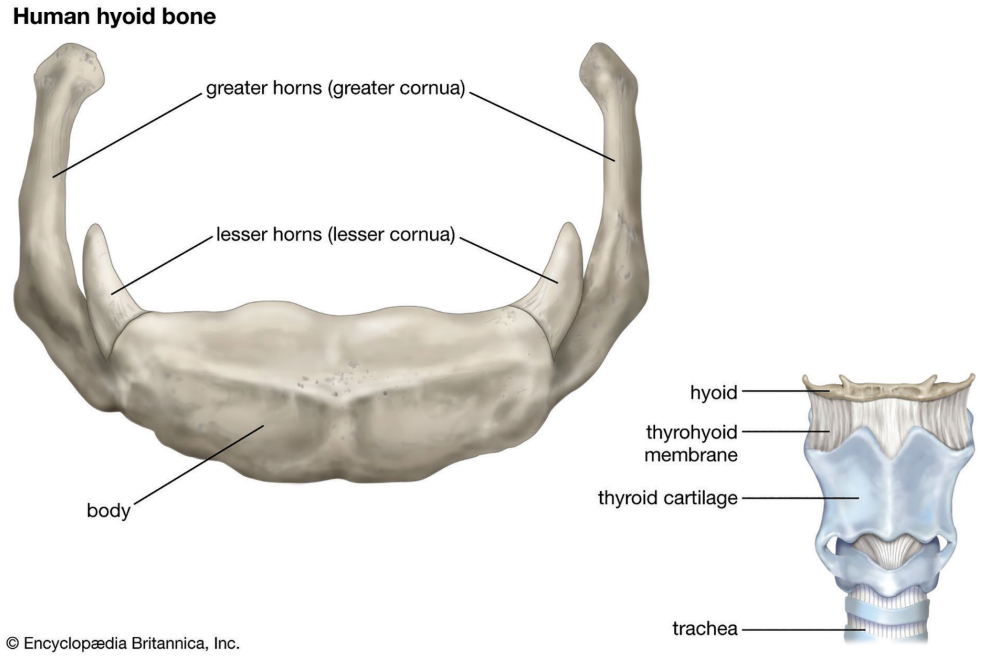

Figure 2. Hyoid bone. From Encyclopaedia Britannica 2023

body was considered sacred due to social and religious issues (5). The opportunity to observe it directly was rare, usually resulting from battle injuries, fight wounds (as in gladiator games) or during surgical operations on the area (6). This historical review surveys a time period of 7 centuries, from 100 BC to 800 AD, a time spam including the Late Hellenistic period and the Late Eastern Roman Empire (Byzantium).

The research aims to unveil data concerning the hyoid bone and describe its anatomy, while simultaneously recording the opinions of various scholars of the eras in question.

Materials and Methods

Our material was derived from the data base Thesaurus Lingua Graeca and was focused on ancient Greek and Byzantine medical literature.

The Greeks

The anatomical examination of the hyoid bone began very early in the history of medicine. Ancient Greek physicians described this anatomic entity of the human body in numerous ancient Greek medical texts, and coined its name. As we may observe, in the work of Rufus of Ephesus (1st – 2nd c. AD), the names of the human anatomical parts are listed and categorized (7). The ancient Greek anatomist informs us that this bone received two different names. The first term used by the majority of the ancient physicians was hyoid, due to the fact that its shape resembled the Hellenic letter "Y" of the ancient Greek alphabet. The second term, as Rufus informs us, was mentioned by Herophilus of Chalcedon (335–280 BC), who named it "παραστάτης" (Greek: 'assistant') due to its position close to the tonsils (Rufus Med. De corporis humani appellationibus 155.1-156.1) (8).

Apart from these terms, in ancient Hellenic medical literature this bone was also given the name "Λαμβδοειδής" (Greek: lambda bone, L-shaped), under the belief that it resembled the Greek letter "Λ" (that is 'L' in Greek) as an eversion of the letter "Υ". In some cases, the term "Υψυλοειδής" (Greek: hypsiloid) was used, as an expanded type of the term "hyoid" (9).

The hyoid bone was of special interest in clinical medicine among ancient Greek physicians. Galen (2nd – 3rd c. AD), the authoritarian anatomist of the Greeks, wrote that the lower part of this bone, reaching the sternum, did not receive any nerves from the VIth cranial nerve, as Galen defines it, that is the glossopharyngeal, vagus, and spinal accessory combined as a single nerve, due to their joint exit through a common foramen (Galen, De nervorum dissectione 2.841.5-8) (10). Meanwhile, he underlined its significance, noting, that despite its small dimensions, a cluster of muscles originates beside it, extending in different directions. These are: all the muscles that extend towards the tongue, the small ones towards the larynx, the long and the narrow ones towards the shoulders, the robust one towards the sternum, and the other oblique ones towards the area of the mentum (Galen, De usu partium 3.591.10-19) (11).

The Byzantines

Although Galen is considered to be a great anatomist, the most detailed presentation of the hyoid bone, shedding light on its anatomical characteristics, is found in the work of the Byzantine physician, Theophilus Protospatharius (7th century AD). This prolific Byzantine physician, in his work on the anatomy of the human body (12), summarized all the ancient anatomical knowledge on the subject, pointing out its topographical area in the neck, its relationship to the nearby musculature, its nomenclature, and finally its clinical role. The actions attributed to the hyoid were emphasized, indicating the fact that all the muscles were linked to the bones which open and close the larynx, as well as the locomotor muscles of the tongue. However, his most important observation was the first understanding of the substance of this bone, writing that it is sometimes as hard as a regular bone, but also softer than cartilage. He was also the first to introduce some data on its shape, noting that each side of the bone (horn) is thinner than the thick middle area, adding that each side is linked to the other by an hymenoid ligament (Theoph. Protospatharius, De corporis humani fabrica libri quinque 3.21.1-21) (13).

Results

The key role concerning the basihyal (body of the hyoid) was found in the fact that many different muscles of the area received part of their name from the hyoid bone. Thus, the terms Sterno-, Omo- Stylo-, Thyreo-, Mylo-, and Genio-hyoideus were introduced to the Latin anatomical nomenclature in the Renaissance and have remained until the present day (14).

Discussion

Modern embryology and gross anatomy testify that the hyoid bone ossifies from six centers. These are the basihyals from which the lateral parts of the body grow, the thyrohyals from which the two centers of the greater horns grow, and the ceratohyals from which the lesser horns grow. It can also be absent, and is sometimes positioned in an area more inferior than its usual position. Moreover, the lesser horns may articulate with the greater ones, while they may be unilaterally or bilaterally absent. Greek and Greco-Roman physicians did not have the opportunity for thorough anatomical examinations of the human body for religious reasons, and the prohibitions of philosophical and spiritual principals. These difficulties rendered accurate description impossible. The more precise delineation of the bone was conducted by Andreas Vesalius. Physicians of the Hellenic antiquity connected the hyoid bone with functions such as speech and swallowing, naming it as a part involved in the movements of the neck (15).

Conclusion

The hyoid bone was observed by the Greeks and was included in all their anatomical works. Books from the late 18th century contain detailed references to the bone, based on Greco-Roman knowledge, which influenced Arab medicine and its

concept of anatomy, while Chinese anatomical ideas did not differ from the ancient Greek approach to the theme (16). Its name derives from its origin in ancient Hellenic medico-philosophy. Its role was accurately described in Greco-Roman medical literature, and entered Western Medicine during the Renaissance.

What Is Already Known on This Topic:
The roots of the name are found in ancient Greek medical literature.

What This Study Adds:
This study presents and discusses the development of the nomenclature and the study of this special bone and how the term has survived until today, highlighting the historical backround.

Authors' Contributions: Conception and design: MR and GD; Acquisition, analysis and interpretation of data: MR, GD and PG; Drafting the article: MR, GD and EM; Revising it critically for important intellectual content: EM; Approved final version of the manuscript: MR, GD, PG and EM.

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Toxic Cardiomyopathy in a Young Patient Treated for Her2-Positive Early Breast Cancer: Case Report and Literature Review

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Abstract

Objective. We present the case of toxic cardiomyopathy in a healthy thirty-eight-year-old female patient treated for Her2-positive early breast cancer. **Case Report.** During the neoadjuvant treatment, the patient received four cycles of AC regimen and four cycles of docetaxel in combination with trastuzumab biosimilar. Two days after she received the ninth dose of trastuzumab biosimilar, she reported feebleness, palpitation, and dyspnoea. A heart ultrasound was performed and was normal without changes in the ejection fraction (EF) compared to previous checks. Three days later she reports worsening of her symptoms that were highly suggestive of heart failure. A cardiologist was consulted who insisted that the patient's symptoms were the consequence of the disease progression. A CT scan showed signs of heart failure. A heart ultrasound was done and the EF dropped to 30%. Drainage of the right pleural cavity was performed and pharmacotherapy for heart failure was started. The treatment led to clinical improvement, but eighteen months later EF is still not back to normal. **Conclusion.** This is a rare case of toxic cardiomyopathy in a young, previously healthy, patient who received anthracyclines followed by trastuzumab biosimilar in combination with taxanes. All the medications this patient received are potentially cardiotoxic. However, the overall presentation is not typical for any of these medications since the patient presented with symptoms and signs of heart failure with significant dilatation of the right atrium, which persists eighteen months after its onset, with only a small increase in the EF. There is also a possibility that the antineoplastic therapy the patient received only facilitated dilatative cardiomyopathy, while the main causative factor was intrinsic or extrinsic.

Key Words: Breast Cancer ▪ Anthracyclines ▪ Trastuzumab ▪ Toxic Cardiomyopathy.

Introduction

Breast cancer has the highest incidence and prevalence among malignant diseases (1). Its rising prevalence can be attributed to better screening methods and the development of innovative therapeutic options that are available for all types of breast cancer. Consequently, the absolute number of patients that experience adverse events (AE) of antineoplastic therapy is increased (2). Most AE are easily controlled and the prevalence of serious AE is relatively low, but they can significantly impact the clinical outcomes. Cardiotoxicity is common in breast cancer patients. Patients treated for breast cancer are more susceptible to heart failure compared to the general population (3) but there is evidence that the incidence of heart failure is higher in patients older than 65 and those with previous cardiovascular disease (CVD) (4).

The objective of this article is to present a rare case of cardiotoxic cardiomyopathy with atypical clinical features in a previously healthy young patient.

Case Presentation

We report the case of a patient who was diagnosed with breast cancer at the age of thirty-eight. She had no comorbidities. In the previous six years, she had been regularly examined by a radiologist

because of positive family history of breast cancer and fibrocystic dysplasia. She underwent an ultrasound examination every four to six months. On several occasions, a fine needle aspiration biopsy (FNAB) was performed and cytological findings were always benign.

In July 2020, the breast ultrasound was without any change in comparison to the previous, but there was a new lymph node in the right axillar region, which was of malignant ultrasound characteristics. The FNAB was performed and malignant cells were found. The CT of the neck, thorax, and abdomen was normal, except for the multicentric expansive lesion of the right breast with a maximal diameter of 10 mm and a pathologic lymph node in the right axillary region. This expansive lesion was previously verified at the ultrasound examination as a fibrous plaque with stationary dimensions of 11×4 mm. A CORE biopsy was performed and moderately differentiated, ER, PgR, and Her2 positive invasive ductal carcinoma was confirmed. Before the onset of the neoadjuvant treatment, heart ultrasound was normal with the ejection fraction (EF) of 60%.

The patient received neoadjuvant chemotherapy, four cycles of the AC regimen (doxorubicin – 60 mg/m^2 and cyclophosphamide – 600 mg/m^2) followed by four cycles of docetaxel (75 mg/m^2) with trastuzumab biosimilar (loading dose of 8 mg/kg, then 6 mg/kg q3w). The dose of medications was reduced after two cycles of therapy according to the AC regimen because of prolonged grade four neutropenia. During the neoadjuvant treatment, in February 2021, the patient had a mild form of COVID-19. The patient experienced only mild rhinopharyngitis that lasted for less than five days. In April 2021, she had a right mastectomy and a prophylactic left mastectomy. A complete pathologic response (pCR) was confirmed. In the adjuvant setting, the continuation of trastuzumab biosimilar for up to one year was planned as well as hormonotherapy (GnRH agonist and tamoxifen) and adjuvant radiotherapy together with regular follow-up.

In July 2021, two days after the patient received the ninth dose of trastuzumab biosimilar, she was referred to an oncologist because of feebleness, palpitation, and dyspnoea. The oncologist suggested an examination by a cardiologist, so the heart ultrasound, which was normal with the EF of 60%, was performed. There was no change in comparison to the baseline ultrasound and ultrasounds that were performed every three months since the onset of systemic treatment for breast cancer. The chest X-ray (Figure 1) showed bilateral pulmonary infiltration with minor pleural effusions bilaterally. The patient was also examined by a pulmonologist that prescribed antibiotics. Three days later, the patient was again referred to an oncologist because of her worsening condition, this time with the obvious clinical signs of heart failure. Even though all symptoms and clinical signs suggested heart failure, in a patient receiving cardiotoxic therapy, the cardiologist insisted that it was impossible since the heart ultrasound had been normal three days before and that the patient's state must have been the consequence of disease progression. In order to prove that the disease progression was the least possible scenario, a CT of the thorax and abdomen

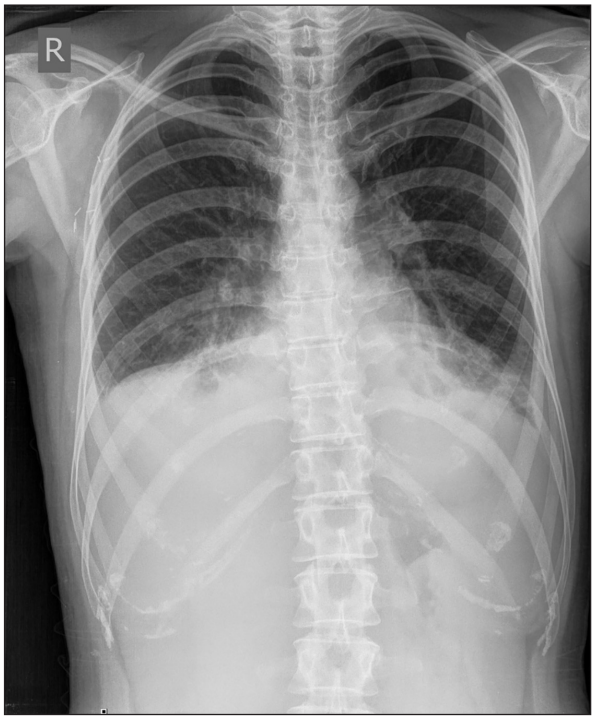

Figure 1. The chest X-ray that demonstrates bilateral pulmonary infiltration and minor pleural effusions.

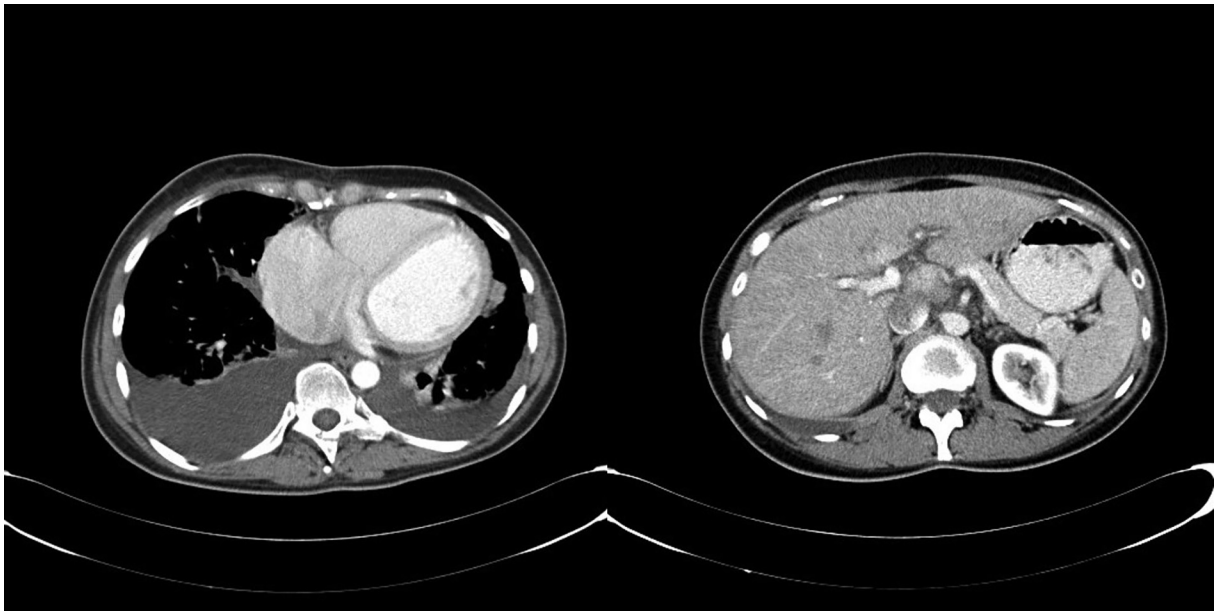

Figure 2. Enlarged heart, pleural effusions, and periportal oedema as signs of heart failure on CT of thorax and abdomen.

was performed (Figure 2). It showed an enlarged heart, especially the right atrium, with pleural effusion bilaterally, periportal edema, and ascites. There were no radiologic signs of dissemination of malignant disease.

In the lab findings, the liver enzymes were three times ULN, with hypoalbuminaemia and consequent hypocalcaemia. Heart biomarkers were normal. Since the D dimer was high (3 mg/mL), a CT angiography was performed and there were no signs of pulmonary thromboembolism. The thoracic drainage was done on the right. The heart ultrasound showed global heart dilatation with global hypokinesia and consequent dilatation of the inferior vena cava as well as the EF of 30%. The pharmacotherapy for heart failure was started with diuretics (furosemide and spironolactone), ACE inhibitor, beta-blocker (carvedilol), and low molecular weight heparin. Instead of tamoxifen, letrozole was prescribed with the continuation of GnRH agonists. The therapeutic response was achieved and after fifteen days the patient was discharged from hospital stable.

When she recovered, she received radiotherapy without any adverse events. Now, the patient is on hormonotherapy and since the EF did not go back to normal, the treatment with trastuzumab was discontinued. She has regular follow-ups, every four months. All her lab and radiological findings are normal, except for the heart ultrasound where the global heart dilatation is still present with the EF of 45%. The patient still tolerates activity poorly. The summary of important clinical events and heart ultrasound findings are shown in Figure 3 and Figure 4.

Sep-2020	Oct-2020 to Apr-2021	Apr-2021	Apr-2021 to Jul-2021	Jul-2021
– Stage II inasive ductal carcinoma (Er, PgR and Her2 positive)	– Neoadjuvant therapy – Mild form of COVID-19 in Feb-2021	– Surgery (pCR)	– Continuation of trastuzumab biosimilar – Adjuvant hormonotherapy from May-2021	– Syptomatic heart failure

Figure 3. The summary of important clinical events.

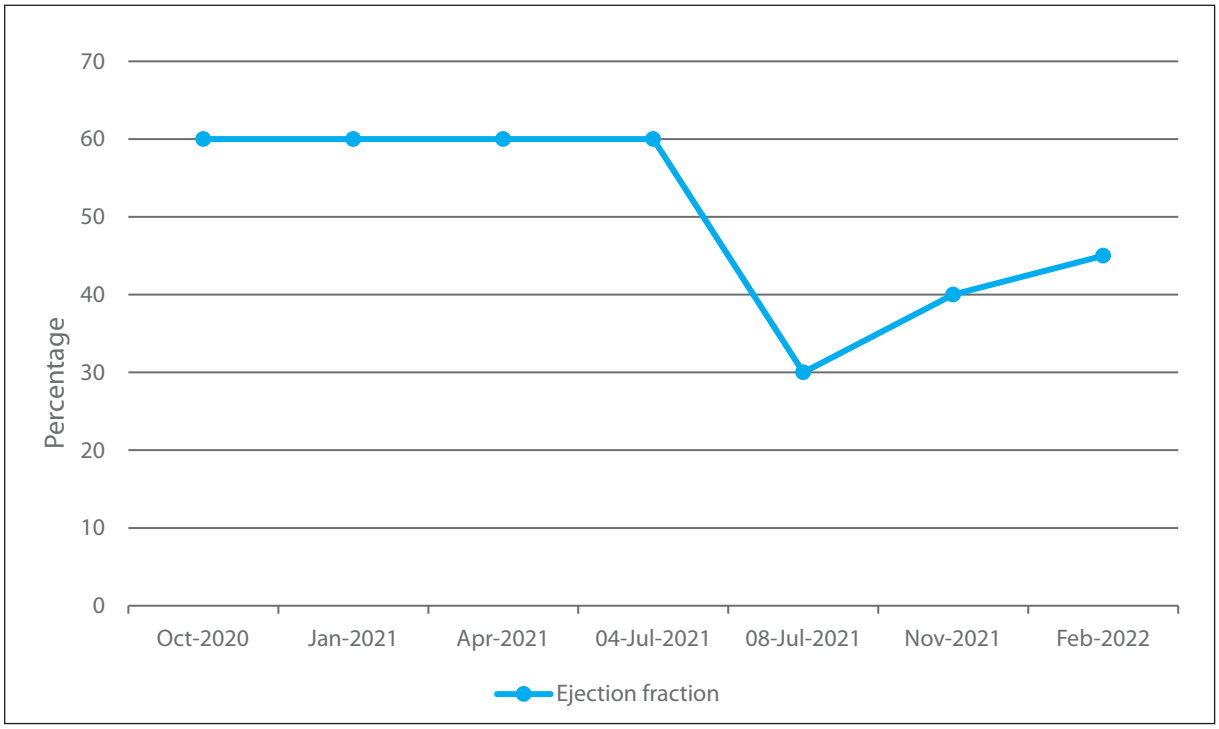

Figure 4. Patient's heart ultrasound findings from the beginning of her treatment until 2022.

Discussion

Cardiotoxicity of antineoplastic therapy led to the fast development of cardio-oncology, which is very important in the treatment of breast cancer, especially Her2-positive breast cancer. A study that included more than 63 000 patients with breast cancer, from 1992-1995, showed that CVDs are the leading cause of death in this population with 15.9%, while breast cancer is the second with 15.1% (5). The incidence of CVDs is higher in women with breast cancer, as well as the mortality caused by them, showed the study that included almost 82 000 patients with breast cancer with a median follow-up of seven years (3). The same study showed that patients treated for breast cancer have a higher risk of developing heart failure, especially patients treated with anthracyclines and trastuzumab with a hazard ratio (HR) of 3.68. HR is higher in patients treated with both anthracyclines and trastuzumab than in patients who only get anthracyclines, 2.53 vs. 1.84. Patients who received adjuvant radiotherapy for breast cancer, as well as those who are treated with aromatase inhibitors, are also at higher risk.

Clinical trials NeoSphere and CLEOPATRA confirmed that the addition of pertuzumab to trastuzumab does not change its safety profile regarding cardiotoxicity (6, 7). The safety profile of the clinical trial TRYPHAENA is comparable to those in NeoSphere and CLEOPATRA (8).

OHERA is an observational study that brought real-world data and showed that the incidence of symptomatic heart failure in patients treated with anti-Her2 therapy is 2.8% with a median onset of 5.7 months since the start of the treatment and complete recovery in 72.6% of patients (4). This study also showed that the incidence of heart failure is higher in patients older than 65 and those with previous CVD. We here have the patient, who is, unlike presented in the results above, young, without comorbidities, with normal heart ultrasound before the onset of the neoadjuvant therapy and EF that was stable during the treatment.

ESMO consensus recommends baseline ultrasound before the start of cardiotoxic treatment as

well as a check-up every three months and active surveillance for at least three years after the end of cardiotoxic treatment (9). Taking into account the fact that this case shows a young patient with no comorbidities, who had heart ultrasounds regularly during the treatment and had no deflection from the baseline EF, not even when the first symptoms occurred, a logical question to arise is whether there is another way to screen the patients with the higher risk of heart failure. The biomarkers, mostly hs-troponins, BNP, and NT pro-BNP, are tested, but ESMO consensus still has not found evidence strong enough to recommend their routine use.

Primary prevention could decrease the incidence of heart failure, which would be particularly important for patients, who, like the patient in this case, are not diagnosed with heart failure while still asymptomatic. Some studies test the cardioprotective effect of certain medications. OVERCOME is a study that showed that the combination of enalapril and carvedilol can decrease the incidence of systolic dysfunction of the left ventricle in patients treated with cardiotoxic chemotherapy (10). MATICORE 101 – Breast showed that bisoprolol decreases the incidence of left ventricle's EF decrease in patients treated with trastuzumab (11).

It is still an open question what led to the heart failure with significant dilatation of the right atrium, which persists eighteen months after its onset, with only a small increase in the EF, in such a young patient. Cardiotoxicity caused by anthracyclines and trastuzumab differs in a way that anthracyclines cause structural damage to cardiomyocytes with consequent cell death, while trastuzumab inhibits signal transmission during DNA reparation (12). Therefore, the cardiotoxicity of anthracyclines is dose-induced and irreversible, while the cardiotoxic effect of trastuzumab is not dose-induced and is usually reversible. One should not a priori omit the possibility that, in this case, dilatative cardiomyopathy is facilitated by the antineoplastic therapy, while the main causative factor was intrinsic (e.g., TTN gene mutation) or some other extrinsic factor (e.g., previous SARS-CoV-2 infection) that we were not able to foresee or prevent.

Conclusion

In conclusion, this is a rare case of a toxic cardiomyopathy in a previously healthy young patient who received anthracyclines followed by trastuzumab biosimilar in combination with taxanes for the treatment of early Her2-positive breast cancer. All the medications this patient received are potentially cardiotoxic. However, the overall presentation is not typical for any of these medications since the patient presented with symptoms and signs of heart failure with significant dilatation of the right atrium, which persists eighteen months after its onset, with only a small increase in the EF. There is also a possibility that the antineoplastic therapy the patient received only facilitated dilatative cardiomyopathy, while the main causative factor was intrinsic or extrinsic.

What Is Already Known on This Topic:
Both anthracyclines and trastuzumab are known to be cardiotoxic. The cardiotoxicity caused by anthracyclines is irreversible, while the cardiotoxicity caused by trastuzumab is reversible in more than 70% of the cases. The incidence of cardiotoxicity is higher in patients older than 65 years and those with CVD. There is evidence that supports the primary prevention of heart failure in patients receiving cardiotoxic therapy.

What This Study Adds:
Something that can be learned from this case is how fibrocystic and fibroadenomatous breast changes, even though they do not show a statistically significant correlation with the incidence of breast cancer, could be misleading during the diagnostic procedure, so the breast ultrasound, which is a diagnostic method of choice in young patients, could be insufficient and needs a correlation with other methods. Secondly, regular follow-up of heart function must not overshadow the importance of clinical signs and symptoms that patients report. Therefore, a medical oncologist should play a central role in the follow-up of cancer patients and always point out possible adverse events of antineoplastic therapy in order to increase the efficacy of collaboration with clinicians of other specialties. In this case, we can observe a bad habit of some clinicians to attribute all symptoms of cancer patients to disease progression. Finally, one should think about primary prevention even in young patients without comorbidities.

Authors' Contributions: Both AH and DK participated equally in every step of the preparation of this article for publication and both AH and DK approved the final version of the manuscript.

Conflict of Interest: The authors declare that they have no conflict of interest.

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Coccygeal Disc Disease as a Possible Cause of Coccygodynia

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Abstract

Objective. The aim of the present study was to describe the causes involved in the pathophysiology of coccydynia, emerging from the coccyx or the anatomical tailbone region. **Case Report.** We present the case of a 64-year-old man with pain in the coccyx and numbness in the perianal area. After clinical examination and imaging evaluation, including plain X-rays and magnetic resonance, coccygeal disc disease was identified. Other findings, such as tumor and fracture were excluded. We decided to undertake conservative management and the pain was eventually relieved. This is the first case report of coccygodynia and perianal numbness attributed to coccygeal disc disease. **Conclusion.** Although there is no standard treatment, coexisting coccygeal disc disease should be always taken into account, with clinical and imaging examinations being considered of major importance to establish both medical diagnosis and treatment.

Key Words: Coccyx ▪ Coccygodynia ▪ Perianal Numbness ▪ Coccygeal Disc Disease.

Introduction

The term coccydynia was initially used by Simpson (1) and later by Foye (2) to describe pain–discomfort localized around the bottom end of the spine, or the lowest (most inferior) site of low back pain, where it was noted that it is produced when the coccyx or the coccygeal joints have been injured, or after prolonged sitting, due to the compression of the surrounding tissues or the muscles attached to the coccyx (1, 2). Moreover, in certain cases it is expressed as pain in the general area of the sacral perianal, in the absence of lower back pain or radiation (3). Nevertheless, fewer than 1% of subjects also manifest lower back pain (4).

The specific pathophysiological mechanisms related to coccygodynia are still vague. However, the majority of cases are linked to recent lumbar spine surgery, epidural injections and rectal surgery, whilst some cases of coccygodynia are idiopathic, or most subjects have a preceding traumatic event, such as falling on their tailbone, or the roots of this problem may even stem from childbirth (5). Posttraumatic coccygeal instability might lead to hypermobility or subluxation of the coccyx with chronic inflammatory alterations that may further lead to sacrococcygeal joint arthritis (6). Microtrauma deriving from inadequate body positioning, such as prolonged periods of motorcycle or bicycle riding, may lead to a chronic sprain of the coccyx (7, 8). It has already been established that female subjects are five times more likely to suffer from chronic coccygodynia than male subjects. This higher risk has been associated with differences in the female anatomy, where the sacrum and coccyx lie more posterior than in male subjects, whilst the coccyx is longer in women (9). Other factors related to an increased risk for this medical condition include an increased body mass index, local tumors and sacrococcygeal joint fusion (10).

In the literature, coccygeal discopathy as a causal factor of coccygodynia has not been previously

noted. To the best of our knowledge, only a few cases have been reported with coccygeal herniation derived from trauma, arthritis, a tumor or after coccygectomy (6, 9, 10).

On the basis of a literature review (2, 3, 7, 8-10), we report a case of coccygodynia and perianal numbness in a healthy man without a previous history of trauma, with a coccygeal disc herniation,

Case Presentation

A 64-year-old, Caucasian male white-collar worker, without a history of trauma, was referred to our department in August 2020 with a complaint of coccygodynia and a verbal report of mild perianal numbness. He had experienced pain in this region over the previous 2 months, and it was typically aggravated by sitting or lying on his back, but was not associated with neurological symptoms. There was no history of difficulty in defecation or micturition. The patient's body mass index was 23 kg/m^2 (specifically, 178 cm in height and 73 kg in weight). His lumbar spine range of motion was normal, with mild pain, especially in end range active flexion and extension, without exacerbation by overpressure or repeated movements. No motor deficits were noted after testing the muscles of the lower extremity, Lasegue's and femoral stretch test were negative bilaterally, and deep tendon reflexes were negative and comparable bilaterally. The overlying skin was normal, however there was mild tenderness localized to the sacrococcygeal joint. The patient reported a medical past of hypertension and hyperlipidemia, both under medication. Besides degenerative changes at level L5-S1, no abnormalities of the coccyx could be identified on plain X-ray. Magnetic resonance imaging (MRI) of the coccyx was performed, which revealed a disc herniation (Figure 1).

Conservative treatment which included the use of oral non-steroidal anti-inflammatory drug (NSAID), (naproxen 500 mg) twice a day for a

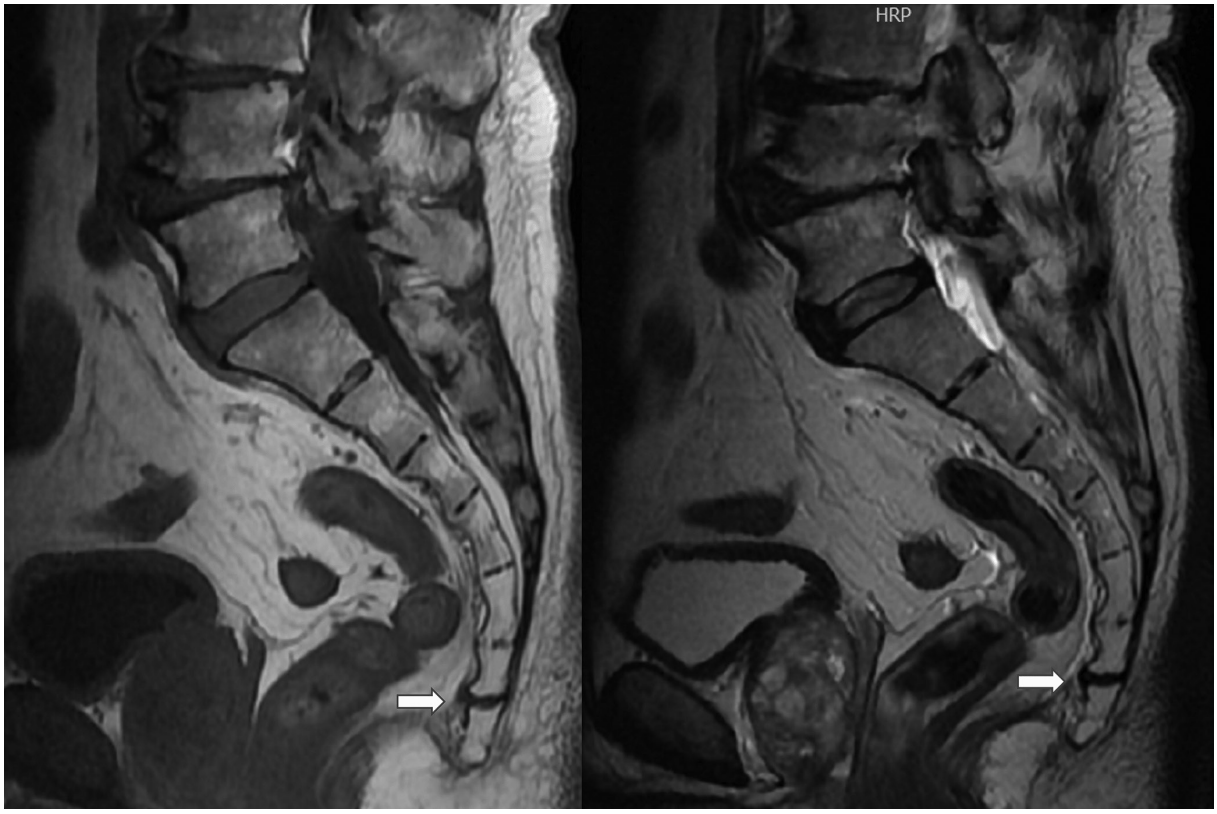

Figure 1. The magnetic resonance imaging in sagittal plane, revealing coccygeal disc disease.

period of 2 weeks was applied, along with seat cushions, and the avoidance of provoking activity, (avoidance of any positions or movements that might exacerbate his symptoms). The patient was also referred for physiotherapy, which included heat and cold application over the site, manual manipulation, massage of the levator ani muscle or the coccygeus muscle, and extracorporeal shockwave therapy, education focused on proper sitting posture, core stabilization, stretching and strengthening of the piriformis and iliopsoas muscles, thoracic mobilization, and pelvic floor muscle rehabilitation (diaphragmatic breathing, perineal bulges, intrarectal manual therapy). After two months of conservative management, with 3 visits per week, his symptoms were relieved and the patient was in good condition with no further symptoms at the follow-up date one year after his initial complaint.

Discussion

This case study presents coccygodynia in a white-collar male with normal BMI, without any history of previous trauma, and it emphasizes that coccygeal disc disease may be related to pain in the coccyx and perianal numbness. Coccygodynia is usually related to trauma, high BMI, sacral bone morphology, infection, posttraumatic arthritis, coccygeal mobility or tumors (7). It can also be associated with nonorganic causes, such as somatization disorder and other psychological disorders (11). Moreover, it can be referred pain from lumbar spine degeneration (7, 12), but in one-third of cases it is idiopathic (13). Coccygodynia may be related to long-term sitting and posture, and may be triggered by defecation, standing, and sexual intercourse. Additionally, it may be worse when rising from a seated position, and leaning backwards while seated. Another possible cause of coccygodynia may be an injury of the coccygeal plexus or its branches. It has already been well established that the coccygeal plexus is derived from the ventral rami nerve roots of the fourth and fifth sacral nerves, and the ventral rami of the first coccygeal nerve. The plexus is found within the ischiococcygeus muscle at the level of the first intercoccygeal joint (13). While there are numerous causes of pain in the coccyx area, a coccygeal disc herniation remains a rare entity. Interestingly, Maigne et al. suggested that common coccygeal pain might be discogenic, on the basis of findings of provocative discography. In their case series, fifteen out of twenty-one subjects had a positive result on provocative discography, whilst 25% of them had coccygeal luxation in the sitting position, which could be reduced in other positions (14). It seems that the presence of dynamic instability demonstrates that the cause of the pain is probably the result of degeneration, as it is also demonstrated in other regions of the spine. However, unlike lumbar discs, coccygeal discs are not known for developing osteophytes or syndesmophytes, due to the fact that they do not sustain compressive loads (14).

The diagnosis of this condition remains clinical, relying primarily on history and physical examination, and it should be investigated thoroughly. Any lower spine or rectal pathology must be recorded and examined appropriately by different specialists, such neurologists, neurosurgeons, orthopedic or colorectal surgeons. The pathophysiological pathway is yet to be established, and may be complex and multifactorial. Conservative treatment, such as rest, NSAIDs, pelvic floor physical therapy and utilizing seat cushions in the sitting position, is usually effective, and several studies have shown that this provides a successful outcome in approximately 90% of patients (3, 15, 16). Our report presents a possible case of coccygeal disc disease, causing pain and perianal numbness, that was treated successfully with conservative management.

Furthermore, imaging of the coccyx, and pinpointing the cause of the pain in the region is quite challenging, as the normal anatomy of the area is complex and can be anatomically variable. Knowledge of the morphological variants is a prerequisite for correct identification of the pathology (17).

Plain X-rays are usually unable to identify the etiology of the pain, but are very useful as a primary investigation tool to rule out or identify

fractures, and visualize the coccyx morphology. The most common cause of coccydynia is trauma, and X-rays are the first imaging modality used. When the radiographs suggest a fracture, a CT scan is recommended for definitive diagnosis.

Another difficulty in the diagnosis is assessing the mobility of the coccyx. This is best evaluated using dynamic X-rays (14, 17). A dynamic plain x-ray can visualize coccygeal mobility and can be very helpful in ruling out abnormal mobility. Hypermobility is defined as 25% or greater flexion while sitting compared to standing, where less than 5 degrees of motion is considered a rigid coccyx (15). Although comparison of sitting and standing films will show radiographic abnormalities in up to 70% of symptomatic coccygodynia cases, in everyday practice dynamic x-rays are not widely used due to the lack of a standardized technique and validated measurements (14).

Regarding MRI, special attention should be paid as many patients with coccygodynia are referred for MRI of the lumbar spine and the scan does not include the lower part of the sacrum or coccyx, thus missing the site of the pain. An area of T2 hyperintensity, with low T1 signal around the sacral or coccygean joint, is a common finding in patients with chronic coccydynia, and is indicative of Modic I changes. In our case, the disc was degenerative with concomitant herniation, and no actual Modic changes were noted.

Limitations of the Study

This study has some potential limitations. Firstly, our patient was unable to complete the MRI scan due to discomfort, and only sagittal views were available. Secondly, the main cause of pain remained unclear. Although, other potential factors previously reported, such as microtrauma caused by inadequate body positioning, or idiopathic coccygodynia may have been present in this patient's case. Even though we could not be certain that our patient's symptoms were caused by degenerative changes to the coccygeal disc, the conservative treatment of our patients with rest, NSAIDs and pelvic floor physical-therapy proved beneficial and relieved his symptoms. Thirdly, static or dynamic lateral films, provocative discography or dynamic MRI were not performed.

Conclusions

Patients suffering from coccygodynia require a thorough medical evaluation, which includes clinical and imaging examinations, according to the current literature. Since there are no standard treatment guidelines, the management of this condition remains challenging for physicians. This report refers to coccygodynia and perianal numbness, with concomitant coccygeal disc disease, treated with conservative methods.

What Is Already Known on This Topic:
Patients suffering from coccygodynia deserve thorough examination, including appropriate imaging, with, in most cases, conservative treatment to be considered as the treatment of choice for such a patient.

What This Case Adds:
Clinical and MR imaging examinations, are both considered to be of great importance to evaluate coccygodynia medically, with coccygeal disc herniation having a preponderant role in the explanation of the occurrence of either coccygodynia or perianal numbness.

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Conflict of Interest: The authors declare that they have no conflict of interest.

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The More Intensive the Diagnostic Workup, the More Likely It Is That the Cause of Coccygodynia Can Be Clarified

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Letter to the Editor[1]

We read with interest Galanakos et al's article about a 64-year-old male with coccygodynia and perianal numbness secondary to a coccygeal prolapse between S6 and S7 (1). With intensive conservative measures over three months, the patient's symptoms disappeared completely (1). It was concluded that imaging is of great importance in elucidating the cause of coccygodynia (1). The study is impressive, but several points require discussion. The major limitation of the study is that various alternative causes of coccygodynia were not sufficiently considered and excluded. Spondylosis, spondylarthrosis, chondrosis, osteochondrosis, foraminal stenosis, uncovertebral arthrosis, vertebrostenosis, osteoporosis, rheumatological disease (rheumatoid arthritis, polyarthritis, Sjögren syndrome, psoriatic arthropathy), immune or infections radiculitis, hypermobile os coccygis, SARS-CoV-2 infection, and varicositas spinalis were not excluded.

Since lumbar spine degeneration is one of the major causes of coccygodynia and since figure 1 shows chondrosis L4/L5, height reduction of the disc L4/5 and L3/L4, listhesis L4/L5, it is imperative to rule out degenerative lumbar disease as the cause of coccygodynia. Another argument in favour of lumbar degenerative disease as the cause of coccygodynia is that physiotherapy was beneficial. A second limitation is that MRI was performed in only one plane. To assess whether prolapse or other pathology actually occurred, it is imperative to also provide images of the axial and coronal planes. In addition, there is no contrast medium. MRI is mandatory to rule out spondylodiscitis, radiculitis, paravertebral abscess, or other causes of inflammation, vasculitis, or a vascularised tumour. A third limitation is that perianal numbness has not been adequately studied. Perianal numbness suggests that sensory functions in this region were impaired, either at the level of the receptors or the level of centripetal signaling to the central nervous system. It is unlikely that central nervous system disease is the cause of coccygodynia and perianal numbness, as this has not previously been described as a CNS manifestation and clinical neurological examination did not provide evidence of CNS disease. A fourth limitation is that no cerebrospinal fluid (CSF) studies have been performed. To rule out radiculitis (e.g. Elsberg syndrome), myelitis, discitis, SARS-CoV-2 infection, or malignant disease, it is imperative to examine the CSF for infectious or immunological disease or malignancies. A fifth limitation is that extravertebral causes of coccygodynia have not been adequately ruled out. The patient did not

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undergo urological examination to rule out benign prostatic hyperplasia, prostatitis, or prostate carcinoma. The results of colonoscopy, rectoscopy, or sigmoidoscopy are not mentioned. A sixth limitation is that no dynamic imaging studies and provocative discography were performed. It would have made sense to carry out the MRI or CT in a laying, sitting, and upright position.

In summary, the excellent study has limitations that should be addressed before drawing final conclusions. Clarifying the weaknesses would strengthen the conclusions and could improve the study. Unless all possible differential causes of coccygodynia have been adequately ruled out in the index patient, coccygodynia should not be attributed to coccygeal prolapse.

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