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Diseases

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Burden of Risk Factors

A Community Based Study

Highlights

Bone Marrow Lymphocyte

Populations of Innate Immunity

Discovering Thoughts, Inventing Future

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Burden of Risk Factors of Common Non-Communicable Diseases in Young Adult Women: A Community Based Study in Delhi

By Ankita Singh, T K Ray & Balraj Dhiman

Abstract- *Introduction:* India is passing through an epidemiological transition in health with high rates of urbanization; which in turn leads to economic improvement and one of the effect of this economic improvement is shift in disease spectrum from communicable to non communicable diseases. Non communicable disease contribute to around 5.87 million (60%) of all deaths in India. Women and men have different levels of exposure and vulnerability to Non communicable diseases risk factors. The present study was done in community setting to identify prevalence and distribution of risk factors for common Non communicable diseases among young adult women.

Methods: A community based cross sectional study conducted in Palam Village of New Delhi. A total of 585 patients were interviewed using a self designed, semi structured, pre designed questionnaire. Waist circumference was measured using non stretchable measuring tape and blood pressure was measured using digital blood pressure apparatus. Digital weighing scale was used to measure weight of study subjects. Association between qualitative variables was done using chi square test.

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Burden of Risk Factors of Common Non-Communicable Diseases in Young Adult Women: A Community Based Study in Delhi

Ankita Singh [α], T K Ray [σ] & Balraj Dhiman [ρ]

Abstract- *Introduction:* India is passing through an epidemiological transition in health with high rates of urbanization; which in turn leads to economic improvement and one of the effect of this economic improvement is shift in disease spectrum from communicable to non communicable diseases. Non communicable disease contribute to around 5.87 million (60%) of all deaths in India. Women and men have different levels of exposure and vulnerability to Non communicable diseases risk factors. The present study was done in community setting to identify prevalence and distribution of risk factors for common Non communicable diseases among young adult women.

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Results: Majority of study subjects were housewives and belong to upper lower and lower middle socioeconomic status. Half of the study subjects were moderately active. Most of the participants have unhealthy dietary habits. Around 2/3rd of study subjects were taking inadequate servings of fruits and vegetables and almost half of them were consuming salt more than the recommended levels. More than half (57.8%) of married subjects were eating salty food and snacks more than once in a week than the unmarried subjects. Out of 411 study subjects who were consuming inadequate amount of fruits and vegetables in a day, 60.3% belonged to nuclear family 39.6% to joint family.

Conclusion: High risk factors of common non communicable diseases among young adult females is seen in Palam village, New Delhi. There is an urgent need to implement population, individual and programme wide prevention and control interventions to lower serious consequences of Non Communicable Diseases.

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I. Introduction

In India high rates of urbanization has led to economic improvement and one of its effect is shift in disease spectrum from communicable to non communicable diseases(1).Non communicable diseases (NCDs), especially cardiovascular disease, diabetes mellitus, and stroke, have emerged as a major public-health problem in India. NCDs kill 41 million people each year, equivalent to 71% of all deaths globally (2). According to Indian Council of Medical Research (ICMR) report contribution of Non-Communicable Diseases (NCDs) to total death in the country was 61.8% in 2016, as compared to 37.9% in 1990 (3).Due to this epidemiological health transition, India's health system is facing dual challenge. Every year, 15 million people die from a NCD between the ages of 30 and 69 years; over 85% of these "premature" deaths occur in low- and middle-income countries(2). In India, 34.3% of total deaths in age group of 15 to 39 years were due to NCDs in 2016 s. NCDs were the dominant cause of death in those 40 years or older (3), but their onset occurs in younger age. The morbidity and mortality in most productive phase of life is posing serious challenges to Indian society and economy (4). Around the world NCDs affects women and men equally (2)Modifiable behaviours, such as tobacco use, physical inactivity, unhealthy diet and the harmful use of alcohol along with metabolic risk factors like raised BP, overweight/obesity, hyperglycemia and hyperlipidemia, all increase the risk of NCDs. Social customs related to physical mobility may reduce women's opportunities for activities thus women are more likely to be obese than men, which leads to their increased vulnerability to non communicable diseases (5) Non communicable diseases result in high health care costs, lost productivity and catastrophic expenses, and even if household has money available for health care, these funds are often spent on men's health needs (6) Deaths of women or men from NCDs during their most productive years (40-60 years) can result in tragedy for families and catastrophic expenditure. The loss of women's labour can push vulnerable families deeper into poverty, particularly in rural areas in developing countries where the number of female-headed households is increasing as men migrate for

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employment. The major impact of adult female mortality on household welfare is well established, including higher mortality amongst small children, food insecurity, children withdrawn from school, increased work burden on children and loss of assets. The burden of NCDs in the family is also borne by girls and women indirectly, as the principal caregivers in many households. Their educational and income earning opportunities are interrupted when having to stay at home to care for a sick family member (2) Moreover, a woman's health status also relates to health and vulnerability to their children. Women's health is therefore critically important to health of future generations (7)

Though many community based studies have been done to assess the prevalence of risk factors of Non communicable diseases but there is still a dearth of such studies among young adult women.

Therefore, the present study was done in community setting to identify prevalence and distribution of risk factors as well as awareness for non communicable diseases among young adult women.

II. Material and Methodology

The present study was a community based cross sectional study, conducted in Palam Village, New Delhi.It is one of the field practice area of department of Community Medicine, Lady Hardinge Medical College, New Delhi.The study was carried out from November 2017 to March 2019. Data was collected from January 2018 to December 2018. The study population comprised of all the women of 15 – 24 years of age who were permanent residents of Palam Village (residing for more than 1 year). The sample size was calculated by the formula $N = 4pq/l^2$where p represents prevalence of obesity (BMI > 30) which is 14.6 % obtained from the previous study done by J. S. Thakur et al on Profile of Risk Factors of Non Communicable Diseases in Punjab, Northern India: Results of a State Wide STEPS Survey. 'l' was allowable error, taken as 20% of p. Therefore, a sample size of 585 individuals was taken.Palam village has a population of 12000 & total number of households is 2400 .Sampling unit was household and study unit was young adult women of age 15 to 24 years. Systematic random sampling was applied with sampling interval of 4 (2400/585=4). Area map was made and first household was selected randomly and then every 4^{th} household was visited till the required sample size was obtained. If eligible subject was not found in the 4^{th} household then adjacent household was visited. If more than 1 eligible girl were residing in the same household, then only one was included in the study by random selection. Information regarding risk factors [Physical inactivity, dietary risk factors, stress and behavioral risk factors (tobacco and alcohol use)] for non communicable diseases was collected bysemi structured interviews schedule consisting of socio demographic characteristics, Global Physical Activity Questionnaire (GPAQ) by WHO to assess physical activity level, dietary assessment by pre designed questionnaire, stress assessment using General Health Questionnaire 12 (GHQ 12), behavioral risk factors by pre designed questionnaire. Non stretchable measuring tape, digital weighing scale, portable stadiometer, digital BP apparatus were used to measure waist and hip circumference, weight, height and blood pressure respectively.

III. Results

A total of 604 households were visited and 596 study subjects were enrolled. Eleven study subjects were excluded (6 refused to give consent and 5 were pregnant), hence the data of 585 subjects was analyzed. Majority (70%) of study subjects were married and Hindu by religion (85.7%). More than half (64.7%) of the study subjects belonged to nuclear family. Most (40%) of study subjects were housewives. Out of those who were employed 6.9% were housemaids and classified as unskilled workers and 21% were working in parlors and boutiques and classified under semi skilled workers. Nearly $1/3^{rd}$ of subjects were students, studying in schools and colleges. 9% of women were illiterate. 11% of subjects studied till primary school and 30% till middle school and 27% studied up to high school. 18% of women studied till intermediate. Most of study subjects (44.4%) belonged to lower middle socioeconomic status followed by upper lower socioeconomic status (43.6%) whereas 1.2% belonged to upper socioeconomic status and 3.2% to lower socioeconomic status. 56% (328) of study subjects were vegetarian and 44% (257) non vegetarian. Majority (70.3%) of them were taking inadequate servings of fruits and vegetables. Excessive salt intake was present among all subjects. 56% of study subjects were adding extra salt to their food and 53.6% were consuming salty snacks and salty foods $\geq$1 day/week. Majority (88%) of individuals were using mustard oil for cooking purpose and 2.4% were changing the brand of cooking oil regularly. Majority (72.5) of the study subjects were moderately active and involved in moderate intensity activities. 27% of study subjects had sedentary lifestyle and 0.5% were heavy workers.8.8% of the study subjects were found to be under stress. In more than half (58.46%) of study subjects waist hip circumference ratio was less than 0.85 indicating absence of abdominal obesity. Overall mean BMI of study population was 21.4 kg/m^2 ($\pm$3.03) with the range 16.8 to 30. Majority of study subjects (53.3%) had BMI within normal range. 34.8% were overweight while 7% were underweight and 4.2% were pre obese and obese.

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IV. Discussion

Although the NCD burden has grown, India still does not have sufficiently detailed data on NCDs for research and policy purposes. Most of the studies that have reported prevalence of risk factors of NCDs included wide range of age groups ranging from 15- 60 years, but the study on risk factors among young adult and more specifically in young females are rare. Most of the times, women are victims of the worse deprivation as a consequence of poor empowerment and discriminatory beliefs and practices. Keeping this in view, the present study was conducted in Palam village, Delhi among young adult women of age 15 to 24 years to find the prevalence of risk factors of common NCDs. In the present study, inadequate intake of fruits and vegetables was found in majority (70.3%) of study subjects. This finding was similar to study done by *Vijayakarthikeyan M 89)* where, prevalence of inadequate servings of fruits and vegetables was 62%. Findings of present study is also in agreement with most of the previously done studies (9,10,11,12,13). However, few studies like Mishra et al (14) and Bhattacharjee et al (15) report a higher prevalence of adequate fruits and vegetables intake as compared to current study. This finding in our study might be due to the reason that, a large proportion of study subjects in our study belonged to underprivileged socioeconomic class (upper lower and lower) who might have found it's consumption highly expensive. This was also evident by the finding in our study that, intake of fruits and vegetables was decreased as socioeconomic status decreased among study subjects and this was found highly significant (p value =0.017). Also, preference was given to male members and children of the family, if at all, fruits were bought in the family. Street food was more favoured by working women and students due to its easy availability in their working and school premises. Dislike of most of the vegetables was commonly found among study subjects. Lack of awareness regarding benefits of fruits and vegetables consumption could also be the reason behind such low prevalence of eating fruits and vegetables.

In our study, all of the study subject reported to consume more than 5g salt per day which was in agreement with NCDs country profile 2018 by WHO in which the mean population salt intake in Indian adult women (age: ≥20 years) was reported 9g per day (2) Sedentary lifestyle was reported in nearly one third of study subjects (27%) and they used to spend their leisure time in watching television or using mobile phones. Majority (72.5%) of study population was engaged in moderate level of physical activity like brooming, mopping, washing clothes, walking to and from work or schools. Only 0.5% were heavy worker. The findings of our study were similar to that of Ketkar et al (10), Gupta et al (16). Unlike our study, Valliyot et al(17) reported moderate level of physical activity in only 42% of study subjects.

Overweight and obesity was found in 39% of study subjects which is in accordance to NFHS 4 data which reports prevalence of overweight and obesity to be nearly 35% among adult women. In our study 34.8 % of subjects were overweight and 4.2% were obese. This prevalence of overweight and obesity could be attributed to inadequate dietary practices and easy availability and affordability of unhealthy food, lack of physical activity. The findings in present study are in concordance with the findings of study done by Sandhu et al in Delhi(18) (33.1% overweight and 6% obese) and Bhagyalakshmi et al in Gujarat (9). Few (8.8%) study subjects were found to be under stress in our study, comparable to findings of Laskar et al (12).Marital conflicts was the major cause of stress as reported by research participants.

As the prevalence of risk factors of NCDs like inadequate fruit and vegetable consumption, excessive salt intake among young adult women i.e. 15 to 24 years has been found very high in our present study, primary prevention has a major role in preventing occurrence of NCDs in later age. So it is recommended to promote healthy lifestyle in this age group female who will further inculcate these practices in their family. In our study, there was high proportion of school and college going students who had sedentary lifestyle. So knowledge regarding healthy lifestyle like physical activity and healthy diet should be inculcated in students through curriculum and teachers should be trained. Consumption of inadequate fruits and vegetables in a day was found in majority of study subjects. One reason might be fruits are considered expensive. Awareness activities regarding intake of seasonal fruits and vegetables in schools as well as in community should be carried out, which are relatively cost effective but equally nutritious. There should be a restriction and its strict implementation on selling of street and junk food in premises of schools and colleges to discourage its use. Many of the schools and colleges have already implemented this, but it have to be further strengthened and promoted. Mass media campaigns, taxes on unhealthy food, subsidies on healthy foods, mandatory food labeling and marketing restrictions on unhealthy food should be done.

Conflict of Interest: None

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Tables

Table 1: Distribution of study subjects according to Socio Demographic profile

Marital status	Number (%)
Married	408 (70.0)
Unmarried	177 (30.0)
Total	585 (100.0)
Religion	**Number (%)**
Hindu	501 (85.7)
Muslim	84 (14.3)
Total	585 (100.0)
Type of family	**Number (%)**
Nuclear family	378 (64.7)
Joint family	206 (35.3)
Total	585 (100.0)

Nature of occupation	Number (%)
Unskilled worker	40 (6.9)
Semiskilled worker	123 (21.0)
House wife	234 (40.0)
Students	188 (32.1)
Total	585 (100.0)
Education	**Number (%)**
Illiterate	53 (9.0)
Primary	64 (11.0)
Middle	175 (30.0)
High school	159 (27.0)
Intermediate	105 (18.0)
Graduate or Post Graduate	29 (5.0)
Total	585 (100.0)
Socioeconomic status	**Number (%)**
Upper	7 (1.2)
Upper middle	45 (7.6)
Lower middle	259(44.4)
Upper lower	256 (43.6)
Lower	18 (3.2)
Total	585 (100.0)

Table 2: Distribution of study subjects according to dietary habits

Dietary information	Quantity and practices	Number (%)
Consumption of Fruits and Vegetables	<5 Servings/ day	411 (70.3)
	≥5 servings per day	174 (29.7)
Salt intake	<5 gm/day	0
	≥5 gm/ day	585 (100.0)
Extra salt added to food	Yes	328 (56.0)
	No	258 (44.0)
Consumption of Salty foods/Snacks	<1 day/ week	271 (46.32)
	1-3 days/ week	194 (33.16)
	3 – 6 days/ week	79 (13.50)
	Daily	41 (7.0)
Cooking oil	Ghee/ Butter	0
	Mustard oil	515 (88.0)
	Refined oil	68 (11.6)
	Olive Oil	2 (0.4)
Change of cooking oil	Yes	14 (2.4)
	No	571 (97.6)

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Table 3: Distribution of study subjects according to physical activity on the basis of GPAQ (Global Physical Activity Questionnaire)

Physical activity	Number (%)
Sedentary	158 (27.0)
Moderate	424 (72.5)
Heavy	3 (0.5)

Table 4: Distribution of study subjects according to stress (assessed by General Health Questionnaire 12) and waist hip circumference ratio

Stress	Number (%)
Absent	533 (91.2)
Present	52 (8.8)
Total	585 (100.0)
WHCR	**Number (%)**
<0.85	342 (58.46)
≥0.85	243 (41.45)
Total	585 (100.0)

Table 5: Distribution of study subjects according to Body Mass Index

Body Mass Index	Number (%)
18.5 to 22.9 (Normal)	314 (53.3)
23 to 24.9 (Overweight)	205 (34.8)
25 to 29.9 and ≥30 (pre obese and obese)	25 (4.2)
<18.5 (Underweight)	41 (7.0)
Total	585 (100.0)

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In Vitro Immunomodulatory Effect of Linalool on *P. gingivalis* Infection

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Federal University

Abstract- *Introduction:* Periodontitis is a multifactorial disease, characterized by an inflammatory response of the periodontal tissues to a dysbiotic biofilm in the subgingival surface. The presence of keystone pathogens, such as Porphyromonas gingivalis, is one of the main causes of dysbiosis, although the host response is preponderant in the beginning and the progression of the disease. The periodontal treatment is based on the mechanic scaling of the biofilm but using of chemicals adjuvants has been preconized. However, there are many restrictions related to the antibiotics and other chemical adjuvants usage, which makes the use of herbal medicines for this purpose very promising. In addition, many herbal medicines have been used in the folk medicine, with various biologic effects.

Objective: To evaluate in vitro the effect of linalool in the periodontitis.

Keywords: *periodontal inflammation, porphyromonas gingivalis, linalool, periodontal treatment.*

GJMR-F Classification: *NLMC Code: QW 920*



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In Vitro Immunomodulatory Effect of Linalool on *P. gingivalis* Infection

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Abstract- Introduction: Periodontitis is a multifactorial disease, characterized by an inflammatory response of the periodontal tissues to a dysbiotic biofilm in the subgingival surface. The presence of keystone pathogens, such as *Porphyromonas gingivalis*, is one of the main causes of dysbiosis, although the host response is preponderant in the beginning and the progression of the disease. The periodontal treatment is based on the mechanic scaling of the biofilm but using of chemicals adjuvants has been preconized. However, there are many restrictions related to the antibiotics and other chemical adjuvants usage, which makes the use of herbal medicines for this purpose very promising. In addition, many herbal medicines have been used in the folk medicine, with various biologic effects.

Objective: To evaluate *in vitro* the effect of linalool in the periodontitis.

Material and Methods: 61 volunteers with and without periodontitis were evaluated. Peripheral blood mononuclear cells were cultured in presence of the crude extract of *Porphyromonas gingivalis* and in the presence of linalool for 48h. The lymphoproliferation and the cell death were evaluated by flow cytometry and the concentration of IL-6, IL-10, IL-17 and IFN-gama were evaluated by enzyme-linked immunoassay (ELISA).

Results and discussion: The individuals with periodontitis produced higher levels of IL-6 than those without the disease, when peripheral blood mononuclear cells were cultured with linalool ($p=0.02$). In addition, the concentration of the four cytokines evaluated were higher in the culture supernatants of the peripheral blood mononuclear cells stimulated with *Porphyromonas gingivalis* ($p<0.01$), when compared to those cultured with linalool only. The linalool alone induced low production of IL-6, IL-10 and IFN-gama than when the peripheral blood mononuclear cells were cultured in presence of the linalool and the *Porphyromonas gingivalis* extract concomitantly ($p<0.01$). Individuals without periodontitis showed higher proliferation rates of T lymphocytes when these cells were cultured with no stimulus ($p=0.04$) or in the presence of the crude extract of *Porphyromonas gingivalis* ($p=0.03$). There was no difference among the stimulus in the apoptosis induction.

Conclusion: The use of linalool determined lower concentrations of cytokines in culture. Linalool showed low toxicity to host cells.

Keywords: periodontal inflammation, porphyromonas gingivalis, linalool, periodontal treatment.

I. Introduction

Periodontitis is an inflammatory disease caused by an interaction between a dysbiotic subgingival biofilm and the host immuno-inflammatory response (HUANG and GIBSON, 2014). It is known that *Porphyromonas gingivalis* (Pg), a bacteria presente in the oral microbioma, induces the production of proinflammatory cytokines, promoting inflammation of the periodontal tissues (HAJISHENGALLIS, 2014) and progressive periodontal breakdown (ZHOU et al., 2017).

This disease has been associated with a variety of other disorders, such as cardiovascular diseases (LOOS et al., 2000), diabetes (MONTEIRO et al., 2017), metabolic syndrome (GOMES-FILHO et al., 2016), respiratory diseases (SOLEDADE-MARQUES et al., 2017), low birth weight (GOMES-FILHO et al., 2007) and erectile dysfunction (SINGH et al., 2017). In addition, some studies have been demonstrated that the periodontal inflammation can be associated with inflammatory autoimmune diseases, such as rheumatoid arthritis (FUGGLE et al., 2016) and systemic lupus erythematous (RUTTER-LOCHER et al., 2017).

Due to the local effects and the association with other disorders, the prevention, treatment and control of the periodontitis are needed. The main treatment involves the mechanic control of the subgingival biofilm through scaling and root planning. However, the chemic control can be added in order to reduce the bacterial pathogenicity (MORO et al., 2017).

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The use of antibiotics and mouthwashes based on chlorhexidine or triclosan are the most commonly used chemical agents in periodontal treatment. Nevertheless, the use of these agents should be restricted due to the toxicity, since they can be converted in toxic compounds by photodegradation and methylation, and due to their ability to bioaccumulate (CORTEZ, 2011). In addition, the systemic use of antibiotics can promote adverse effects, such as bacterial resistance, a very important problem in public health (HAJISHENGALLIS and LAMMONT, 2014).

Because of these disadvantages, the study of phytotherapy, specially the use of extracts and essential oils has been increased (MANAOUZE et al., 2017). Furthermore, the results from the use of natural products in integrative medical practices as adjuvants to pain, inflammatory processes and anxiety disorders treatments have reinforced this search (OLIVEIRA et al., 2017).

Among the components of the essential oils of several plants, such as lavender, sage, rosewood and basil, stands out linalool, a monoterpene alcohol (CHENG et al., 2017) that possess analgesic, anti-inflammatory, anticonvulsant and neuroprotective properties (ELISABETSKY et al., 1995; PARK et al., 2016). *In vitro* studies showed that linalool can inhibit the growth of *Porphyromonas gingivalis* (JUIZ et al., 2016).

Thus, the present study aimed to evaluate the *in vitro* effect of linalool in the production of IL-6, IL10, IFN-γ e IL-17, as lymphoproliferation and the cell death in context of periodontitis.

II. Material and Methods

This research was approved by the Institutional Review Board of Feira de Santana State University through CAAE no 46267915.8.0000.0053.

The *in vitro* experimental study was conducted through the analysis of peripheral blood mononuclear cells (PBMC) of 61 individuals attended at the School of Dentistry of the Feira de Santana State University, Bahia, Brazil. The exclusion criteria consisted of individuals with a history of systemic diseases, current gestation, previous periodontal treatment, smoking, antibiotic and anti-inflammatory use in the six and two months before data gathering, respectively.

A pilot study was performed to determine the minimum error of 950 pg/mL and standard deviation of IL-6 levels of 1260 pg/mL. The level of significance and power of the test adopted was of 5% and 80%, respectively, in a ratio of 1: 4. In addition, 10% was added to predict losses.

After the periodontal evaluation, which included probing depth, clinical attachment level and bleeding on proing, the individuals were separated into two groups: 12 individuals with the diagnosis of periodontitis (P) and 49 individuals without periodontitis (WP).

Individuals with periodontitis were the participants who had at least four teeth with at least one site with probing depth greater than or equal to 4 mm; clinical attachment loss greater than or equal to 3mm; and bleeding on probing concomitantly (GOMES-FILHO et al., 2007).

Linalool compound was obtained commercially (Sigma, SP, BR), racemic mixture (+/-), lot STBD6780V. *Porphyromonas gingivalis* strain ATCC 33277 was grown in Brucella broth supplemented with 0.5% yeast extract, 0.1% hemin, 0.1% menadione and 0.05% L-cysteine under anaerobic conditions (85% N2, 10% H2, 5% CO2). The immunogenic extract was produced by a protocol standardized by Trindade et al (2008).

Previosly to the execution of sandwich-type Enzyme-Linked Immunosorbent Assay (ELISA), cell culture was performed in 24-well plates (10^6 cells per well) with Roswell Park Memorial Institute (RPMI) culture medium with 1% antibiotic/antimycotic and 10% fetal bovine serum. Cultivation was carried out at 37°C, in humidified atmosphere and in the presence of CO_2. The PBMC of the individuals were cultured with the following stimulus conditions: (1) cells in RPMI medium as a negative control (white); (2) addition of pokeweed mitogen (PWM) as a positive control at 2.5 µg/mL; (3) addition of the extract of *Porphyromonas gingivalis* at a concentration of 0.5 µg/mL; (4) addition of 10 µg/mL linalool previously standardized from the cellular cytotoxicity test, a 3% methanol/DMSO solution was used to solubilize the compounds and (5) addition of linalool at a concentration of 10 µg/mL and extract of *Porphyromonas gingivalis* at the concentration of 0.5 µg/mL.

Following cell culture, for cytotoxicity assessment, PBMC were distributed in 96 well plates in RPMI culture medium with 1% antibiotic/antimycotic and 10% fetal bovine serum. The linalool was added at different concentrations 100 µg/mL, 50 µg/mL, 25 µg/mL, 10 µg/mL, 5 µg/mL, 2 µg/mL and 1 µg/mL. After 48 h, the plate was removed and centrifuged at 1000 rpm for 5 minutes at 4°C. The supernatant was aspirated and then 100µl of the solution of MTT-tetrazolium [3- (4,5-dimethazol-2-yl-2,5-diphenyltetra-zolium bromide)] (Sigma Chemical Co., St. Louis, MO, USA) at 6 mg/mL was added to the well. The cells were again incubated for 4 hours in a humid chamber at 37° C and 5% CO2, then centrifuged at 1000 rpm for 5 minutes at 4°C. After removal of the culture medium, 100 µL of dimethylsulfoxide (DMSO, Sigma-Aldrich Co., SP, BR) was added to the well. The reading absorbance value was determined at wavelength at 570 nm using a microplate reader. The assay was carried out in six-fold. Compounds that allowed a cell viability greater than or equal to 90% were considered non-toxic.

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The concentrations of the cytokines, IL-6, IL-10, IL-17 and IFN-gama in cell culture supernatants after 48 hours of culture, in the presence of pokeweed mitogen (PWM) (2.5 µg/mL); of the antigen, extract of *Porphyromonas gingivalis* (0.5 µg/mL); of linalool (10 µg/mL) and linalool (10 µg/mL) in addition to the *Porphyromonas gingivalis* extract (0.5 µg/mL) were measured by enzyme immunoassay using commercially available kits (R & D, Systems, Minneapolis, USA). The assays were performed using 96-well flat-bottomed adsorption polystyrene plates (COSTAR, Coornig Life Science, Tewksbury, MA, USA). All steps were also performed according to the manufacturer's instructions. After development with tetramethylbenzidine (TMB) and quenching of the reaction with H2SO4, optical density was determined in ELISA Reader (ELx 800 - Bio-Tek) adjusted to a wavelength in the 450 nm range.

Previosly to the evaluation of apoptosis/necrosis induction by flow cytometry, PBMC were cultured in 24-well culture plates (10^6 cells per well) for 18 h at 37°C, 5% CO2 in RPMI medium containing antibiotic and antimycotic, in presence of pokeweed (2.5 µg/mL); dexametasona (1µM); of the extract of *Porphyromonas gingivalis* (0.5 µg/mL); of linalool (10 µg/mL) and linalool (10 µg/mL) with the extract of *Porphyromonas gingivalis* (0.5 µg/mL), added concomitantly. After incubation, the programmed cell death identification assay was performed using a detection kit, by identification of phosphatidylserine expression in the cell membrane, by the addition of annexin V conjugated to fluorescein isothiocyanate (FITC), (SIGMA-USA) in concentration of 25 µg/mL in cells post-cultured with the antigens for 5 min at room temperature.

For detection of necrotic cells, 50 µg/mL propidium iodide was added to the cells one minute prior to acquisition to read the reaction on the flow cytometer (Facscalibur, Franklin Lakes, USA).

For the verification of the cell proliferation capacity, the PBMC were labeled with the cell proliferation marker 5-carboxy-29, 79 dichlorofluore-sceoxyacetatesuccinimidyl ester (CFSE) (Invitrogen, Carlsbad, USA), diluted in dimethylsulfoxide (DMSO) added to the well at a final concentration of 2 mM, then they were incubated for 10 minutes at 37°C. After this step, labeled PBMC were distributed at a concentration of 10^6 per well in cell culture dishes in RPMI culture medium added 1% antibiotic/antimycotic and 10% fetal bovine serum in the presence of poke weed (2.5 µg/mL); of the extract of *Porphyromonas gingivalis* (0.5 µg/mL); of linalool (10 µg/mL) and linalool (10 µg/mL) and *Porphyromonas gingivalis* extract (0.5 µg/mL), concomitantly. The negative control contains only with culture medium. Cultivation was carried out at 37°C, humidified atmosphere and in the presence of CO2. After 48 h the samples were collected and read by flow cytometry (Facscalibur, Franklin Lakes, USA).

The distribution of the data was tested by using the Kolmogorov-Smirnov test. The comparison between the groups was performed with the Student's T-parametric test for the data that presented normal distribution and with the non-parametric Mann-Whitney test for those who did not present normality in the distribution. Following the same criterion, the comparison between the stimuli was done using the ANOVA test with Games-Howell posthoc for normal distribution and the Kruskall-wallis test, followed by the Bonnroni-corrected Mann-Whitney test, in absence of normal data distribution.

III. Results and Discussion

Participants in this study were 61 individuals who met the eligibility criteria. The group with periodontitis (P) was composed of 12 participants (19.68%), while the group without periodontitis (WP) was composed of 49 participants (80.32%). The mean age of participants in the P group was 43.67±10.23 years, while the mean age of the participants in the WP group was 32.29±8.88 years. Regarding the sex of the volunteers, 58.33% (07) and 59.18% (29) of the participants in the P and WP groups were female, respectively. There was a statistically significant difference in mean age ($p = 0.000$), but this difference did not remain in relation to gener ($p=0,957$).

The periodontal condition of individuals can be seen in Table 1. There was a statistically significant difference between the two groups in all the clinical descriptors evaluated. Except for the number of teeth present, the other descriptors were larger in individuals with periodontitis. To ensure the quality of cellular responses obtained in the present study, cellular toxicity tests were performed, which revealed potential cytotoxic activity of linalool at a concentration of 100 µg/mL (Figure 1). The results discarded the significant cytotoxic effect generated by linalool at the concentrations of 10, 5, 2 and 1 µg/mL in PBMC. Although no statistical difference, there was a cytotoxic tendency in the concentration of 25 µg/mL. Based on these data, the 10 µg / mL concentration was chosen for use in the experiments. It is worth mentioning that, in osteoclasts, linalool present in *Ocimum basilicum* oil was considered non-toxic at concentrations less than or equal to 300 µg/mL (JUIZ, 2013; JUIZ et al., 2016).

The cell death assay showed no statistically significant difference among the diverse condition of growing tested in this study (namely, without stimulus, dexamethasone, PWM, Pg, linalool and Pg + linalool), in the quantity of viable cells ($p = 0.369$), cells in initial apoptosis, ($p = 0.681$), in late apoptosis ($p = 0,892$) and in necrosis ($p = 0.098$) process (Figure 2).

In general, the cells had high viability rates, regardless of the culture conditions. In contrast, a previous study, showed the capability of linalool to

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induce apoptosis in osteoclast culture at concentrations of 50 µg/mL (JUIZ, 2013; JUIZ et al., 2016).It's important to note that the extract of *Porphyromonas gingivalis* was expected to induce higher rates of apoptosis, since the results found by TRINDADE et al. (2012), showed that the extract of *Porphyromonas gingivalis* induced higher levels of apoptosis. The most observed form of death was the initial apoptosis, in which the cell shows the inversion of the membrane, without the loss of its integrity when there is exposure of the phosphatidylserine (TRINDADE et al., 2012).

Additionally, individuals with periodontitis presented higher rates of viability among dexamethasone-stimulated cells (p=0.033) than individuals without the disease (Figure 3), showing some showing some ability to protect from apoptosis death, what may have occurred due to the greater expression of BCL 2 (CARVALHO-FILHO et al., 2013) or increase of HSP in the cytoplasm (MASCARENHAS and ROCHA, 2018). However, the mechanism of this protection remains unclear.

In the cell proliferation analysis, pokeweed, a polyclonal activator of T lymphocites (ROITT et al., 2003) induced higher rates of these cells proliferation than *p. gingivalis* (P=0.005), linalool (P=0.001) and linalool + Pg (P=0.008). However, when the other populations of lymphocytes were evaluated, pokeweed demonstrated low capacity of inducing proliferation (p=0,001), as demonstrated in figure 4.

In relation to the difference between P and WP groups (Figure 5), individuals without periodontitis had a higher rate of proliferation of T lymphocytes when they were cultured without stimulus (p = 0.043), or in the presence of Pg (p = 0.032). In the other lymphocyte populations, proliferation rates were higher in individuals with periodontitis than in those without the disease in the following culture conditions: without stimuli (p = 0.026), in the presence of linalool (p = 0.033) and in the presence of linalool and Pg (p = 0.043). It is worth noting that *P. gingivalis* induced lymphoproliferation in peripheral blood mononuclear cells in a previous evaluation (TRINDADE et al., 2012), but the behavior of linalool in this biological response is still poorly studied.

Regarding to the cytokines evaluation, it was observed that *P. gingivalis*, linalool and linalool+PG induced the production of IL-6, IL-10, IL-17 and IFN-γ, since the levels of these four cytokines were higher when cells were cultured with the above-mentioned stimuli than the cells cultured without stimulus (p = 0.000). Moreover, cells submitted to the concomitant stimulation of Pg and linalool had lower levels of IL-17 in relation to the cells under stimulation only of Pg (p = 0.031) and linalool (p = 0.015). However, the concomitant stimulation with Pg and linalool induced higher production of IL-6, IL-10 and IFN-γ than the stimulus alone with linalool (p=0.000).

Comparing the concentrations of cytokines produced by PBMCs from individuals without periodontitis (WP) and diagnosed with periodontitis (P), there was a statistically significant difference in IL-6 levels when cells were stimulated with linalool alone, that is, individuals with periodontitis produced higher levels of IL-6 than those without the disease (Figure 7a), (p = 0.018). There were no differences when IL-10, IL17 and IFN- γ levels were compared between the WP and P groups (Figures 7b, 7c and 7d).

Expression of the IL-6 gene is increased when macrophages are infected with *Porphyromonas gingivalis* (GMITEREK et al., 2016), which occurred when linalool was used in PBMC cultures concomitantly with the bacteria extract, indicating that this substance had a limited modulating effect. However, the decrease in IL-6 production by the presence of linalool may be a favorable factor for the control of periodontitis, since IL-6 is a cytokine involved in bone resorption because it acts unfavorably on the RANK-RANKL-OPG axis (SINGH et al., 2012).

The antinflamatory activity of linalool activity was previously demonstrated in a study with diabetic rats, which had their serum IL-6 and insulin concentrations reduced when linalool was given (DEEPA e ANURADHA, 2011). On the other hand, prior contact of the diseased individuals with key pathogens of periodontal dysbiosis, such as *Porphyromonas gingivalis*, may have induced a more effective memory immune response with the increase, therefore, in the production of IL-6 (HAJISHENGALLIS, 2014; TRINDADE et al., 2013). Periodontitis is associated with the G allele in the IL-6 gene (position -174), which seems to confer a phenotypic profile of high production of this cytokine. Under stimulation of *Porphyromonas gingivalis* PBMC of individuals with periodontitis produced more IL-6 than individuals without the disease (TRINDADE et al., 2013), which may explain these higher levels observed in this study, even with the use of linalool.

With respect of IFN- γ, the Th1 profile signature cytokine, cells cultured with the extract of Porphyromonas gingivalis produced high concentrations of IFN-gamma and co-cultivation with linalool was not able to inhibit this production. Although *Porphyromonas gingivalis* is an extracellular microorganism, studies have shown its penetration into macrophages (GMITEREK et al., 2016) and fibroblasts (BENGTSSONA et al., 2015), and can be recognized by toll-type receptors 7 (TLR-7), increasing NFkB expression, which may promote the polarization of the immune response to this profile. The reduced concentration of these cytokine in the culture supernatant in the presence of linalool demonstrate that it is not inducing the immunoinflammatory response characteristic of this profile, which may be favorable, since IFN- γ is a potent macrophage activator and induces bone resorption (SILVA et al., 2015).

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The presence of linalool in conjunction with the extract of Porphyromonas gingivalis was also not able to inhibit the production of IL-10, a cytokine that plays an important role in regulating the immune response and contributes to attenuation of tissue destruction (ZHANG et al., 2014; TRINDADE et al., 2012). Cells cultured only with linalool at the concentration employed did not produce high concentrations of this cytokine. The use of some monoterpene compounds, such as linalool, in splenocyte culture increased the IL-10/IL-2 secretion ratio but decreased the levels of IL-2. Thus, treatments with monoterpere compounds, including linalool, have an anti-inflammatory potential *in vitro* (KU e LIN, 2013).

As aforementioned *P. gingivalis,* linalool alone and linalool used with the extract of *P. gingivalis* induced higher concentrations of IL-17 than the non-stimulated cells. The participation of this cytokine in the pathogenesis of periodontitis has been reported (ZENOBIA e HAJISHENGALLIS, 2015) and *Porphyromonas gingivalis* can induce its production by host cells (CHENG et al., 2016). Since IL-17 is a cytokine with kinetics characterized by later peak concentrations (CHEN et al., 2015), it is possible that differences between individuals with and without periodontitis could be observed in longer cultures. In addition, except for IL-6, groups with and without periodontitis showed no differences in cytokine production, possibly due to the small number of diseased individuals evaluated.

IV. Conclusions

Linalool has been shown to be poorly aggressive to host cells, but its use as adjuvant in periodontitis control needs to be better studied, with higher concentrations and more empowered sample size in distinct groups. In addition, studies are required to verify its hepatotoxic capacity and to determine optimal concentrations in possible topical administration vehicles.

Competing interests

There is no conflict of interest to declare.

Authors' contributions

Ana Carla Montino Pimentel: Data collection

Ellen Karla Nobre dos Santos: Discussion and writing of the manuscript

Geraldo Pedral Sampaio: Execution and analysis of flow cytometry.

Marcia Tosta Xavier: Design of the study, discussion and writing of the manuscript

Michelle Miranda Lopes Falcao: Discussion and writing of the manuscript

Patricia Mares de Miranda: Data collection

Paulo Cirino de Carvalho-Filho: Study design, data collection, writing.

Raimon Rios da Silva: Data collection

Rebeca Pereira Bulhosa Santos: Study design, data collection, writing

Roberto Meyer: Discussion of the results

Soraya Castro Trindade: Study design, data collection, writing, data analysis, discussion of results.

Yuri Andrade de Oliveira: Data collection

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Table 1: Clinical findings of individuals with periodontitis (P) and without periodontitis (WP), BRAZIL, 2018.

CLINICAL FINDINGS	GROUP WP n=49	GROUP P n=12	P VALUE*
	(média ±DP)	(média ±DP)	
Number of teeth	26.00±3.39	21.17±4.74	0.00
Number of sites with BOP	13.88±15.05	46.17±21.34	0.00
Number of sites with PD≥4mm	1.10±1.59	12.75±7.07	0.00
Number of sites with CAL≥3mm	18.49±21.42	58.00±21.12	0.00
Number of sites with CAL≥5mm	1.37±4.09	10.42±6.88	0.00

WP=Withohut Periodontitis; P= Periodontitis; SD: Standard deviation; BOP: Bleeding on probing; PD: Probing depth; CAL: Clinical attachment level; P< 0,05.

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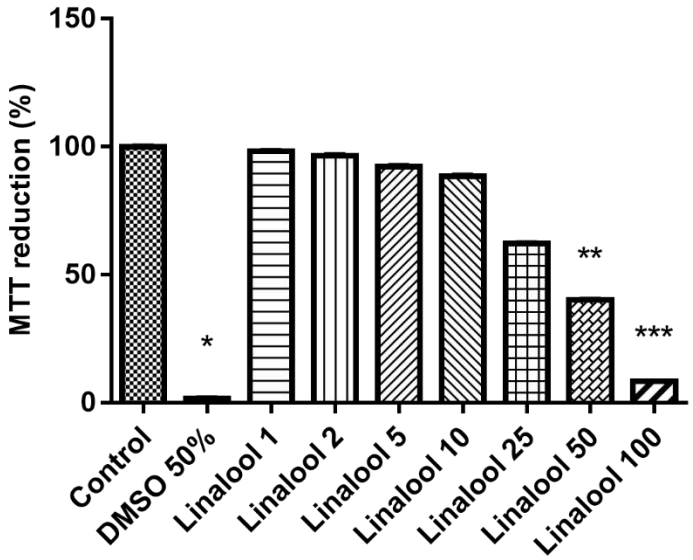


Figure 1: Cytotoxic effect of peripheral blood mononuclear cells (PBMC). Cell viability assessment of PBMC for 48 hours at 37 ° C and 5% CO2 by the technique of MTT-tetrazolium [3- (4,5-dimethazol-2-yl-2,5-diphenyltetrazolium bromide)]. * p, ** p, *** p <0.05

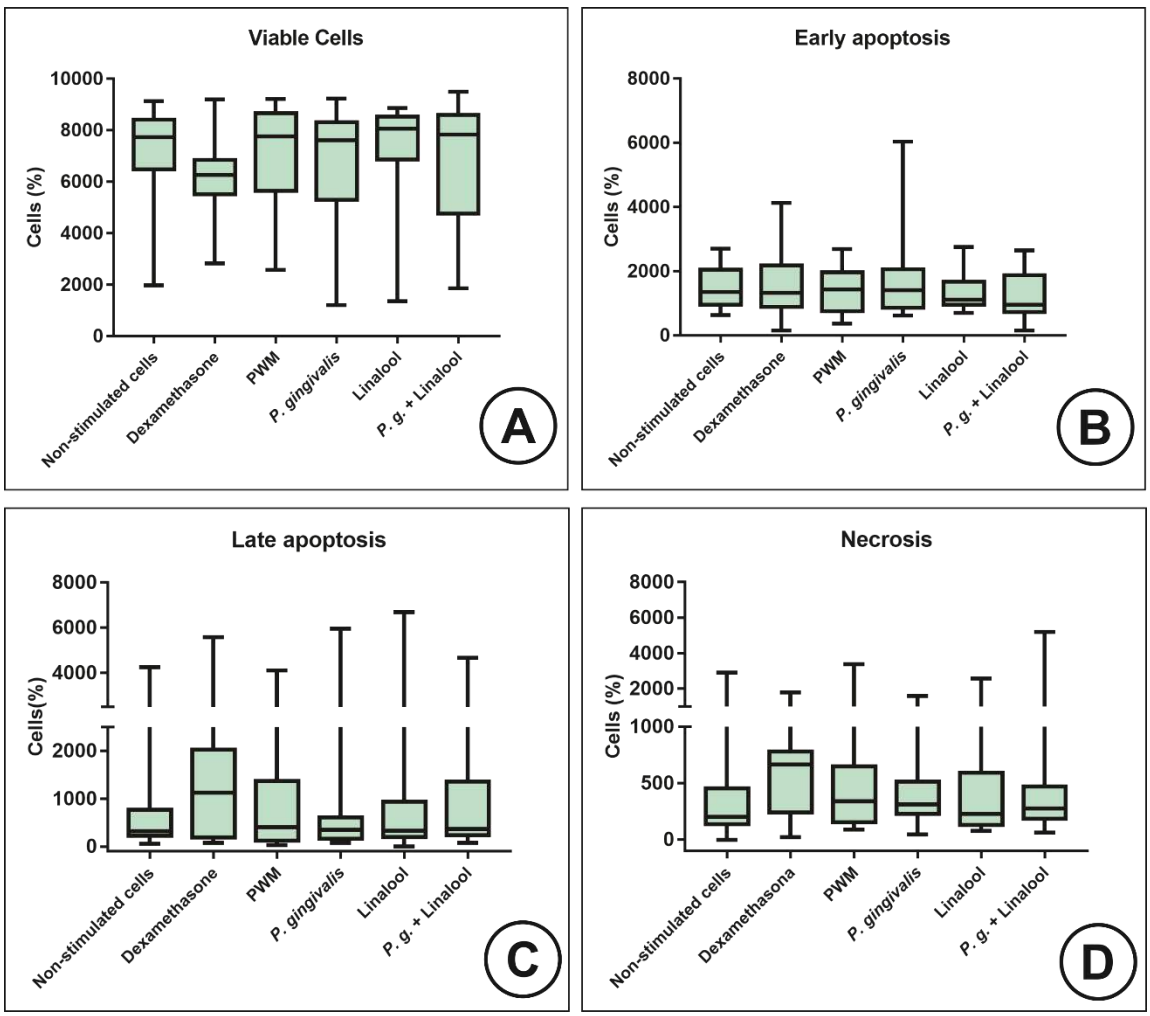


Figure 2: Evaluation of cell death (%), in PBMC, evaluated by flow cytometry after 18-hour stimulation. A: Viable cells; B: Initial apoptosis; C: Apoptosis late and D: Necrosis

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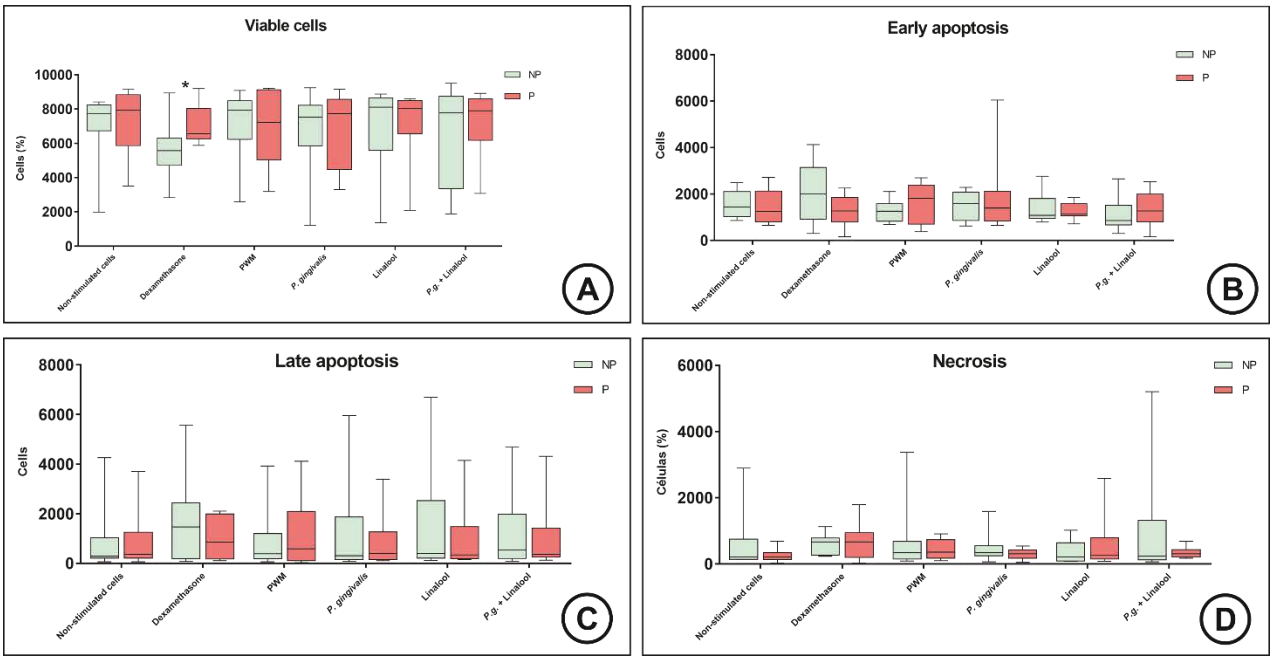


Figure 3: Evaluation of cell death (%), in PBMC of individuals without periodontitis (SP) and with periodontitis (CP), evaluated by flow cytometry after 18-hour stimulation. A: Viable cells; B: Initial apoptosis; C: Late apoptosis and D: Necrosis

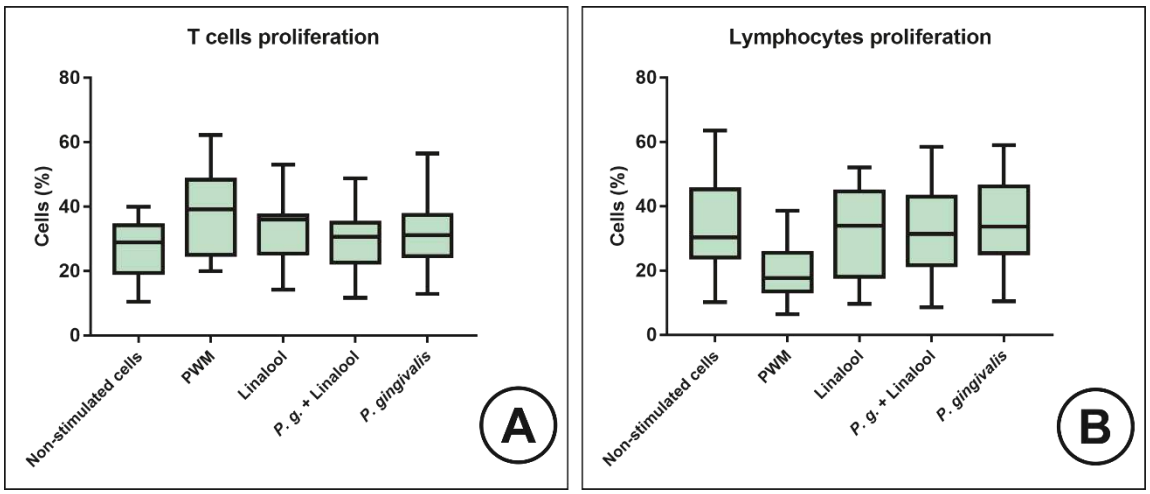


Figure 4: PBMC proliferative response after 48-hour stimulation, assessed by flow cytometry. A: T lymphocyte population; B: Population of other lymphocytes

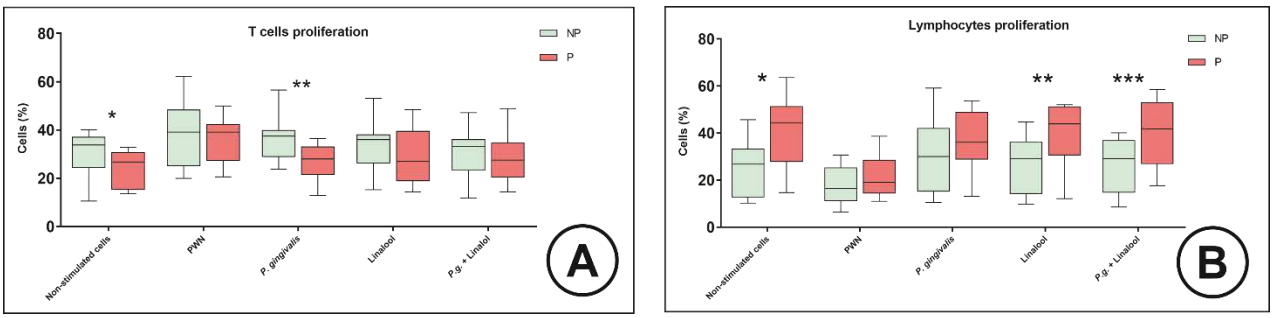


Figure 5: PBMC proliferative response of individuals without periodontitis (SP) and with periodontitis (PC), after 48-hour stimulation, evaluated by flow cytometry. A: T lymphocyte population; B: Population of other lymphocytes

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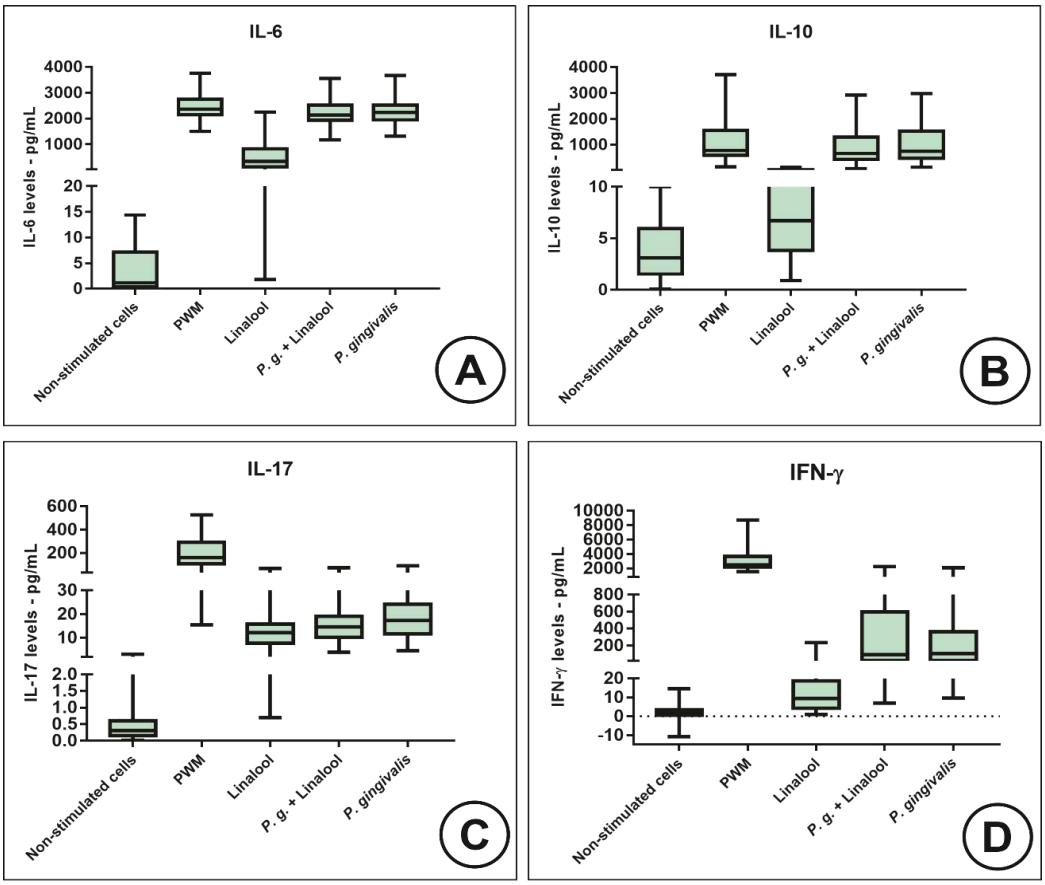


Figure 6: Cytokine production in culture supernatants in PBMC of all individuals. A: IL-6; B: IL- 10; C: IL-17 and D: IFN-gamma

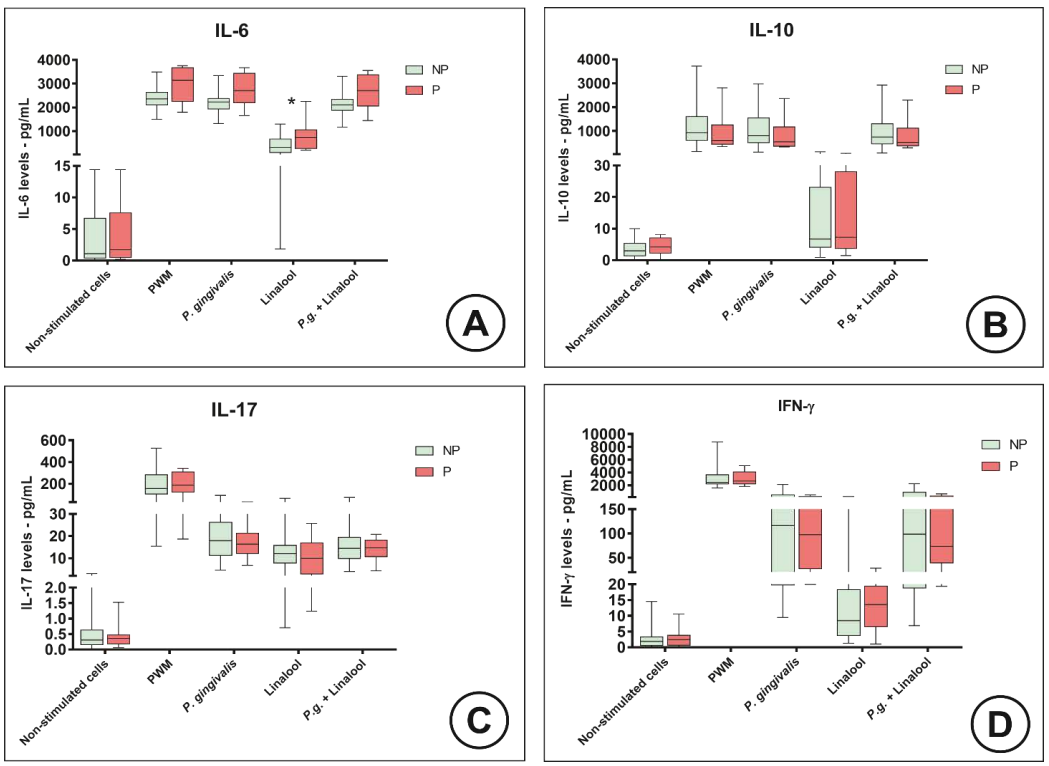


Figure 7: Cytokine production by PBMC in culture supernatants of individuals without periodontitis (SP) and with periodontitis (PC), A: IL-6; B: IL-10; C: IL-17 and D: IFN-gamma

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Salmonella Hepatitis - A Case Report

By M. Z. Hussain & S. K. Chowdhury

Abstract- Salmonella hepatitis is a rare complication of Typhoid fever in tropics. Typhoid fever is an infectious disease associated with high morbidity and mortality. The classical pattern of presentation is continuous fever with a “step ladder” rise, headache, abdominal pain, diarrhea, constipation, and relative bradycardia. In a rare instance, Typhoid fever presented with the feature of acute hepatitis like hepatomegaly and deranged liver functions with hepatic encephalopathy. After taking proper informed consent, we have reported a young patient who presented with low-grade fever, diarrhea, and jaundice. Subsequently he was found to have acute salmonella hepatitis secondary to typhoid fever. Recognition of Salmonella hepatitis is of clinical importance as it can mimic acute viral hepatitis. Early institution of specific therapeutic intervention and supportive care improve the prognosis in these patients.

Keywords: *typhoid fever, salmonella hepatitis, typhoid hepatitis.*

GJMR-F Classification: *NLMC Code: QW 138.5.S2, QW 170*

SALMONELLAHEPATITISACASEREPORT

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Salmonella Hepatitis - A Case Report

M. Z. Hussain [α] & S. K. Chowdhury [σ]

Abstract- Salmonella hepatitis is a rare complication of Typhoid fever in tropics. Typhoid fever is an infectious disease associated with high morbidity and mortality. The classical pattern of presentation is continuous fever with a "step ladder" rise, headache, abdominal pain, diarrhea, constipation, and relative bradycardia. In a rare instance, Typhoid fever presented with the feature of acute hepatitis like hepatomegaly and deranged liver functions with hepatic encephalopathy. After taking proper informed consent, we have reported a young patient who presented with low-grade fever, diarrhea, and jaundice. Subsequently he was found to have acute salmonella hepatitis secondary to typhoid fever. Recognition of Salmonella hepatitis is of clinical importance as it can mimic acute viral hepatitis. Early institution of specific therapeutic intervention and supportive care improve the prognosis in these patients.

Keywords: typhoid fever, salmonella hepatitis, typhoid hepatitis.

I. Introduction

Typhoid fever, a type of enteric fever caused by gram-negative bacteria Salmonella typhi. There is various type of bacteria, but S.typhi can only live in human. The usual manifestations are fever, headache, anorexia, abdominal pain, diarrhea, constipation, skin rash, relative bradycardia. It is a common infectious disease of tropics, associated with high morbidity and mortality. Each year, typhoid and paratyphoid fever, respectively, cause an estimated 26 million and 5 million illnesses globally. [1] It spreads through the consumption of contaminated food and water. Populations that have lack access to potable water, and adequate sanitation and hygiene are most affected. Incidence is highest in southern Asia and sub-Saharan Africa [2]. Various authors have highlighted the protean manifestations of this common tropical infection. Systemic complications ranging from intestinal perforation, hepatitis to neurologic manifestations have been well documented [3]. It usually starts as an acute systemic disease without localization and is clinically indistinguishable from other infections, including malaria, bacterial, and viral infections. After being ingested of the contaminated food or water organism invade the intestinal mucosal cell and secret sif A inside host cell. Which manipulate the immune function of the host cell and help multiplication of organism and release toxin and manifest inflammatory feature. Hepatic involvement could be considered important, as it may be associated with a higher relapse rate.[4] However, cholestasis secondary to typhoid fever also reported in a few instances. Typhoid fever usually diagnosed by the presence of an organism in blood and stool and urine. High-risk groups and complicated cases need an antibiotic to treat. Typhoid fever with a clinical picture of acute hepatitis is a rare complication. We report a young patient who presented with low-grade fever, diarrhea, and jaundice and had acute hepatitis secondary to typhoid fever.

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II. Case Report

Our patient, 30 years old serving soldier of a paramilitary force admitted in a tertiary level hospital with a history of low-grade fever and diarrhea of seven days duration on 5 September 2019. He was reasonably well 7 days back. There were no histories of cough, chest pain, dyspnea, abdominal pain, vomiting, dysuria, skin rashes, pruritus, dark color urine, clay color stool, or any hemorrhagic manifestations. He was not known to have any significant diseases like hypertension, diabetes mellitus, ischemic heart disease, or bronchial asthma. There was no history of surgical intervention or tooth extraction and hospital admission. He denied drug abuse, blood transfusion, tattooing, unprotected sexual activity, and intravenous drug intake and alcohol consumption. He did not take any herbal and indigenous medicine. His family history of jaundice, congenital hyperbilirubinemia, and hemolytic anemia was negative. On examination, he was ill-looking, mildly dehydrated, and febrile. There was no edema, cyanosis, clubbing, koilonychia, leuconychia. His vitals were within the normal range. His relevant systemic examination was unremarkable. His routine blood picture shows ESR was 25 mm/h and the CRP was elevated 90 mg/l. ICT for malaria and thick and thin film for malaria parasites were negative. RFT and coagulation profile were normal. LFT shows a mild elevation of transaminase (AST-83U/L, ALT-114 U/L, ALP-196U/L) and S bilirubin (1.6gm/dl). Relevant investigations (Blood C/S, urine C/S, Hepatic viral markers and febrile antigen, NS1 antigen for dengue and serology for leptospirosis) were sent to rule out Viral hepatitis, Enteric fever, Leptospirosis, Dengue fever. The patient was treated empirically with IV fluid and antipyretic. Two days later, he has developed a yellow color of the sclera and vague right upper abdominal discomfort. His liver enzymes showed worsening (AST- 792 U/l, ALT-2893U/L), and LDH level

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was 378 U/L. His jaundice was apparent clinically, with a total bilirubin level of 6.14m mg/dl. The serum ALT: Lactate dehydrogenase (LDH) ratio was < 9. There was no evidence of viral hepatitis, Leptospirosis, and Dengue from the investigation report. The serological test for enteric fever was strongly positive in 1:320 dilution for both "O" and "H" antigens. His blood culture yielded growth of Salmonella typhi after 72 hours incubations, with antibiotic sensitivity to Ciprofloxacin (MIC <0.5 mg/L) and Ceftriaxone (MIC <0.25 mg/L). The strain was resistant to Vancomycin. Injection Ceftriaxone 2 gm twice daily was started after getting blood culture reports. With the above treatment, the patient showed rapid clinical improvement along with a gradual decline in liver transaminases levels. He got released from hospital after twenty days with follow up advice after one month. The patient report to the outpatient department after one month with liver function test which shows normal transaminase level.

III. Discussion

Typhoid fever is a disease of high prevalence in Southeast Asia and Subsaharan Africa. Any febrile patient in this region demands evaluation for Typhoid and Malaria. As this disease manifest without any localization feature, other possibilities like a viral infection, acute enterocolitis, malaria, dengue, flu, other febrile illness needs exclusion before starting treatment. The confirmatory laboratory diagnostic tool is blood culture, other test like urine, feces, duodenal content culture may use but those can be positive in carrier state also. Now a day various diagnostic tools like ELISA test to measure anti lipopolysaccharides antibody, Rapid diagnostic test to detect Ig M & IgG antibody, real-time PCR assay, TUBEX, and TPTEST are used.[5]The factors predisposing to varying degrees of hepatic injury in typhoid fever are not known. Possibly, there is an interplay of the micro-organism factors and immunity, which causes liver injury.[6] Histopathological study and immune histochemistry of the liver biopsy reveals typhoid nodules, cloudy swelling, ballooning degeneration, moderate fatty change, and mononuclear cell infiltrate in few focal areas and intact bacilli in the liver parenchyma. [7] Approximately 21 to 60% patient shows a mild increase of transaminase level without hepatomegaly.[8] severe hepatic involvement in typhoid fever is a rare complication. Hepatic involvement clinically presents with vague discomfort in the right upper abdomen, enlarged liver, hepatic encephalopathy without asterixis. Only 4 to 5% patient shows severe hepatic derangement simulating acute viral hepatitis. [9] Early recognition of this clinical condition and institution of specific therapy is particularly important in tropical countries where malaria and viral hepatitis are quite common and clinical features are indistinguishable.[10] As in our setting typhoid fever and viral hepatitis are common ailment and Salmonella hepatitis is a rare incident so, we like to highlight the case to reduce causality in the future. The timely institution of antimicrobial therapy has reduced typhoid case-fatality rates from 15%–20% to less than 1%. [11]Other clues that raise the possibility of Salmonella hepatitis include fever, relative bradycardia, jaundice at the peak of fever, and left shift of WBCs. The serum ALT/LDH ratio (ALT: LDS <9) is the best discriminator between both entities. In some cases rise alkaline phosphatase and 5-adenosine nucleotidase, hypofibrinogenemia, low prothrombin index and low platelets observed.[12]

IV. Conclusions

Salmonella hepatitis responds well to specific antibiotic therapy and jaundice resolves with clinical improvement within a few days. The clinical course can be severe, with a mortality rate as high as 20%, particularly with delayed treatment or in patients with other complications of salmonella infection. So as Typhoid fever is a common infection in a certain area of the globe, the recognition of salmonella hepatitis and other complication is of clinical importance.

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Bone Marrow Lymphocyte Populations of Innate Immunity in Breast Cancer Patients

By N. N. Tupitsyn, V. A. Mkrtchyan, A. D. Palladina & I. K.Vorotnikov

Abstract- The innate immunity system plays an important role in antitumor protection, and more and more attention has been paid to its study recent years. However, the interrelation of the effect or subpopulations of the cells of the innate immunity in bone marrow with clinical parameters is poorly studied. The paper presents data on the composition of innate immune cells in the bone marrow of 64 patients with operable breast cancer, as well as 10 women with benign processes in the mammary gland. As result a significant correlation between the molecular subtype of cancer and the level of B1- lymphocytes was identified. Breast cancer patients levels of NK cells (CD56 + CD3- and CD16 + CD3-) in the bone marrow were significantly higher in patients with low proliferative activity (Ki-67 less than 20%), compared to patients having a high tumor proliferation index. Populations of NK cells were interrelated with erythropoiesis in patients with breast cancer and were significantly higher in cases of reduced basophilic and polychromatophilicnormoblasts.

Keywords: innate immunity. breast cancer, tcrγδ cells, b1-lymphocytes, nk cells, hematopoiesis.

GJMR-F Classification: NLMC Code: WC 524, WH 380



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Bone Marrow Lymphocyte Populations of Innate Immunity in Breast Cancer Patients

N. N. Tupitsyn [α], V. A. Mkrtchyan [σ], A. D. Palladina [ρ] & I. K.Vorotnikov [ω]

Abstract- The innate immunity system plays an important role in antitumor protection, and more and more attention has been paid to its study recent years. However, the interrelation of the effect or subpopulations of the cells of the innate immunity in bone marrow with clinical parameters is poorly studied. The paper presents data on the composition of innate immune cells in the bone marrow of 64 patients with operable breast cancer, as well as 10 women with benign processes in the mammary gland. As result a significant correlation between the molecular subtype of cancer and the level of B1-lymphocytes was identified. Breast cancer patients levels of NK cells (CD56 + CD3- and CD16 + CD3-) in the bone marrow were significantly higher in patients with low proliferative activity (Ki-67 less than 20%), compared to patients having a high tumor proliferation index. Populations of NK cells were interrelated with erythropoiesis in patients with breast cancer and were significantly higher in cases of reduced basophilic and polychromatophilicnormoblasts. A relationship was also found between bone marrow populations of plasma cells and B1-lymphocytes.

Keywords: innate immunity. breast cancer, tcrγδ cells, b1-lymphocytes, nk cells, hematopoiesis.

I. Introduction

The innate immunity system plays an important role in antitumor immunity, and more and more attention has been paid to its study in recent years. However, the interrelation of the effect or subpopulations of the cells of the innate immunity in bone marrow with flow and prognosis of oncologic diseases is poorly studied. The significance of innate immunity has been proven in sarcomas [1,2], gastric cancer [3], melanoma and other tumors. At present, innate immunity has the leading importance in oncology[4].

A significant number of works have been devoted to studying the characteristics of immunity in breast cancer. The role of tumor-infiltrating lymphocytes and, in particular, CD8 + cells in the prognosis of the disease at early stages (N0) has been proven [5]. Number of antitumor immunity effectors was increased in the bone marrow of patients [6].

Cellular lymphoid effectors of innate immunity are NK cells, B1-(CD5 +) lymphocytes, TCRγδ lymphocytes.

B1-lymphocytes in normal bone marrow can make up 5% of lymphocytes or less. They produce pentameric (IgM) antibodies which recognize tumor cells when interacting with their tumor-associated glycans, transport lipids into the tumor cells, and thus play the role of antibody effectors of innate immunity leading to lipoapoptosis [7,8].

TCRγδ - lymphocytes are considered to be one of the most "mysterious" subpopulations in immunology. They participate in many processes during the immune response, both innate and acquired, but further studies are still needed to uniquely determine their mechanism of action and clinical role. It has been shown that they produce many cytokines, chemokines, are capable of both presenting antigen and cytotoxicity. It is known that a change in the number of TCRγδ-lymphocytes has diagnostic and prognostic significance in some stages of rhabdomyosarcoma in children. [2] The works of the last ten years have shown that antiresorptive drugs (bisphosphonates) lead to an increase in the concentration of TCRγδ-lymphocytes, thereby causing an additional antitumor effect. [9,10]

Specific recognition of tumor cells by antibodies-effectors of innate immunity produced by B1-lymphocytes occurs when these antibodies interact with tumor-associated glycans of malignant cells [7]. Immunodeficiencies based on a deficiency in the blood serum of patients with breast cancer antibodies to tumor-associated Le^c glycan are described [11]. An important role in the mechanism of action of NK cells is played in cases of loss of HLA-I class molecules on cancer cells during tumor progression [5].

The bone marrow is the organ in which the generation and maturation of cells of innate and acquired immunity occurs. Some of them accumulate in the bone marrow, and the levels, as well as the subpopulation of these cells in malignant tumors, differ from the norm. According to our data, both the

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subpopulation of innate immune cells in the bone marrow and erythropoiesis in patients with malignant tumors have a number of peculiarities [12.13]. However, innate immunity in the bone marrow of patients with breast cancer has not been studied. That study is undoubtedly an urgent task, as it will allow a deeper understanding of the immune mechanisms of controlling the prolonged persistence of disseminated tumor cells in the bone marrow of these patients. This, in turn, can serve as the basis for the development of methods for influencing the immune system of the bone marrow in order to eradicate disseminated tumor cells.

II. Materials and Methods

The study was conducted in 64 patients with operable breast cancer. The age of women is from 28 to 77 years, the median is 56 years. 2 women with tumors in situ participated, 20 – with stage IA, 21 – with stage IIA, 10 – with stage IIB, 6 – with stage IIIA, 2 – with stage IIIC, and three patients had their stage which was not determined (treatment to the Oncology Research Center after non-radical operations in other institutions; when the histological preparations were reviewed, the diagnosis was confirmed, but there was no reliable information about the primary

All patients underwent morphological examination of the bone marrow (myelogram).

An immunological study of bone marrow subpopulations was performed by multicolor flow cytometry, the antibody panel for the study is presented in table 1.(Table1)

Table 1: Panel of monoclonal antibodies

Sample No.	Fluorochromes and antibody specificity							
	FITC	PE	PerCP-Cy5	PerCP-Cy7	APC	APC-H7	V450	V500
1.	CD20	CD95	CD27	CD5	CD3	CD19	CD4	CD45
2.	CD22	CD38	CD27	CD5	CD3	CD19	-	CD45
3.	CD4	CD25	CD3	TCRγδ	CD5	CD8	CD2	CD45
4.	CD16	CD45RO	CD3	CD56	CD94	CD8	CD2	CD45
5.	CD16	HLA-DR	CD3	CD56	CD94	CD8	CD2	CD45
6.	CD16	CD7	CD3	CD56	CD94	CD8	CD2	CD45
7.	Perforin	Granzyme	CD3	CD56	CD94	-	-	CD45
8.	CD57	CD26	CD3	CD56	CD94	-	-	CD45

Studies of subpopulations of bone marrow lymphocytes were performed in the gate of CD45 ++ cells with low side light scattering characteristics of the laser beam (SSC low). Samples 1 and 2 are destined to study the innate link of B-cell immunity (B1-cells). Sample 3 is a characteristic of TCR γδ-lymphocytes. Samples 4-6 are a characteristic of NK and NKT lymphocytes. Sample 7 is an assessment of the cytotoxic potential of T cells and NK cells. Sample 8 is additional markers of the characteristics of the subpopulations of T and NK cells.

Cell collection and recording of the corresponding files was performed on a FACSCANTO II flow cytometer. Data analysis was performed using the FCS 3 program.

Statistical data processing was performed using the SPSS program.

III. Results

One of the main tasks of the work was to study the indicators of innate immunity based on the levels of lymphoid cell subpopulations in bone marrow in breast cancer compared with benign processes, as well as to study these subpopulations in breast cancer, depending on the clinical and biological characteristics of the tumor.

From the number of B-cells of innate immunity, we studied mature (CD45 ++) B1-lymphocytes (CD19 +, CD20 +) of bone marrow expressing the CD5 molecule on the membrane. The natural killer cells (NK cells) studied in the work, included 2 subpopulations (CD56 + CD3 - and CD16 + CD3-), of course, mature T-cells (CD3 +) expressing these receptors (CD16, CD56) were studied along with it. In addition, TCRγδ-lymphocytes were studied from the number of T-cells of innate immunity.

Comparison of indicators for breast cancer and benign processes did not reveal significant differences. As a comparison, we evaluated the levels of mature T and B bone marrow lymphocytes in patients with breast cancer, which also did not differ. Only T-cells, of the studied subpopulations, expressing CD16 were slightly higher (differences are close to authentic, $p=0.055$) in patients with breast cancer compared with benign processes in the mammary gland: $3.4\pm0.89\%$ ($n=49$) and $1.45\pm0.4\%$ ($n=9$), $p=0.055$. For the remaining subpopulations, no differences were found.

When analyzing the clinical characteristics of the tumor and bone marrow subpopulations of TCRγδ-lymphocytes and B1-lymphocytes (CD19 or CD20) + CD5 +, we did not establish any correlation between the innate cellular immunity indices and tumor size, N index, histological type of tumor, the fact of lymph nodes damage, and localization metastases (axillary, subclavian, parasternal).

No significant differences were found in the level of bone marrow subpopulations of innate immunity cells in patients with breast cancer depending on the

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receptor status of tumor cells (estrogen, progesterone receptors, Her2 /neu).

Interesting and reliable interrelations between the subpopulation composition of bone marrow lymphocytes were found depending on the levels of proliferative activity of cancer cells (Ki-67), these data are presented in table 2. (Table 2).

Table 2: Subpopulations of cells of innate immunity in the bone marrow, interrelated with the level of proliferative activity (Ki-67) of cancer cells

Subpopulation of lymphocytes	KI67, %	N	Average	Std average error	p
CD56+CD16+ (%% among NK-cells)	>= 20,00	6	77,4500	4,20894	0,021
	< 20,00	4	91,8000	2,59551	
CD16+CD3-	>= 20,00	29	11,0890	1,20889	0,028
	< 20,00	17	15,8312	1,79148	
CD16+CD3+	>= 20,00	18	2,4067	,37303	0,045
	< 20,00	6	6,4883	3,27183	
CD56+CD3-	>= 20,00	30	9,4407	1,10127	0,009
	< 20,00	18	14,5806	1,60064	
CD3+	>= 20,00	32	62,9394	2,04285	NS
	< 20,00	19	62,0311	1,59818	
CD20+	>= 20,00	32	16,7394	1,54649	NS*
	< 20,00	21	13,1729	1,92918	

NS – differences are not statistically significant.

As it can be seen from the table, both studied subpopulations of NK cells (CD16 + CD3-, CD56 + CD3-) were reliably higher in patients with a low proliferative index (less than 20% Ki-67 + of tumor cells). Particularly significant differences were noted in CD56 + CD3-lymphocytes: 9.4% and 14.6%, p = 0.009.

It is interesting to note that the population of bone marrow NK-cells expressing both markers (CD16 + CD56 + CD3-) in all cases of breast cancer was dominant among NK-cells, but significantly prevailed in cancer with low proliferative activity (92% and 77 %, p = 0.021). This is a new feature that has not been previously described. Of course, the number of observations here is small (6 and 4), and it is necessary to continue the collection of material for more reliable information.

It is important to emphasize that the levels of T-lymphocytes expressing the CD16-receptor were significantly higher in patients with low proliferative activity of breast cancer cells. As a comparison, the table shows the levels of mature T and B bone marrow lymphocytes in patients with breast cancer, which did not differ depending on the proliferative index.

Molecular subtypes of breast cancer varied in levels of innate immune cells in the bone marrow. Significant differences were obtained when comparing the levels of B1-lymphocytes with luminal B Her2-negative and luminal B Her2-positive types, p = 0.032. The maximum levels of B1-cells were noted in these cases in the presence of the Her2 receptor (table 3).

Table 3: Levels of the cells of innate immunity in molecular subtypes of luminal B mammary tumors

	Molecular sub type	N	Average	Std average error	p
CD5+B-cells	luminal B (Her2-negative)	20	4,0030	0,79890	0,032
	luminal B (Her2-positive)	10	10,2170	3,60638	
TCRγδ-cells	luminal B (Her2-negative)	17	4,0147	0,52155	0,57
	luminal B (Her2-positive)	12	4,6567	1,11408	

When comparing the luminal B Her2-positive subtype to Her2-positive subtype lacking expression of estrogen and progesterone receptors, the same tendency for B1-lymphocytes remains, however, the data are unreliable (p = 0.066) due to the small number of observations in Her2 + receptor-negative group (n = 2).

Thus, there is a clear selectivity in the bone marrow indices of innate immunity depending on the molecular subtype of breast cancer. The percentage of B1-lymphocytes is the highest with the luminal B Her2-positive subtype.

One of the interesting and promising areas of bone marrow research in recent years has been the

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study of hematopoiesis and, in particular, erythropoiesis in tumors.

Levels of basophilic normoblasts were increased in comparison with the norm in only one patient (1.6%), in most cases (50 out of 62, 80.7%), this indicator was decreased, the normal range of oxyphilicnormoblasts was noted in 11 patients (17,7%). Polychromatophilicnormoblasts were increased in 4 patients (6.5%), decreased in 53.2% of cases and were within normal limits in 25 patients (40.3%). A completely different picture was observed regarding oxyphilicnormoblasts. These cells were increased in most patients (67.7%; 42 patients), and in the remaining cases were within normal limits - 20 patients, 32.3%. In general, the sum of erythroid cells was increased in 8.1% of cases (5 patients), was within normal limits in 36 patients (58.1%) and was reduced in 20 patients (32.3%).

We evaluated how the changes in erythropoiesis are related to the levels of cells of innate immunity, primarily NK-cells in the bone marrow of patients with breast cancer.

It is interesting to note that among the evaluated markers, only subpopulations of NK cells were associated with basophilic normoblast levels. Higher values of NK cells for both indicators were canceled in patients with a decrease in basophilic normoblasts. For the population of CD16 + CD3-, the indices in cases of a decrease in basophilic normoblasts amounted to 13.6 ± 1.2% (n = 36), in cases with a normal content of these cells – 7.9 ± 1.6% (n = 8), p = 0.013. For the population of CD56 + CD3-: 12.2 ± 1.2% (n = 38) and 7.5 ± 1.2% (n = 8), p = 0.012.

Similarly, polychromatophilicnormoblast levels were associated only with these two populations of NK cells. Higher levels of CD16 + CD3-cells were marked with a decrease in polychromatophilicnormoblasts in comparison with those at normal levels of these cells: 15.4 ± 1.3% (n = 26) and 9.1 ± 1.6% (n = 16), p = 0.004. Similar figures for the population of CD56 + CD3-cells: 13.7 ± 1.3% (n=27) and 8.5 ± 1.4% (n=17), p = 0.013.

No significant differences in NK cells were obtained for oxyphilicnormoblasts. A population with coexpression of CD16 and CD56 on NK cells predominated in patients with normal levels of oxyphilicnormoblasts compared with a group of patients with elevated levels of these cells, however, the number of observations was small: 89.7 ± 2.9% (n = 4) and 75 , 4 ± 4.3 (n = 5), p = 0.036.

Thus, our data indicate that NK cells of both subpopulations (CD56 + CD3- and CD16 + CD3-), as well as T / NK lymphocytes with the CD16 + CD3 + phenotype, prevail in patients with breast cancer with low proliferative activity, as the levels of proliferative activity rise, the content of these subpopulations in the bone marrow of patients reduces.

There is an interesting fact of the interrelation of nucleated cells of the erythroid series with the levels of NK cells of the bone marrow of patients with breast cancer. At reduced levels of basophilic and polychromatophilicnormoblasts, the content of NK cells was significantly higher.

When assessing the correlation of TCRγδ-lymphocytes with other subpopulations of bone marrow lymphocytes, reliable interconnections were established only with CD5 + B cells: R = 0.28; p = 0.044; n = 52. It is interesting to note that this subpopulation, as well as TCRγδ lymphocytes, belongs to innate immunity, which is of undoubted interest. These data were obtained by analyzing the entire patient population - breast cancer patients and patients with benign changes in breast tissue. Therefore, it was of interest to evaluate the presence of correlations in these 2 groups separately.

Indeed, there was no correlation between CD5 + B lymphocytes and TCRγδ lymphocytes (p> 0.05) in patients with breast cancer. On the contrary, the correlation between these two subpopulations was very high in patients with benign diseases: R = 0.757; p = 0.03; n = 8. Thus, a kind of “imbalance” occurs between the cells of innate immunity in the bone marrow in breast cancer, and the high correlation of TCRγδ-lymphocytes with CD5 + B-lymphocytes is lost.

However, it is important to keep in mind that despite the high correlation coefficients and the reliability of the relationship, the number of patients in the comparison group with benign processes is small (10 patients), and therefore further accumulation of material is necessary.

The correlation of TCRγδ-lymphocytes with the cell types and indices allocated in the myelogram, as a whole, was absent for the studied group of patients. Similarly, there were no corresponding associations in patients with breast cancer. It is interesting to note that in benign breast diseases, an inverse reliable correlation was established between TCRγδ-lymphocytes and the erythroid cell maturation index: R = -0.688; p = 0.04; n = 9.

In general, a significant correlation between B1-(CD5 +) lymphocytes and eosinophilicmyelocytes (R = 0.331; p = 0.012; n = 57), as well as with plasma cells (R = 0.399; p = 0.002; n = 57) was observed in the examined group. Patients with a reduced or normal content of segmented neutrophils showed significantly higher levels of these cells (CD5 + B-lymphocytes) compared with cases of increased segmented neutrophils: 8.1 ± 1.8% (n = 39) and 2.0 ± 0, 5% (n = 19); p = 0.002. In patients with breast cancer, the same interdependence was noticed: for eosinophilicmyelo-cytes, R = 0.365; p = 0.011; n is 48; for plasma cells, R = 0.409; p = 0.004; n = 48. The average levels of CD5-positive B-lymphocytes were also significantly higher in patients with normal or reduced values of segmented neutrophils in comparison to

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cases of increase in these cells: 8.5 ± 2.0% (n = 33) and 1.7 ± 0.47% (n = 16), p = 0.003. The indicated correlations were not observed in patients with benign processes: the only inverse correlation between the population of CD5 + B-lymphocytes was established with the number of monocytes: R = -0.953; p = 0.002, n = 9.

IV. Discussion

In recent years, innate immunity has attracted a lot of attention from oncologists. The discovery of a specific mechanism for the destruction of tumor cells - lipoapoptosis - marked a new stage in the development of immuno-oncology (8). This can be called a turn to the humoral immunity, or rather - to the innate component of this link of immunity - B1-lymphocytes. It is natural pentamerIg M-antibodies that are able to specifically bind to tumor-associated glycans of cancer cells and transport lipids in them, leading to the death of malignant cells.

Deficiencies of antibodies to tumor-associated glycans in breast cancer have been proven in approximately 35% of cases (14). Breast cancer with the expression of some tumor-associated carbohydrates on the membrane (e.g., Le^C) is characterized by poor prognosis in the early stages (15,16).

Natural IgM antibodies are produced by B1(CD5+)-lymphocytes, for this reason we have paid considerable attention in the work to this particular population of bone lymphocytes.

The role of NK-cells in tumors has been the subject of a large number of publications. In the context of immunophenotypic characteristics, some differences in the subpopulations of NK cells in cancer patients are described. In general, pronounced NK cell tumor infiltration is usually associated with a better prognosis. This has been demonstrated for lung and stomach tumors, colorectal cancer, and head and neck tumors. However, there is evidence that there is no correlation between NK cell levels and prognosis, or even, on the contrary, the association of NK cell infiltration with a more aggressive, advanced stage of the tumor process, in particular with breast cancer [17-20]. Obviously, these contradictions may well be explained by differences in the receptor repertoire of tumor-infiltrating NK cells, which drastically affects their functions. The functional inferiority of NK cell subpopulations revealed in cancer patients is naturally reflected in the change in the immunophenotypic characteristics of NK cell subpopulations. Thus, tumor-infiltrating NK cells of non-small cell lung cancer show a particular immunophenotype and were characterized by weak expression or complete absence of CD57, DHAM, NKp30 NKG2A antigens. While the expression of CD127 was distinct, and an increase in the proportion of these cells was associated with tumor progression [21]. Certain features of the immunophenotype of NK cells isolated from pleural effusion in cancer patients were also identified [22,23]. There is no unified concept regarding tumor-infiltrating NK cells, and their biological features, as well as prognostic significance, require detailed study.

Immature NK cells arise from a precursor in the bone marrow and are characterized by the expression of CD56 + CD94 +/- NKG2A / C-KIR-. Further differentiation consists in increasing expression levels of CD56 ++. At this stage, the cells do not yet express CD16, are characterized as NKG2A +, NKG2C +/-, KIR-. Further, the expression levels of CD56 become weak, CD16 appears; NKG2A + NKG2C +/- KIR +/-. The next stage of differentiation is the occurrence of KIR diversity: cells still express CD16, NKG2A is lost; NKG2C +/-, KIR receptors are stably expressed (KIR +). At the terminal stage of NK cell differentiation, adaptive NKG2C ++ cells similar to memory cells arise. They retain the expression of CD16, NKG2A are absent, the cells are iKIR +. This is the stage of clonal expansion and survival of NK cells [24].

Bone marrow NK cells, which we described in this study in patients with breast cancer, were mainly quite mature cells coexpressing CD56 and CD16, and this fraction was in all cases prevailing among NK cells and significantly more pronounced in patients with a low index of proliferation of tumor cells. It is not entirely clear today whether this means that as breast cancer progresses, levels of effector (CD16-positive) NK cells decrease.

In general, NK cells of both subpopulations (CD56 + CD3- and CD16 + CD3-) decreased as the proliferative activity of breast cancer cells increased, and this parallelism is probably due to coexpression of these molecules on the patient's bone marrow NK cells.

Other patterns are noted for T / NK lymphocytes. Here, a decrease was noted only for cells with the CD16 + CD3 + phenotype (but not CD56 + CD3 +), which prevailed in patients with breast cancer with low proliferative activity, and decreased as the levels of proliferative activity increased.

According to B. Fisher [25], about 35% of breast cancer patients have clinically detectable metastases during the detection of the primary tumor, in addition, another 30–35% of patients have micrometastases, which subsequently manifest clinically. Therefore, the number of studies and publications on macro- and micrometastases of cancer is growing: the detection and study of their correlations with clinical parameters. For this, new methods are used which are much more sensitive than the examinations included in the "gold standard": from PET-CT and MRI studies [26-28] to the study of bone marrow aspirates using multicolor flow cytometry, immunocytochemical [29] and other cytological methods [30,31]. Bone marrow is one of those organs where single tumor cells

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and micrometastases are most often found, both in an active and in a "dormant" state. This is due to the intensity of blood supply to the bone marrow and its components - immune, stromal, hematopoietic cells of different degrees of maturity, to many different growth factors and other cytokines [32]. Therefore, it seems necessary to study the populations of bone marrow cells in cancer patients in the presence and absence of micrometastases.

The study of hematopoiesis in patients with breast cancer revealed a number of patterns that we had noted in earlier studies with squamous cell carcinoma of the head and neck, melanoma, and also with lymphomas [12,13]. A decrease in the populations of basophilic and polychromatophilicnormoblasts and an increase in oxyphilic forms have been established. It is important to note that such observations often occurred in cases of bone marrow involvement in the tumor process, for example, with melanoma [13]. In this study, we did not provide data on the presence of breast cancer micrometastases in the bone marrow; there was no lesion in all cases at the morphological level. A completely new fact described in this work was the establishment of the interrelation of altered erythropoiesis with levels of NK cells in the bone marrow.

In this work, a reliable inversely proportional relation of CD5 + B lymphocytes with myeloid cells — eosinophilicmyelocytes and segmented neutrophils — was shown for breast cancer.

According to the myelogram an increase in the level of plasma cells was significantly more often detected with an increased level of B1-lymphocytes in the bone marrow. It was previously established that the presence of accumulations of plasma cells can be attributed to the earliest manifestation of the presence of tumor cells in the bone marrow: bone marrow micrometastases were immunocytologically determined in 100% of patients in whose punctures accumulations of plasma cells were registered [33]. Thus, it can be assumed that an increase in the level of B1-lymphocytes is associated with a higher probability of micrometastatic damage to the bone marrow by a tumor.

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Cow's Milk Protein Allergy in Children with in herited Epidermolysis Bullosa

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Objective: Evaluate the features of the IgE response to cow's milk protein (CMPA) in children with EB.

Methods: We examined 94 patients with EB - 72 children with dystrophic EB and 22 - with EB simplex. Assessment of the total wound burden and disease severity was based on "The Epidermolysis Bullosa Disease Activity and Scarring Index" (EBDASI). Diagnosis of CMPA was based on a generally accepted set of clinical and laboratory methods (natural history of disease, clinical manifestations, elimination diet, provocation tests).

Keywords: *inherited epidermolysis bullosa, children, cow's milk protein allergy, IgE.*

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Cow's Milk Protein Allergy in Children with inherited Epidermolysis Bullosa

Аллергия к белкам коровьего молока у детей с врожденным буллезным эпидермолизом

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Results: Sensitization to cow's milk proteins, casein, and serum protein is found to be statistically significant for children with a dystrophic EB compared to EB simplex ($p < 0.05$). In the group of children with dystrophic EB, CMPA was diagnosed in 13 patients, which was 18.1%, and clinically significant CMPA was diagnosed in 25% of cases. Food-adverse reactions were detected in two patients with EB simplex (9.1%).

Conclusion: Children with a dystrophic EB are characterized by a higher frequency of sensitization to milk proteins and clinically significant CMPA.

Keywords: inherited epidermolysis bullosa, children, cow's milk protein allergy, IgE.

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Резюме- Обоснование: **Нарушение эпидермального барьера, которое наблюдается у детей с врожденным буллезным эпидермолизом (ВБЭ) с первых дней жизни, может стать причиной сенсибилизации, в том числе пищевой.**

Цель исследования: **оценить особенности IgE-ответа на белки коровьего молока и клинических проявлений аллергии на белки коровьего молока (АБКМ) у детей с ВБЭ.**

Методы: **Обследовано 94 пациента с ВБЭ – 72 ребенка с дистрофической и 22 – с простой формой заболевания. Оценка общей раневой нагрузки на организм и определение тяжести заболевания проводилась на основании «индекса активности заболевания БЭ и рубцевания» (EBDASI). Диагностика АБКМ основывалась на общепринятом комплексе клинико-лабораторных методов (анамнез, характер клинических проявлений, диагностическая безмолочная диета, открытая провокационная проба). Уровень специфических IgE к белкам коровьего молока и его фракциям (казеину, β-лактоглобулину, бычьему сывороточному альбумину) определяли с помощью метода непрямой иммунофлуоресценции (ImmunoCAP 250). Результаты. Сенсибилизацию к белкам коровьего молока, казеину, бычьему сывороточному альбумину выявляли статистически значимо чаще у детей с дистрофической формой ВБЭ по сравнению с простой ($p<0,05$). В группе детей с дистрофической формой ВБЭ АБКМ была диагностирована в 13 наблюдениях, что составило 18,1%, клинически значимая АБКМ – в 25% случаев. Среди детей с простой формой ВБЭ клинические реакции на пищу были выявлены у двух пациентов (9,1%).**

Заключение: **Для детей с дистрофической формой ВБЭ характерна более высокая частота сенсибилизации к молочным белкам и клинически значимой АБКМ.**

Ключевыеслова: врожденный буллезный эпидермолиз, дети, аллергия на белки коровьего молока, IgE.

I. Введение

Врожденный буллезный эпидермолиз (ВБЭ) — это группа редких наследственных заболеваний, для которых характерно нарушение межклеточных связей в эпидермисе или эпидермально-дермальном соединении, приводящее к образованию пузырей на коже и/или слизистых оболочках даже при

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незначительном травмировании [1]. Наиболее тяжелую группу составляют пациенты с дистрофической формой ВБЭ. Основными клиническими проявлениями этой формы заболевания являются пузыри и/или эрозии на коже и слизистых оболочках. Эпителизация эрозивных дефектов может происходить с формированием рубцовой ткани (чаще атрофической) и милиумов [2]. В желудочно-кишечном тракте процессы рубцевания приводят к заращению вестибулярных складок, утрате уздечки, анкилоглоссии, стриктурам пищевода, что сопровождается эпизодами дисфагии, явлениями гастроэзофагеального рефлюкса, запорами [3, 4].

Потеря кожей и слизистыми оболочками барьерных свойств обусловливает избыточное поступление антигенов, в том числе аллергенов пищевого и непищевого происхождения. Однако вопросы пищевой сенсибилизации и пищевой аллергии у данной категории пациентов изучены недостаточно. Так, поиск публикаций (Web of Science и PubMed) позволил обнаружить лишь одну работу H. Marcelo и соавт. [5], в которой впервые описан клинический пример ребенка с дистрофической формой ВБЭ и наличием эозинофильных инфильтратов в сочетании с повышенным уровнем общего IgE. Также опубликовано наблюдение контактного дерматита у ребенка с ВБЭ [6]. В то же время, одновременное нарушение и кожного и эпителиального барьера, которое у детей с ВБЭ имеет место с первых дней жизни, может стать причиной сенсибилизации к такому важному аллергену детского возраста, которым является белок коровьего молока, с последующим развитием клинически значимой аллергии.

Цель исследования: оценить особенности IgE-ответа на белки коровьего молока и клинических проявлений аллергии на белки коровьего молока (АБКМ) у детей с ВБЭ

II. Методы

a) Дизайн исследования

Проведено открытое нерандомизированное наблюдательное проспективное исследование оценки особенностей АБКМ у детей с ВБЭ в период с 2016 по 2018 гг.

b) Критерии включения

дети любого возраста обоих полов с установленным диагнозом буллезного эпидермолиза (Q81.0 — простая форма ВБЭ, Q81.2 — дистрофическая форма ВБЭ).

c) Критерии исключения

отказ родителей/законных представителей ребенка от участия в исследовании.

Исследование проведено на базе отделения кожных болезней Национального медицинского исследовательского центра здоровья детей (Москва). Работа выполнена в рамках научной темы «Оптимизация общих подходов к диагностике, лечению и реабилитации хронических дерматозов у детей». Исследование одобрено локальным Этическим комитетом.

Все дети с ВБЭ проходили комплексное обследование в соответствии с международными регламентирующими документами по ведению больных с данной патологией [1].

Оценка общей раневой нагрузки на организм и определение тяжести заболевания проводилась на основании «индекса активности заболевания БЭ и рубцевания» (EBDASI).

Диетологический анамнез включал в себя информацию о видах вскармливания, сроках введения продуктов и блюд прикорма и реакции на эти продукты, особенностях питания матери во время беременности и лактации, особенностях рациона питания ребенка. Для более эффективного сбора анамнестических данных использовали адаптированную сокращенную форму структурированного вопросника, рекомендованного EAACI для оценки анамнеза при подозрении на пищевую аллергию [7].

При наличии проявлений атопического дерматита верификацию диагноза осуществляли, опираясь на критерии Hanifin и Rajka [8].

При наличии клинических данных за аллергию на белки коровьего молока назначали диагностическую безмолочную элиминационную диету продолжительностью 30 дней. На этот период из рациона исключали все молочные продукты, говядину, телятину, а также другие «подозреваемые» причинно-значимые продукты при наличии соответствующих клинических данных [9]. Через 1 мес проводили открытую провокационную пробу или диагностическое введение молочного продукта [9] для подтверждения или исключения АБКМ. Тактика диетотерапии детей с АБКМ определялась в соответствии с актуальными клиническими рекомендациями [10].

Взятие крови на анализы (генетический, иммунологический) осуществляли в плановом порядке, при заборе крови для осуществления основного плана обследования, без дополнительной венепункции.

С целью верификации диагноза ВБЭ использовали метод массивного параллельного секвенирования. В панель были включены 24 гена, мутации в которых вызывают симптомокомплекс буллезного эпидермолиза. Поиск нуклеотидных последовательностей генов проводился в биоинформационной базе данных NCBI (National Center forBiotechnological Information, USA). Для поиска консервативных участков использована программа BLAST [11]. Последовательности олигонуклеотидов подбирались с помощью программы Beacon Designer 8.10. Проверка специфичности пар праймеров проводилась с помощью программы Primer-BLAST [12]. Диагноз дистрофического буллезного эпидермолиза устанавливался при наличии различных мутаций в гене коллагена 7 типа (COL7A1), простой формы буллезного

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эпидермолиза – при наличии мутаций в генах: KRT5, KRT14, TGM5, DSP, PLEC, COL17A1.

Иммунологические и аллергологические методы обследования включали в себя определение в сыворотке крови содержания общего IgE, уровня специфических IgE (sIgE) к белкам коровьего молока и его фракциям (казеину, β-лактоглобулину, бычьему сывороточному альбумину) с помощью метода непрямой иммунофлуоресценции на автоматическом анализаторе ImmunoCAP250 (UniCAP System, Thermo Fisher Scientific, ранее Phadia AB). Применялись референсные значения для специфических IgE-антител согласно инструкции производителя тест-системы: 0,35-0,7 kUA/l - I (Низкий) класс сенсибилизации, 0,7-3,5 kUA/l II (Средний) класс сенсибилизации, 3,5-17,5 kUA/l, III (Умеренно высокий) класс сенсибилизации,17,5-50 kUA/l – IV(Высокий) класс сенсибилизации, 50-100 kUA/l - V (Очень высокий) класс сенсибилизации, выше 100 kUA/l – VI (Предельно высокий) класс сенсибилизации.

Размер выборки предварительно не рассчитывали. Обработку полученных данных проводили при помощи пакета статистических программ SPSS 20 («IBM», США). Полученные количественные данные проверялись на соответствие нормальному распределению с помощью критерия Шапиро-Уилка. Для выборок, не подчиняющихся нормальному распределению, определяли медиану, а также 25 и 75 перцентили. При оценке различий для признаков, распределение которых отлично от нормального, использовали критерий Манна-Уитни. Разницу значений считали значимыми при $p < 0,05$.

III. Результаты

a) Объекты (участники) исследования

В исследование были включены 94 пациента с ВБЭ: 72 детей с дистрофической формой ВБЭ в возрасте от 11 месяцев до 16 лет 2 месяцев, из них мальчиков 45,8%, и 22 ребенка с простой формой ВБЭ в возрасте от 2 месяцев до 9 лет 2 месяцев, из них мальчиков 45,4% (табл. 1).

Таблица 1: Общая характеристика больных ВБЭ [*Me* (Q_1; Q_3)]

Характеристика	Простая форма ВБЭ (n=22)	Дистрофическая форма ВБЭ (n=72)
Возраст, годы	5,6 (2,11; 7,3)	8,9 (3,6; 13,9)
Индекс EBDASI	12,3 (7,1; 16,4)	109,7 (78,2; 143,9)

b) Особенности IgE-ответа в подгруппах детей с ВБЭ в зависимости от формы заболевания

Содержание общего IgE в группе детей с простой формой ВБЭ не соответствовало возрастной норме у 3 детей, при этом не превышало 1000 kUA/l. У одного из детей с повышенным уровнем IgE была диагностирована пищевая аллергия, у другого — бытовая сенсибилизация.

В группе пациентов с дистрофической формой ВБЭ уровень общего IgE превышал возрастные показатели в 36 (50%) наблюдениях, при этом у 10 (13,8%) детей уровень IgE был в выше 1000 kUA/l (рис. 1), у 6 — выше 3000 kUA/l. Для большинства детей с дистрофической формой ВБЭ с повышенным уровнем IgE были характерны более высокие значения площади поражения кожи, площади инфицированных участков кожи.

c) Характер сенсибилизации к молочным белкам у детей с различными формами ВБЭ

Частота выявления повышенных уровней специфических IgE у детей с простой и дистрофической формой ВБЭ представлена в табл. 2.

Таблица 2: Частота выявления сенсибилизации у детей с простой и дистрофической формой ВБЭ

Аллерген	Простая форма ВБЭ (n=22)		Дистрофическая форма ВБЭ (n=72)		*p**
	sIgE, Me (25; 75)	частота сенсибилизации	sIgE, Me (25; 75)	Частота сенсибилизации	
Молоко	0,04 (0,02; 0,11)	1 (4,5%)	0,09 (0,05; 0,27)	12 (16,6%)	0,013
α-лактальбумин	0,015 (0,01; 0,02)	1 (4,5%)	0,04 (0,02; 0,1)	11 (15,3%)	0,009
β-лактоглобулин	0,02 (0,01; 0,06)	0	0,04 (0,02; 0,07)	11 (15,3%)	0,21
Казеин	0,02 (0,01; 0,04)	0	0,04 (0,02; 0,1)	6 (8,3%)	0,009

*Примечание. * — статистически значимые различия между номинальными значениями sIgE, вычисленные с помощью критерия Манна — Уитни.*

Выявление повышенных уровней sIgE к пищевым белкам при отсутствии явной клинической картины АБКМ расценивалось как сенсибилизация. Ребенку назначалась диагностическая безмолочная диета с последующим диагностическим введением молочного продукты (открытая провокационная проба), на основании чего или подтверждалась, или исключалась клинически значимая АБКМ.

Оценка клинических проявлений пищевой аллергии у детей с различными формами ВБЭ.

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Анализ наследственности в группе детей с дистрофической формой ВБЭ и АБКМ выявил, что лишь у 6 детей имелся отягощенный семейный анамнез по аллергическим заболеваниям.

Учитывая особенности клинической картины буллезного эпидермолиза, заключающиеся в поражении как кожного покрова, так и слизистых оболочек ротовой полости и кишечника, верификация диагноза пищевой аллергии у данной категории пациентов весьма затруднительна. Тем не менее при тщательном сборе анамнеза, а также выявлении четкой причинно-следственной связи появления симптомов с приемом того или иного вероятного причинно-значимого продукта удается выделить спектр подозреваемых пищевых белков. Большинство родителей пациентов указывали на усиление зуда при приеме определенных продуктов или появление новых, не характерных для основного заболевания, высыпаний. Важную дополнительную информацию давали результаты диагностической элиминационной диеты и открытой провокационной пробы. В результате пищевая аллергия была диагностирована у 2 (9,1%) детей с простой формой и у 18 (25%) детей с дистрофической формой ВБЭ. В группе детей с *дистрофической формой ВБЭ* IgE-опосредованная АБКМ была диагностирована в 13 наблюдениях, что составило 18,1%. Проявления АБКМ выражались в виде кожных и гастроинтестинальных симптомов. Усиление зуда после употребления того или иного молочного продукта, появление пятнисто-папулезной сыпи при дистрофической форме ВБЭ отмечалось у 18,1%. У 4 детей с дистрофической формой заболевания имелись кожные проявления, соответствующие критериям диагностики атопического дерматита (5,5%). Проявления атопического дерматита купировались или значительно регрессировали после исключения из питания ребенка причинно-значимого продукта.

Гастроинтестинальные проявления АБКМ – срыгивания, разжиженный, непереваренный стул, стул со слизью, на фоне приема молочных продуктов отмечались при дистрофической форме ВБЭ 16,6% детей. Важно также отметить, что после назначения безмолочной диеты у 4 детей с АБКМ прошли запоры, которые рассматривались до этого как проявление основного заболевания.

В группе детей с дистрофической формой заболевания помимо АБКМ отмечалась также аллергия на злаки (n = 5), фрукты и овощи (n = 5), яйца (n = 3).

Среди детей с простой формой ВБЭ клинические реакции на молочные продукты были выявлены у двух пациентов. У обоих детей молочный белок был единственным причинно-значимым аллергеном. Симптомы АБКМ носили эпизодический характер, купировались исключением причинно-значимого продукта.

sIgE к молочному белку и его фракциям выявлялись на уровнях, соответствующих классу сенсибилизации от низкого до умеренно высокого (табл. 3). В ряде случаев делалось заключение о наличии не-IgE-опосредованной аллергии на молочные белки.

Таблица 3: Характер распределения классов сенсибилизации sIgE к пищевым аллергенам в группе детей с дистрофической формой ВБЭ и клиническими проявлениями АБКМ (число детей).

Аллерген	Класс сенсибилизации						
	0	I	II	III	IV	V	VI
Молоко	4	1	3	2	2	1	0
α-лактальбумин	3	3	2	1	2	0	0
β-лактоглобулин	5	1	2	2	1	1	0
Казеин	5	1	3	0	2	0	0

IV. Обсуждение

Дистрофические формы буллезного эпидермолиза характеризуются значительным повреждением кожных покровов и у подавляющего числа больных - также и слизистой оболочки ЖКТ. Это приводит одновременно к нарушению барьерных свойств кожи и ЖКТ и создает условия для избыточного поступления во внутреннюю среду организма антигенов, в том числе и пищевого происхождения. Механизм развития транскутанной сенсибилизации сложен и включает в себя следующие основные моменты. В начале происходит проникновение аллергена через дефектный эпидермальный барьер и его воздействие на кератиноциты эпидермиса, которые затем в ответ синтезируют и выделяют провоспалительные цитокины (IL1β, IL6, IL33) и тимусный стромальный лимфопоэтин (TSLP), которые активируют врожденный иммунный ответ, стимулируют процессы созревания дендритных клеток и инициируют дифференцировку наивных Т-клеток преимущественно в клетки Th2-типа [13]. Стоит отметить важную роль TSLP, заключающуюся также во влиянии на миграцию дендритных клеток к коже (индукция хемокиновых рецепторов CCR7 и CXC через CXCR4-зависимый путь) и выделении активированными TSLP дендритными клетками важного медиатора кожной сенсибилизации – OX40L [14]. Th2-лимфоциты, в свою очередь, экспрессируют ряд аллергенспецифических цитокинов (IL4, IL5, IL10, IL13), стимулирующих созревание и дифференцировку В-лимфоцитов в IgE-продуцирующие плазматические клетки [15].

Поскольку молочный белок является одним из основных белков из «большой восьмерки» аллергенов, с

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которым ребенок сталкивается с первых месяцев жизни, представляло интерес изучение частоты сенсибилизации к белкам коровьего молока и особенностей клинических проявлений аллергии на молочные белки у детей с ВБЭ. Аллергия на белки коровьего молока имеет важное клиническое значение, особенно в раннем детском возрасте, когда от правильного подбора смеси в значительной степени зависит успех всего лечения.

Настоящее исследование подтвердило, как высокую частоту сенсибилизации к белкам коровьего молока, так и высокую частоту клинически значимой аллергии у детей с ВБЭ. АБКМ была выявлена у 25% детей с дистрофической формой ВБЭ, в 18,1% случаев она носила IgE-опосредованный характер (72,2% от всех детей с клинически значимой АБКМ). У детей с простой формой ВБЭ клинически значимая АБКМ выявлена в 9,1% случаев. Сенсибилизацию к белкам коровьего молока, казеину, бычьему сывороточному альбумину диагностировали статистически значимо чаще у детей с дистрофической формой ВБЭ по сравнению с простой ($p<0,05$). Такая высокая встречаемость сенсибилизации к белкам коровьего молока и АБКМ среди детей с данной формой заболевания, по всей видимости, связана с обширными нарушениями целостности кожных покровов, их воспалением, а также поражением слизистой оболочки желудочно-кишечного тракта; что в свою очередь обусловливает избыточное поступление антигенов, в том числе пищевых аллергенов, и, как следствие, — формирование пищевой сенсибилизации [16, 17].

Аллергологическое обследование выявило также высокие уровни общего IgE у 50% детей с дистрофической формой ВБЭ, что встречалось статистически значимо чаще, чем в группе детей с простой формой ВБЭ, $p<0,05$. Причина обнаружения очень высоких - выше 1000 kUA/l (у 10 детей - 13,8%) и крайне высоких уровней (более 3000 kUA/l у 6 детей) общего IgE требует дальнейшего изучения.

Анализ клинических данных показал, что пищевая аллергия вносит свой вклад в клиническую картину заболевания и должна обязательно диагностироваться и учитываться в комплексной терапии этой сложной категории больных. Наличие сенсибилизации к пищевым аллергенам при отсутствии клинической картины пищевой аллергии также достаточно часто встречается у больных с дистрофической формой ВБЭ и при отсутствии реакций не требует исключения молочных продуктов из рациона ребенка. Напротив, клинически подтвержденная диагностической диетой и открытой провокационной пробой АБКМ, даже при отсутствии сенсибилизации к молочным белкам, должна трактоваться в соответствии с клинической картиной как не-IgE-опосредованная форма пищевой аллергии [9,10].

Следует отметить, что вопросы пищевой сенсибилизации и пищевой аллергии у данной категории больных не изучены. Полученные нами данные говорят о том, что наличие пищевой аллергии следует активно выявлять и учитывать при составлении рациона и осуществлении нутритивной поддержки этих тяжелых больных.

Ограничением исследования являются в первую очередь небольшая группа пациентов, и это объясняется тем, что ВБЭ относится к редким заболеваниям. Еще одним ограничением является то, что в настоящей работе проанализированы клинический и иммунологический ответ только на один пищевой продукт – коровье молоко, как основной аллерген детского возраста. Особенности реакций на другие продукты, а также на антигены окружающей среды требуют дальнейшего изучения.

Представляем вашему вниманию клинический случай, демонстрирующий развитие сенсибилизации и формирование пищевой аллергии у ребенка с дистрофической формой врожденного буллезного эпидермолиза.

Девятимесячная девочка поступила в отделение дерматологии с диагнозом: рецессивный дистрофический ВБЭ, белково-энергетическая недостаточность умеренной степени (рис. 1, 2, 3). ВБЭ был диагностирован с рождения, имеется подтверждение мутации в гене COL7A1. С трехмесячного возраста на коже наблюдались эритематозно-сквамозные высыпания, сопровождающиеся выраженным зудом и усиливающиеся при добавлении в рацион новых продуктов. Кожный патологический процесс носил распространенный характер (была поражена кожа головы, лица, щеки, уши, подбородок, шея, туловище, верхние и нижние конечности, руки и ноги) и был представлен пятнами, волдырями, эрозиями, корками, чешуйками, рубцами. Кроме того, у этого пациента отмечался сниженный аппетит. Мы использовали систему ImmunoCAP 250 для определения уровня специфических IgE к следующим пищевым аллергенам: молоко, казеин, яичный белок, говядина, свинина, мясо индейки, пшеница, ячмень, глютен, соя, дрожжи, яблоко, банан, груша.

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Рисунок 1, 2, 3: Пациентка в возрасте 9 месяцев. Кожный патологический процесс распространенный, имеет симметричный характер, представлен сливающимися эритематозными ярко-красными пятнами неправильной формы, четкими границами, с экссудацией, пузырями с прозрачным содержимым, эрозиями неправильной формы, экскориациями, корками смешанного характера. Отмечается мелкопластинчатое шелушение.

В результате была выявлена сенсибилизация к белкам коровьего молока, яйцу и глютену. Общий уровень IgE в сыворотке был> 3000 ед/мл. После назначения элиминационной безмолочной, безбелковой диеты с использованием гипоаллергенной смеси на основе аминокислот наблюдалась выраженная положительная динамика. У пациента наблюдалось улучшение как нутритивного статуса, так и кожного покрова: прибавка в весе за 7 дней составила 380 г (8 г/кг/день); также было отмечено значительное

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уменьшение зуда и выраженности эритематозного компонента высыпаний.

Благодаря комплексной терапии отмечалось значительное улучшение. Однако, высокий уровень сенсибилизации к пище в дальнейшем сохранялся (таблица 4), несмотря на соблюдение элиминационной диеты (рис. 4, 5).

Таблица 4: Результаты теста ImmunoCAP 250 через 5 лет

ImmunoCAP (аллерген)	Results
Пищевая смесь (треска, креветки, голубая мидия, тунец, лосось)	Positive
Смесь грибковых аллергенов mx2 (Penicilium notatum, Cladosporium herbarum, Aspergillus fumigatus, candida albicans, Altermaria alternata, Helm.hal.)	Positive
Смесь домашней пыли hx2 (hollister-stier labs,dermatophagoides pteronyssinus, dermatophagoides farinae, blatella germanica)	Positive
Коровье молоко	53,2 kUA/l (V)
Свинина	2,27 kUA/l (II)
Говядина	21,8 kUA/l (IV)
Мясо кролика	4,48 kUA/l (III)
Мясо индейки	52,7 kUA/l (V)
Треска	0,86 kUA/l (II)
Дрожжи	0,86 kUA/l (II)
Пшеница	>100 kUA/l (VI)
Ячмень	51,7 kUA/l (V)
Банан	15,1 kUA/l (III)
Груша	15,1 kUA/l (III)





Рисунок 4, 5: Та же пациентка спустя 5 лет. Кожный патологический процесс распространенный, симметричный, представлен сливающимися между собой эритематозными пятнами неправильной формы с четкими границами, расположен на коже туловища и конечностей. Отмечается умеренная лихенификация в области сгибов верхних конечностей

V. Заключение

По-видимому, из-за редкости заболевания и небольшого количества пациентов с дистрофической формой ВБЭ, данные по коморбидности пищевой аллергии и ВБЭ не суммировались и не представлены в научной литературе (Web of Science и PubMed). Согласно полученным нами данным, коморбидность пищевой аллергии и ВБЭ достаточно характерна для дистрофических форм заболевания. Наличие коморбидной АБКМ приводит к утяжелению течения основного заболевания и требует внимания со стороны

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специалистов, занимающихся данной патологией. Важно, что несостоятельность кожного барьера у детей может приводить к сенсибилизации не только пищевыми аллергенами, но и аллергенами других групп, как показано в приведенном клиническом примере, И это требует дальнейшего изучения.

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Конфликт интересов

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12. Know what you know: Always try to know what you know by making objectives, otherwise you will be confused and unable to achieve your target.

13. Use good grammar: Always use good grammar and words that will have a positive impact on the evaluator; use of good vocabulary does not mean using tough words which the evaluator has to find in a dictionary. Do not fragment sentences. Eliminate one-word sentences. Do not ever use a big word when a smaller one would suffice.

Verbs have to be in agreement with their subjects. In a research paper, do not start sentences with conjunctions or finish them with prepositions. When writing formally, it is advisable to never split an infinitive because someone will (wrongly) complain. Avoid clichés like a disease. Always shun irritating alliteration. Use language which is simple and straightforward. Put together a neat summary.

14. Arrangement of information: Each section of the main body should start with an opening sentence, and there should be a changeover at the end of the section. Give only valid and powerful arguments for your topic. You may also maintain your arguments with records.

15. Never start at the last minute: Always allow enough time for research work. Leaving everything to the last minute will degrade your paper and spoil your work.

16. Multitasking in research is not good: Doing several things at the same time is a bad habit in the case of research activity. Research is an area where everything has a particular time slot. Divide your research work into parts, and do a particular part in a particular time slot.

17. Never copy others' work: Never copy others' work and give it your name because if the evaluator has seen it anywhere, you will be in trouble. Take proper rest and food: No matter how many hours you spend on your research activity, if you are not taking care of your health, then all your efforts will have been in vain. For quality research, take proper rest and food.

18. Go to seminars: Attend seminars if the topic is relevant to your research area. Utilize all your resources.

19. Refresh your mind after intervals: Try to give your mind a rest by listening to soft music or sleeping in intervals. This will also improve your memory. Acquire colleagues: Always try to acquire colleagues. No matter how sharp you are, if you acquire colleagues, they can give you ideas which will be helpful to your research.

20. Think technically: Always think technically. If anything happens, search for its reasons, benefits, and demerits. Think and then print: When you go to print your paper, check that tables are not split, headings are not detached from their descriptions, and page sequence is maintained.

21. Adding unnecessary information: Do not add unnecessary information like "I have used MS Excel to draw graphs." Irrelevant and inappropriate material is superfluous. Foreign terminology and phrases are not apropos. One should never take a broad view. Analogy is like feathers on a snake. Use words properly, regardless of how others use them. Remove quotations. Puns are for kids, not grunt readers. Never oversimplify: When adding material to your research paper, never go for oversimplification; this will definitely irritate the evaluator. Be specific. Never use rhythmic redundancies. Contractions shouldn't be used in a research paper. Comparisons are as terrible as clichés. Give up ampersands, abbreviations, and so on. Remove commas that are not necessary. Parenthetical words should be between brackets or commas. Understatement is always the best way to put forward earth-shaking thoughts. Give a detailed literary review.

22. Report concluded results: Use concluded results. From raw data, filter the results, and then conclude your studies based on measurements and observations taken. An appropriate number of decimal places should be used. Parenthetical remarks are prohibited here. Proofread carefully at the final stage. At the end, give an outline to your arguments. Spot perspectives of further study of the subject. Justify your conclusion at the bottom sufficiently, which will probably include examples.

23. Upon conclusion: Once you have concluded your research, the next most important step is to present your findings. Presentation is extremely important as it is the definite medium though which your research is going to be in print for the rest of the crowd. Care should be taken to categorize your thoughts well and present them in a logical and neat manner. A good quality research paper format is essential because it serves to highlight your research paper and bring to light all necessary aspects of your research.

Informal Guidelines of Research Paper Writing

Key points to remember:

- Submit all work in its final form.
- Write your paper in the form which is presented in the guidelines using the template.
- Please note the criteria peer reviewers will use for grading the final paper.

Final points:

One purpose of organizing a research paper is to let people interpret your efforts selectively. The journal requires the following sections, submitted in the order listed, with each section starting on a new page:

The introduction: This will be compiled from reference matter and reflect the design processes or outline of basis that directed you to make a study. As you carry out the process of study, the method and process section will be constructed like that. The results segment will show related statistics in nearly sequential order and direct reviewers to similar intellectual paths throughout the data that you gathered to carry out your study.

The discussion section:

This will provide understanding of the data and projections as to the implications of the results. The use of good quality references throughout the paper will give the effort trustworthiness by representing an alertness to prior workings.

Writing a research paper is not an easy job, no matter how trouble-free the actual research or concept. Practice, excellent preparation, and controlled record-keeping are the only means to make straightforward progression.

General style:

Specific editorial column necessities for compliance of a manuscript will always take over from directions in these general guidelines.

To make a paper clear: Adhere to recommended page limits.



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Mistakes to avoid:

- Insertion of a title at the foot of a page with subsequent text on the next page.
- Separating a table, chart, or figure—confine each to a single page.
- Submitting a manuscript with pages out of sequence.
- In every section of your document, use standard writing style, including articles ("a" and "the").
- Keep paying attention to the topic of the paper.
- Use paragraphs to split each significant point (excluding the abstract).
- Align the primary line of each section.
- Present your points in sound order.
- Use present tense to report well-accepted matters.
- Use past tense to describe specific results.
- Do not use familiar wording; don't address the reviewer directly. Don't use slang or superlatives.
- Avoid use of extra pictures—include only those figures essential to presenting results.

Title page:

Choose a revealing title. It should be short and include the name(s) and address(es) of all authors. It should not have acronyms or abbreviations or exceed two printed lines.

Abstract: This summary should be two hundred words or less. It should clearly and briefly explain the key findings reported in the manuscript and must have precise statistics. It should not have acronyms or abbreviations. It should be logical in itself. Do not cite references at this point.

An abstract is a brief, distinct paragraph summary of finished work or work in development. In a minute or less, a reviewer can be taught the foundation behind the study, common approaches to the problem, relevant results, and significant conclusions or new questions.

Write your summary when your paper is completed because how can you write the summary of anything which is not yet written? Wealth of terminology is very essential in abstract. Use comprehensive sentences, and do not sacrifice readability for brevity; you can maintain it succinctly by phrasing sentences so that they provide more than a lone rationale. The author can at this moment go straight to shortening the outcome. Sum up the study with the subsequent elements in any summary. Try to limit the initial two items to no more than one line each.

Reason for writing the article—theory, overall issue, purpose.

- Fundamental goal.
- To-the-point depiction of the research.
- Consequences, including definite statistics—if the consequences are quantitative in nature, account for this; results of any numerical analysis should be reported. Significant conclusions or questions that emerge from the research.

Approach:

- Single section and succinct.
- An outline of the job done is always written in past tense.
- Concentrate on shortening results—limit background information to a verdict or two.
- Exact spelling, clarity of sentences and phrases, and appropriate reporting of quantities (proper units, important statistics) are just as significant in an abstract as they are anywhere else.

Introduction:

The introduction should "introduce" the manuscript. The reviewer should be presented with sufficient background information to be capable of comprehending and calculating the purpose of your study without having to refer to other works. The basis for the study should be offered. Give the most important references, but avoid making a comprehensive appraisal of the topic. Describe the problem visibly. If the problem is not acknowledged in a logical, reasonable way, the reviewer will give no attention to your results. Speak in common terms about techniques used to explain the problem, if needed, but do not present any particulars about the protocols here.

The following approach can create a valuable beginning:

- Explain the value (significance) of the study.
- Defend the model—why did you employ this particular system or method? What is its compensation? Remark upon its appropriateness from an abstract point of view as well as pointing out sensible reasons for using it.
- Present a justification. State your particular theory(-ies) or aim(s), and describe the logic that led you to choose them.
- Briefly explain the study's tentative purpose and how it meets the declared objectives.

Approach:

Use past tense except for when referring to recognized facts. After all, the manuscript will be submitted after the entire job is done. Sort out your thoughts; manufacture one key point for every section. If you make the four points listed above, you will need at least four paragraphs. Present surrounding information only when it is necessary to support a situation. The reviewer does not desire to read everything you know about a topic. Shape the theory specifically—do not take a broad view.

As always, give awareness to spelling, simplicity, and correctness of sentences and phrases.

Procedures (methods and materials):

This part is supposed to be the easiest to carve if you have good skills. A soundly written procedures segment allows a capable scientist to replicate your results. Present precise information about your supplies. The suppliers and clarity of reagents can be helpful bits of information. Present methods in sequential order, but linked methodologies can be grouped as a segment. Be concise when relating the protocols. Attempt to give the least amount of information that would permit another capable scientist to replicate your outcome, but be cautious that vital information is integrated. The use of subheadings is suggested and ought to be synchronized with the results section.

When a technique is used that has been well-described in another section, mention the specific item describing the way, but draw the basic principle while stating the situation. The purpose is to show all particular resources and broad procedures so that another person may use some or all of the methods in one more study or referee the scientific value of your work. It is not to be a step-by-step report of the whole thing you did, nor is a methods section a set of orders.

Materials:

Materials may be reported in part of a section or else they may be recognized along with your measures.

Methods:

- Report the method and not the particulars of each process that engaged the same methodology.
- Describe the method entirely.
- To be succinct, present methods under headings dedicated to specific dealings or groups of measures.
- Simplify—detail how procedures were completed, not how they were performed on a particular day.
- If well-known procedures were used, account for the procedure by name, possibly with a reference, and that's all.

Approach:

It is embarrassing to use vigorous voice when documenting methods without using first person, which would focus the reviewer's interest on the researcher rather than the job. As a result, when writing up the methods, most authors use third person passive voice.

Use standard style in this and every other part of the paper—avoid familiar lists, and use full sentences.

What to keep away from:

- Resources and methods are not a set of information.
- Skip all descriptive information and surroundings—save it for the argument.
- Leave out information that is immaterial to a third party.



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Results:

The principle of a results segment is to present and demonstrate your conclusion. Create this part as entirely objective details of the outcome, and save all understanding for the discussion.

The page length of this segment is set by the sum and types of data to be reported. Use statistics and tables, if suitable, to present consequences most efficiently.

You must clearly differentiate material which would usually be incorporated in a study editorial from any unprocessed data or additional appendix matter that would not be available. In fact, such matters should not be submitted at all except if requested by the instructor.

Content:

- Sum up your conclusions in text and demonstrate them, if suitable, with figures and tables.
- In the manuscript, explain each of your consequences, and point the reader to remarks that are most appropriate.
- Present a background, such as by describing the question that was addressed by creation of an exacting study.
- Explain results of control experiments and give remarks that are not accessible in a prescribed figure or table, if appropriate.
- Examine your data, then prepare the analyzed (transformed) data in the form of a figure (graph), table, or manuscript.

What to stay away from:

- Do not discuss or infer your outcome, report surrounding information, or try to explain anything.
- Do not include raw data or intermediate calculations in a research manuscript.
- Do not present similar data more than once.
- A manuscript should complement any figures or tables, not duplicate information.
- Never confuse figures with tables—there is a difference.

Approach:

As always, use past tense when you submit your results, and put the whole thing in a reasonable order.

Put figures and tables, appropriately numbered, in order at the end of the report.

If you desire, you may place your figures and tables properly within the text of your results section.

Figures and tables:

If you put figures and tables at the end of some details, make certain that they are visibly distinguished from any attached appendix materials, such as raw facts. Whatever the position, each table must be titled, numbered one after the other, and include a heading. All figures and tables must be divided from the text.

Discussion:

The discussion is expected to be the trickiest segment to write. A lot of papers submitted to the journal are discarded based on problems with the discussion. There is no rule for how long an argument should be.

Position your understanding of the outcome visibly to lead the reviewer through your conclusions, and then finish the paper with a summing up of the implications of the study. The purpose here is to offer an understanding of your results and support all of your conclusions, using facts from your research and generally accepted information, if suitable. The implication of results should be fully described.

Infer your data in the conversation in suitable depth. This means that when you clarify an observable fact, you must explain mechanisms that may account for the observation. If your results vary from your prospect, make clear why that may have happened. If your results agree, then explain the theory that the proof supported. It is never suitable to just state that the data approved the prospect, and let it drop at that. Make a decision as to whether each premise is supported or discarded or if you cannot make a conclusion with assurance. Do not just dismiss a study or part of a study as "uncertain."

Research papers are not acknowledged if the work is imperfect. Draw what conclusions you can based upon the results that you have, and take care of the study as a finished work.

- You may propose future guidelines, such as how an experiment might be personalized to accomplish a new idea.
- Give details of all of your remarks as much as possible, focusing on mechanisms.
- Make a decision as to whether the tentative design sufficiently addressed the theory and whether or not it was correctly restricted. Try to present substitute explanations if they are sensible alternatives.
- One piece of research will not counter an overall question, so maintain the large picture in mind. Where do you go next? The best studies unlock new avenues of study. What questions remain?
- Recommendations for detailed papers will offer supplementary suggestions.

Approach:

When you refer to information, differentiate data generated by your own studies from other available information. Present work done by specific persons (including you) in past tense.

Describe generally acknowledged facts and main beliefs in present tense.

The Administration Rules

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Please read the following rules and regulations carefully before submitting your research paper to Global Journals Inc. to avoid rejection.

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Topics	Grades		
	A-B	**C-D**	**E-F**
Abstract	Clear and concise with appropriate content, Correct format. 200 words or below	Unclear summary and no specific data, Incorrect form Above 200 words	No specific data with ambiguous information Above 250 words
Introduction	Containing all background details with clear goal and appropriate details, flow specification, no grammar and spelling mistake, well organized sentence and paragraph, reference cited	Unclear and confusing data, appropriate format, grammar and spelling errors with unorganized matter	Out of place depth and content, hazy format
Methods and Procedures	Clear and to the point with well arranged paragraph, precision and accuracy of facts and figures, well organized subheads	Difficult to comprehend with embarrassed text, too much explanation but completed	Incorrect and unorganized structure with hazy meaning
Result	Well organized, Clear and specific, Correct units with precision, correct data, well structuring of paragraph, no grammar and spelling mistake	Complete and embarrassed text, difficult to comprehend	Irregular format with wrong facts and figures
Discussion	Well organized, meaningful specification, sound conclusion, logical and concise explanation, highly structured paragraph reference cited	Wordy, unclear conclusion, spurious	Conclusion is not cited, unorganized, difficult to comprehend
References	Complete and correct format, well organized	Beside the point, Incomplete	Wrong format and structuring

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