Recent Developments in Medicine and Medical Research

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Editor(s)

Dr. Pietro Scicchitano

Department of Cardiology, Hospital “F. Perinei” Altamura (BA), Italy.
Email: piero.sc@hotmail.it, pietrosc.83@libero.it;

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Preface

This book covers key areas of medicine and medical research. The contributions by the authors include microdermabrasion, intralesional steroid, aluminium oxide crystals, exclamation marks, cardiac PR-interval, Cardiac QT-interval, electrical cardiac systole, palpitations, tachycardias, Sudden cardiac death, snoring, obstructive sleep apnea, chronic unreduced shoulder dislocation, neglected dislocation, conservative shoulder treatment, open shoulder reduction, bone marrow cells, fibrosis, electromagnetic radiation, apoptosis, cells "lifespan", isolated hepatomegaly, liver abscess, congestive cardiac failure, hepatocellular carcinoma, Ischemic heart disease, radionuclide imaging, gastric cancer, bone metastasis, repeated biopsy, lung cancer, tumor characterization, traumatic fibroma, fosfomycin, disc – diffusion, agar dilution, nutrigenomics, nutrition, periodontal disease, nutrient-gene interactions, manual liquid based cytology, cervix, immunomarkers, conventional Pap smear, meningitis, epidemics, Neisseria meningitides, neurons, artificial intelligence, robotic surgery, prototypes, Human MGMT gene, mobile genetic elements, composite cluster structures, regulatory elements, promoter, alternative promoters, Skincare products, nanoparticle. This book contains various materials suitable for students, researchers and academicians in the field of medicine and medical research.

Chapter 1
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A New Combination Therapy for Scalp Alopecia Areata: An Approach to High Negative Pressure Microdermabrasion with Intralesional Steroids

M. I. Sheikh[1*]

DOI: 10.9734/bpi/rdmmr/v1/4025F

ABSTRACT

This paper aims to present a study of a relatively new combination procedure to treat AA. In our study we combined high negative pressure microdermabrasion (MD) with intra lesional steroid (ILST) injection. We compared the results of this combination with ILST alone also. Alopecia Areata (AA) is an auto immune condition that affects the skin's hair-bearing areas. The prevalence of alopecia areata has been rising in recent years. This condition affects 0.1-0.2%2 of humans, occurring in both men and women. AA occurs in people who are apparently healthy and have no existing skin disorders. Local irritants, such as capsicum lotion, topical steroids, and oral immuno suppressants, as well as oral and injectable steroids, are already in use. Hair regrowth is variable, either uniform or bushes-like, and recurrence is common.

Keywords: Alopecia areata; microdermabrasion; intralesional steroid; aluminium oxide crystals; exclamation marks

ABBREVATIONS:

MD : Microdermabrasion
ILST : Intra Lesional Steroid

1. INTRODUCTION

In AA the affected skin usually appears like smooth bald skin spots (spot baldness) which may show broken hair known as short stubs (exclamation marks) [1]. In this combination therapy, during each session MD was carried out followed by ILST [2,3].

1.1 Microdermabrasion Can Result in

a. Very effective and controlled irritation of scalp (contact immunotherapy).
b. Cleansing of plugged follicular canals.
c. Assistance in uniform distribution of injected ILST.
d. Improves blood flow (indirectly helping immunotherapy).

The ILST: This provides immunosuppression [4–6] and is given in a dose according to the area of scalp involved.

2. MATERIALS AND METHODS

This combination therapy of MD followed by ILST constituted one session. Each patient had 6 sessions every 15-20 days. MD was performed with aluminium oxide crystals with a specially

[1]Consultant dermatologist, Alhamd Skin Clinic, Pakistan.
**Corresponding author: E-mail: iftikhar96@hotmail.com;*

designed hand piece (made in Pakistan) that had a wide hole at its tip, suctioning almost 1cm square chunk of skin and shot a crystal jet in a fan like movement abrading a fine layer of skin. The wide bore tip of hand piece of MD machine moved linearly over AA area of scalp at slow speed with high negative pressure (Fig. 1).

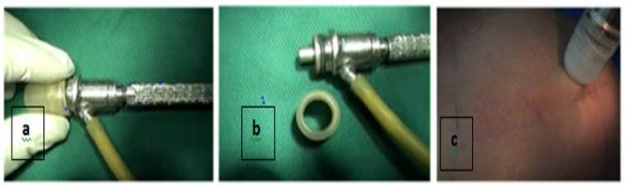

Fig. 1. The specially designed hand piece for MD, with wide hole at tip, suctioning almost 1cm square chunk of skin due to high negative pressure and abrading fine layer of skin by shooting crystal and producing very affective skin irritation (A) Hand Piece. (B) Wide Hole Tip. (C) High Negative Pressure Suctioning Skin While Hand Piece Moving Linearly.

This was followed by injection of 0.02cc (0.8mg) of undiluted ILST to an approximately 2 inch square area or AA on the scalp with 30G needle (Fig. 2).

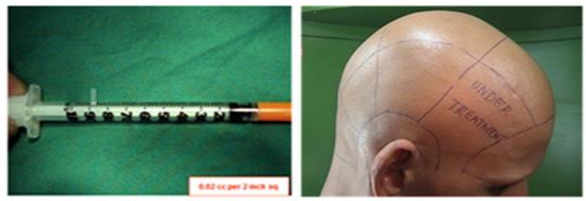

Fig. 2. The syringe shows the amount of undiluted ILST between 2 adjasent white arrows, and the scalp shows the area of AA divided into 2" square boxes. 0.02cc (0.8mg) of ILST is given into the center of each square

Inclusion criteria:

a. Willing patient only and otherwise healthy.
b. Non scaring alopecia.
c. No concomitant infection.

Exclusion criteria:

a. Patients with unhealthy skin of the scalp due to eczema, fungus or other infections.
b. Scarring alopecia.
c. Pregnancy.
d. Diabetes.

Pre-procedure:

a. Photos of all patients were taken at the start and during treatment sessions for reference, to assess the progress, to record improvement and to evaluate the result of this treatment.
b. All patients not gave any other medicine.
c. All patients given detailed information about MD, ILST and consent taken.

So a study of this combination was done in 70 patients, in both sexes, in different age groups from January 2013 to January 2016. A comparison was also done with ILST alone (Table 1).

Table 1. This table shows the study protocol

Study period proposed 3 year	**From January 2013 to January 2016, follow up continued to date**
Total number of patient 70	50 patients treated with combination of MD+ILST
Males 45 female 25	20 patients treated with ILST alone
Age 5-50 years	Mean age 20 years
Treatment duration 3-5 months	Mean treatment duration 4 months
Each patient had six sessions every 15 to 20 days.	

3. RESULTS AND DISCUSSION

With our combination treatment (MD+ILST), all patients had remarkable improvement. Hair regrowth was usually obvious after third or fourth session. There was usually enough growth in 5-6 sessions. The regrowth of hair was fast full and very uniform (Fig. 3).

Fig. 3. The combination of MD+ILST gives uniform and full growth (after 4th Session)

With ILST alone the growth was slower, less uniform, like bushes, unevenly distributed, with areas of no grow (Fig. 4) (Fig. 5).

Fig. 4.The ILST alone without MD gives uneven hair regrowth like bushes

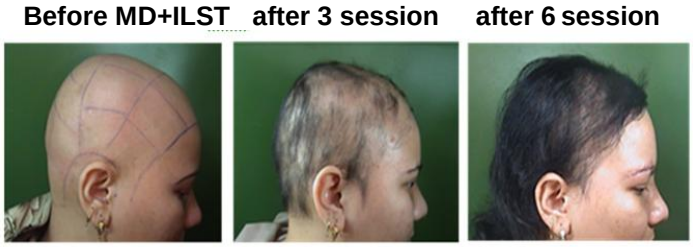

Fig. 5. The combination of MD+ILST

3.1 We Proposed Result Criteria as Following

a. Complete response (80-100%) All patches full, uniform re growth and no recurrence before 6 months.

b. Partial response (50-80%) Most patches had enough growth, but the growth was not full, less uniform and there was recurrence in less than 6 months.
c. No response (<50%) Hair growth was not optimum and new lesions continued.

3.2 Our Results of MD + ILST for the 50 Patients Were

a. Complete response in 35 patients (70%).
b. Partial response in 10 patients (20%).
c. No response in 5 patients (10%).Table 2.

Table 2. Recurrence rate after stopping treatment

Recurrence for all 50 patients (MD+ILST)		
After 4-6months	After 6-12months	After 12-24months
5 Patients (10%)	9 Patients (18%)	**12** patients (24%)

4. CONCLUSION

Our results indicate that MD+ILST are safe and very effective treatment for AA. MD produces controlled irritation of bald skin, suctioned follicular plugs, improved blood flow and enabled uniform distribution of ILST. The ILST provides immunosuppression. The recurrence rate after stopping treatment is from 10-24% from 6 months to 24 months.

ACKNOWLEDGEMENTS

Special thanks to Shehla Bajwa, Rabia Nisar and Zainab Nisar for their untiring efforts in making this study.

COMPETING INTERESTS

Author has declared that no competing interests exist.

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Chapter 2
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Breijo's Pattern: A Shortened of Cardiac Electrical Activity

Francisco Ramón Breijo- Márquez[1*]

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ABSTRACT

The decrease of the cardiac electrical systole – short PR and QTc intervals in the same electrocardiogram, also known as "Electrocardiographic Breijo Pattern" - is increasingly studied by several authors. The vast majority of the time it can be overlooked in an electrocardiogram tracing. People who had this kind of electrocardiographic pattern had also suffered from a wide variety of symptoms. Nocturnal tachycardias, dizziness, seizures, and unexplained syncopal accesses were the main symptoms common to all patients. More than 127 cases have been studied and cross-checked by our research team so far. Its diagnosis is indispensable in eluding of the most heartbreaking consequence: avoidable death. Despite the fact that for many authors, the cardiac electrical systole comprises only from the beginning of the Q wave to the end of the T wave – that is, depolarization and repolarization of the ventricles, the atria are also part of it. Consequently, the P wave, as well as the PR segment, must be a part of the electrical cardiac systole. When there is a shortening of the PR interval along with a shortening of the QT interval, we should talk about: Decrease of cardiac electrical systole.

Keywords: Cardiac PR-interval; Cardiac QT-interval; electrical cardiac systole; palpitations; tachycardias; Sudden cardiac death.

1. INTRODUCTION

This peculiar electrocardiographic pattern is denominating the Breijo Pattern:

A PR interval less than 0.120 seconds along with a QTc interval less than 0.360 seconds.

It is typical in this type of patients, carriers of the Breijo pattern, some common peculiarities in all of them.

1. Unspecific symptoms that are considered mild, such as:

   a) Palpitations, usually nocturnal, which awaken the patient from the natural sleep. Profuse nocturnal sweating.
   b) Light-headedness feelings misinterpreted.

2. A perception of chest pain very unspecified, not irradiated and whose electrocardiographic study is regarded, in the vast majority of cases, as untypical or normal, since coronary alterations are not observed.
3. A personal background, in childhood, of seizures treated with anti-epileptic drugs without the presence of any brain disorder on the electroencephalogram.
4. Low levels of lythemia. (This is a typical and constant feature on all patients).
5. A preference for young age (up to 40) and male sex.

[1]Department of Clinical Cardiology, Boston Hospital, USA.
**Corresponding author: E-mail: frbreijo@gmail.com;*

2. MAIN DOCUMENT

In 2008, Breijo-Marquez et al. [1,2,3] presented an electrocardiographic pattern, in which both the PR and QT intervals were shorter in milliseconds than what is regarded as acceptable limits.

They called this phenomenon as *"Decrease of electrical cardiac systole"* [1] since both, depolarization and repolarization, atrial and ventricular, are lower in their standard lengths. (PR interval and QT interval). Both the short PR interval and the short QT interval are being extensively studied and sought in routine practice on a daily basis – but always as isolated entities, not together in the same ECG tracing [4,5].

It is well known that, in an electrocardiogram, there are different waves, intervals, and segments. These are the follows:

A/Waves: P, Q, R, S, T. (U-wave in some occasions) B/ Intervals: PR (for other PQ authors). QRS. QT. RR. C/ Segments: ST fundamentally.

In spite of the repeated repetition of the image, we put it below in an attempt to gain a better understanding (Fig. 1)

The P- wave reflects atrial depolarization (contraction).

The PR- interval corresponds to the delay between the end of atrial depolarization (contraction) and the beginning of ventricular depolarization (contraction); its length must be between 0.120 seconds and 0.200 seconds. The Q wave is a negative deflection in the ECG resulting at the beginning of ventricular depolarization (first wave in QRS complex). The T wave is a reflection of ventricular repolarization.

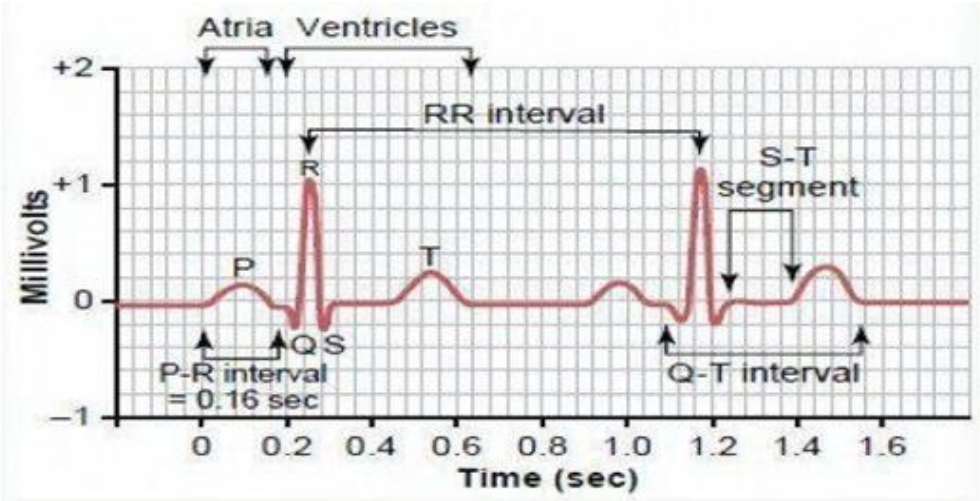

Fig. 1. Normal electrocardiogram tracing: Waves. Intervals and Segments

The QT interval includes a complete ventricular depolarization and repolarization (full ventricular cycle); its length must be between 0.400 and 0.450 seconds (depending on authors and their conveniences, since some authors have studied and published in different journals what the correct length of the QTc interval should be. Even they have not agreed with their different conclusions. We agree to Gollop, these values may vary; for us and with a broader context, the standard QTc values are between 0.400 and 0.450 seconds in length).

There are many formulas to measure the amount of these ranges; the most used are Bazett and Fridericia yet.

Like the R-R interval, the QT interval is dependent on the heart rate in an obvious way (the faster the heart rate, the shorter the R-R Interval and QT interval) and may be adjusted to improve the detection of patients at increased risk of ventricular arrhythmia. The length of the PR interval, of the QRS complex, of the ST segment and the corrected QT interval, are very all- important and must be valued in all cases. The PR interval must be greater than 120 milliseconds and lower than 200 milliseconds. Otherwise, we would find a "short PR" if this is fewer than 120 milliseconds). If greater than 200 milliseconds would be the denominated like an Auricle-ventricular block in any of its variants. The QRS complex should have a maximum length of 0.10 seconds. If it were longer lasting, we would be in front of a branch block in its different modalities (complete or incomplete).

Several formulas are used to correct the QT interval (QTc). The most used are those of Bazett and Fridericia.

However, for these authors, typical values would be between 0.40 and 0.44 seconds, regardless of the person's age and sex. The discrepancies among the different authors about the typical values of corrected QT are immense. The great controversy that persists to this date is about which should be considered as an average length of the QT interval since it is related to the heart rate; that is, the QT value is frequency-dependent. These controversies are producing an authentic catastrophe when it comes to cataloging when it is or not a short QTC [6-10]. For us,- according with Gollop [11] any QT value corrected interval less than 0.360 seconds must be considered as "short QT". The most commonly used formulas are as follows (Table 1).

When the lengths of the different waves, intervals, and segments are greater or lesser than the values considered as normal, the heart is much more vulnerable to deadly arrhythmias (any of these may be truly lethal, and accesses to ventricular fibrillation may develop). As we have already written, Breijo et al. published a new electrocardiographic pattern consisting of a short PR and QT intervals in the same electrocardiogram tracing.

People who had this kind of electrocardiographic pattern had also suffered from a wide variety of symptoms. Nocturnal tachycardias, dizziness, seizures, and unexplained syncopal accesses were the main symptoms common to all patients. Absolutely all them were diagnosed as people with epilepsy and treated with specific drugs for epilepsy all patients; however, the electroencephalographic registers did not provide any visualization for epileptic focus in any of assessed patients [1]. Of course, the results with such treatment were null.

Table 1. QT heart rate correction formulas

QT Heart Rate Correction Formulas	
Exponential	Formula
Bazett	QT/ RR1/2
Fridericia	QT/ RR1/3
Linear	Formula
Framingham	QT + 0.154 (1-RR)
Hodges	QT + 1.75 (HR- 60)

The patient age ranged from 16 to 40 years. The male gender was predominant. All previous electrocardiographic studies were considered within average ranges (the shortening of either the PQ interval or corrected QT interval went unnoticed).

As we have previously written, the typical features of the Breijo pattern are: 1/ A PR interval of fewer than 120 milliseconds (short PR) 2/ A QTc interval fewer than 360 millisecond. Both on the same electrocardiographic tracing.

We agree with Gollop et al. [11] on when the QTc interval duration ought to be considered as "short". Gollob has written over 61 cases of Short QT Syndrome. Their cohort of 61 cases was predominantly male (75.4%) and had a mean QTc value of 0.306 seconds with values ranging from 0.248 to 0.381 seconds in symptomatic cases. For Gollob et al., the overall median age at clinical

presentation was 21 years [IQR: 17 to 31.8 years) with a value of 20 years (IQR: 17 to 29 years) in males and 30 years (IQR: 19 to 44 years) in females].These authors developed, about the ECG characteristics of the general population, and in consideration of clinical presentation, family history and genetic findings, a highly sensitive diagnostic using a scoring system. This "scoring system" includes (Table 2).

We have seen cases of a short QT interval (QTc ≤ 0.350 seconds) in asymptomatic patients and without a positive family history thereto for congenital (and non-genetic) character.

We also think is worthy to be mentioned an interesting paradoxical ECG phenomenon called *deceleration-dependent shortening of QT interval* (shortening of QT interval associated with a decrease in heart rate) should also be considered in a differential diagnosis [1-3]. In order to know precisely if the corrected QT value-by the different existing formulas-is in ranges, we use the Boston diagram**,** which we present below (Fig. 2).

3. THE ELECTROCARDIOGRAPHIC"BREIJO PATTERN"

As we have written in earlier pages, the first case of Breijo Pattern was published in the International Journal of Cardiology in 2008 [1]. The patient was a 37-year-old male, born in Mexico, D.F. Since his childhood, he had suffered from tonic-clonic seizures and was treated with antiepileptic drugs (concretely with valproic acid) but without any epileptogenic focus showing up on his electroencephalogram. Since then, the patient has referred multiple accesses of nocturnal palpitations, accompanied by intense sweating. Unstable gait sensation. He liked to play sports, but at the minimum effort, he was impressed anew severe palpitations that impede him to continue with it.

Table 2. Gollop's score Patients are deemed high-probability (≥ to 4 points), intermediate probability (3 points) or low probability (≤ 2 points)

QTc in milliseconds	
<370	1
<350	2
<330	3
J point-T peak interval	
<120	1
Clinical History	
Sudden cardiac arrest	2
Polymorphic VT or VF	2
Unexplained syncope	2
Atrial fibrillation	2
Family History	
1st or 2nd degree relative to SQTS	2
1st or 2nd degree relative to sudden death	1
Sudden infant death syndrome	1
Genotype	
Genotype positive Mutation of undetermined signif- icance in a culprit gene	2
	1

The patient was very worried about his heart. and visited numerous specialists in the field. He underwent a lot of diagnostic tests, and all of them were considered as in average range. The doctors believed him to be a patient with intense anxiety and hypochondriasis. The patient had reported about two attacks of total loss of consciousness without loss of sphincters. This was regarded as a vase-vagal etiology. A thorough compilation of patients with this kind of symptoms such as childish convulsions without an adequate response to conventional treatment for epilepsy, bouts of nocturnal tachycardia with sudden character, and syncopal events related to the effort. An exhaustive study of personal antecedents and of his current clinical situation was made. An exhaustive measurement of intervals, segments, and electrocardiographic waves also was made. By

way of example, we will expose the electrocardiogram tracing of this patient: A 37-year-old man with much nocturnal tachycardia crisis (since childhood) and three syncopal events observed and related to physical stress. In his family background, two sudden deaths were found: father died at age 55 years of sudden cardiac, and a brother died at 22 months by sudden infant death. He was diagnosed in his Reference Hospital (where he was transferred by emergency services) with supraventricular tachycardia to 195- 200 beats per minute, with narrow QRS complexes. Severe diaphoresis, with the paleness of skin and mucous. A severe arterial hypotension to 90/50 mm. Hg. Cardiac auscultation was in normal ranges but with a rapid rhythm. Tachypnea to 20 cycles/minute. A grade Stuporous (Glasgow 15/15). The neurological examination was within normal ranges without focalizations. Central and peripheral pulses were palpable, symmetric and synchronous. The patient was diagnosed with nonspecific supraventricular tachycardia (Fig. 3).

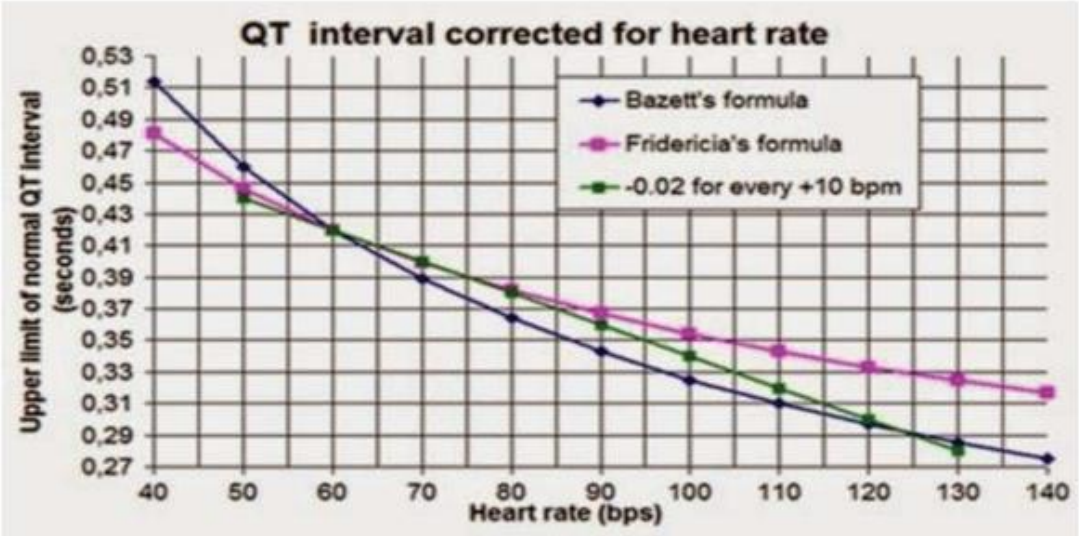

Fig. 2. Boston diagram. Heart rate in bpm (coordinates) and length of QT interval (in abscissa) are perfectly exposed. Of all the current layouts, this is the one we consider as the most reliable and the most accurate

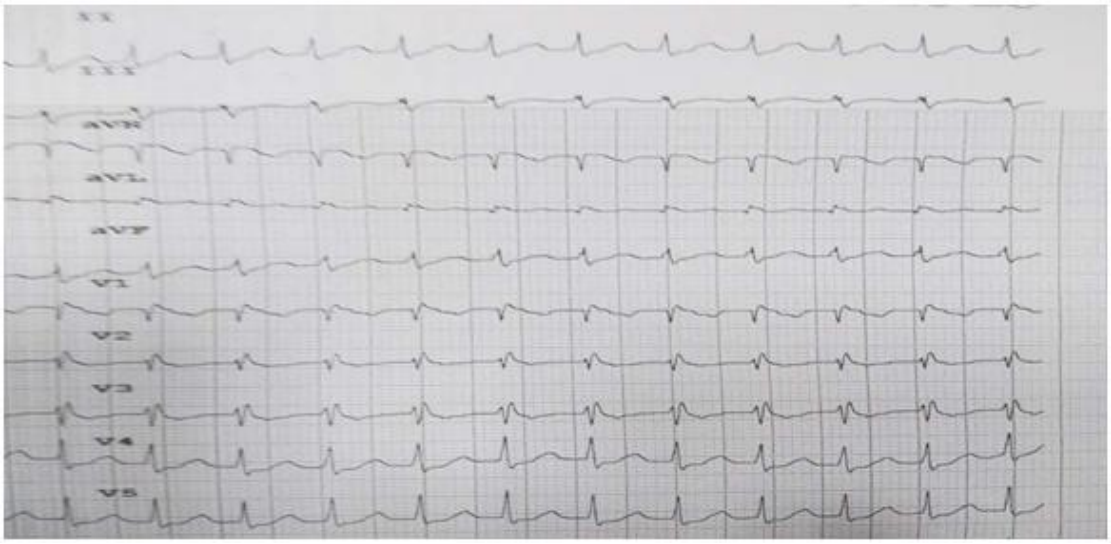

Fig. 3. This supraventricular tachycardia disappeared using the administration of two doses of Adenosine iv

In bolus, with six mgrs. each one in 1 minute (Fig. 4). A Hospital discharge was made after full stabilizing of acute process and patient was derived from your cardiologist outpatient, with the following diagnosis. A paroxysmal supraventricular tachycardia and Crisis of anxiety: The patient was transferred to our Hospital because he had a similar event as the exposed, after the first visit with his outpatient cardiologist. There, the patient was adequately assessed with electrocardiogram, echocardiogram, blood levels of ions and cardiac markers as well as electrophysiological study (EEF) (Fig. 4). He was negative for high levels of Troponin (I- T), CK, CPK-MB and however he was positive for low levels of lithium-ion (<0.1 mEq/L). Nevertheless, in an in-depth and careful study of his basal electrocardiogram, we were able to assess the existence of a short PR and QTc interval. Below, we present the first electrocardiogram of the patient that we were able to assess. (Despite the fact that we practice a full series of tests on the patient, the most significant in this exposure is the electrocardiography and the Holter studies) (Figs. 5, 6).

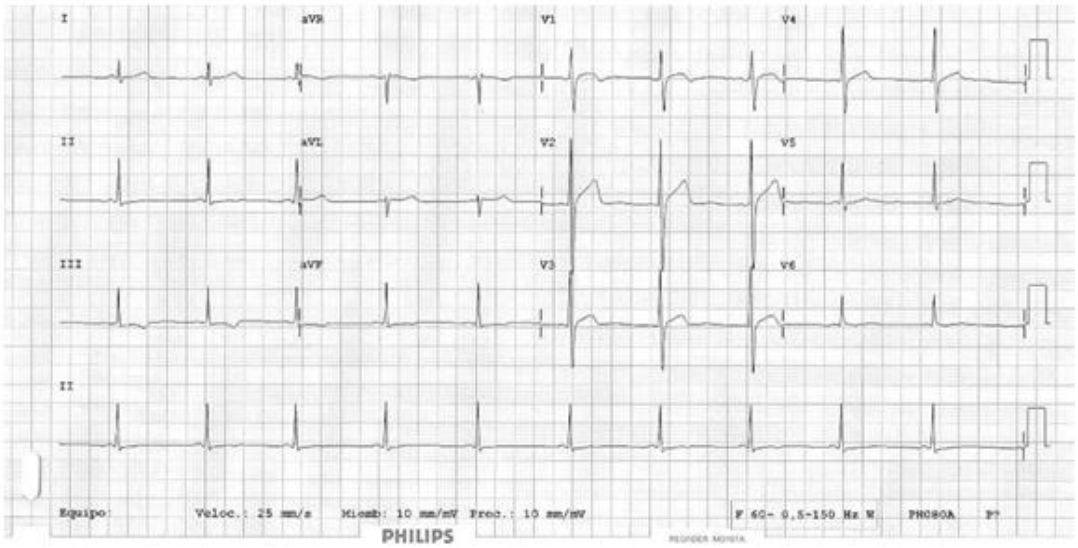

Fig. 4. Electrocardiographic tracing after administering Adenosine. The full basal electrocardiogram tracing to 60 bpm can be seen the short PR-interval (< 0.120 seconds) together to the short QT-interval (< 0.350 seconds.). Chiefly in inferior and precordial leads

4. DIFFERENTIAL DIAGNOSIS

A Differential diagnosis is imperative to do it so with any electrocardiographic entity that has a shortened PR interval.

These are, fundamentally (Table 3)

1. Wolff-Parkinson-White (W. P. W.).
2. Low-Ganong-Levine (L.G.L.).
3. Mahaim.

A Breijo Pattern along with a *Wellens Pattern* can be valued in the image [12, 13].

The "Broken heart syndrome" (Takotsubo) and the Breijo Pattern are correctly appreciated in the following image. [14, 15]. This "Breijo Pattern" we have assessed both in isolation and associated with other kinds of cardiac pathologies. Such as *"Wellens Pattern", Wolf -Parkinson- White syndrome* and in "*Takotsubo's Disease".* As it can be seen in the images below (Fig. 7).

The *"Broken heart syndrome"* (Takotsubo) and the Breijo Pattern are correctly appreciated in the following image [14-15](Fig. 8).

Table 3. Differential diagnosis, based on the characteristics of the different intervals and complex

ENTITY.	PR-interval.	QRS complex.	QTc -interval
WPW	Short.	Wide (δ-wave)	Normal.
L.G.L	Short.	Normal.	Normal.
Breijo Pattern	Short.	Normal.	Short.
Mahaim	Normal or Short.	Normal or wide	Normal.

We have also known the existence of a *Wolf-Parkinson-White syndrome* associated with an electrocardiographic Breijo pattern**,** as can be seen below [16,17] (Fig. 9).

5. SOME SIGNIFICANT IMAGES, TYPICAL OF THE BREIJO PATTERN

A typical image of a Breijo Pattern in precordial left leads. Measured PR interval: 0.988 seconds Calculated QTc interval: (Table 4).

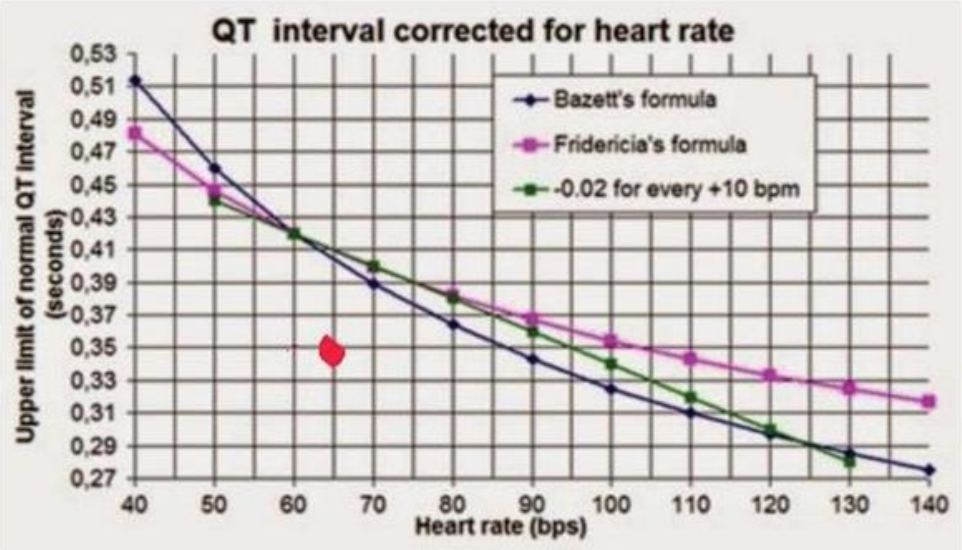

Fig. 5. On the Boston Diagram, it would be 350 milliseconds (red marked)

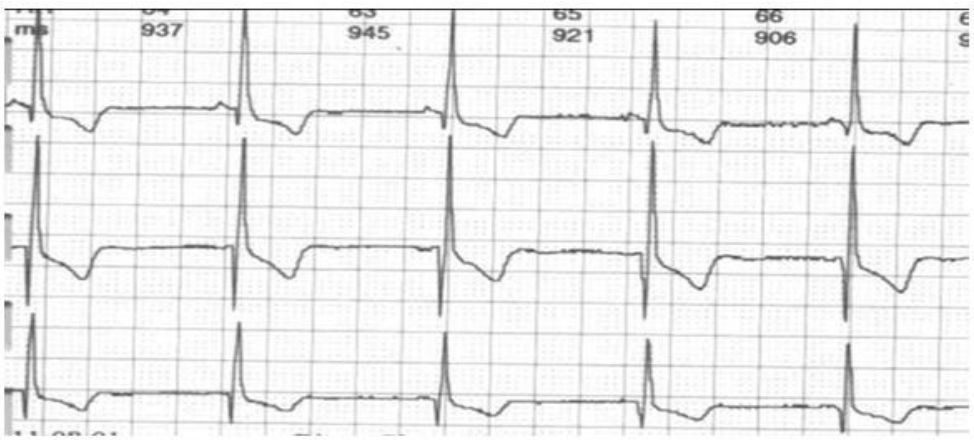

Fig. 6. Holter Study Same features than Fig 1. PQ-interval: 0.100-0.110 seconds=Short PQ-interval. QTc (Bazzet) 0.339-0.340 seconds (< 0.350 seconds) = Short QT-interval. QTc (Fridericia) 0.332 seconds (< 0.350 seconds) = Short QT-interval

In the Boston Diagram at 68 bpm (Figs. 10, 11). The last electrocardiogram performed with a Breijo Pattern, in a male person who unfortunately died due to not being able to be recovered from a sudden death.

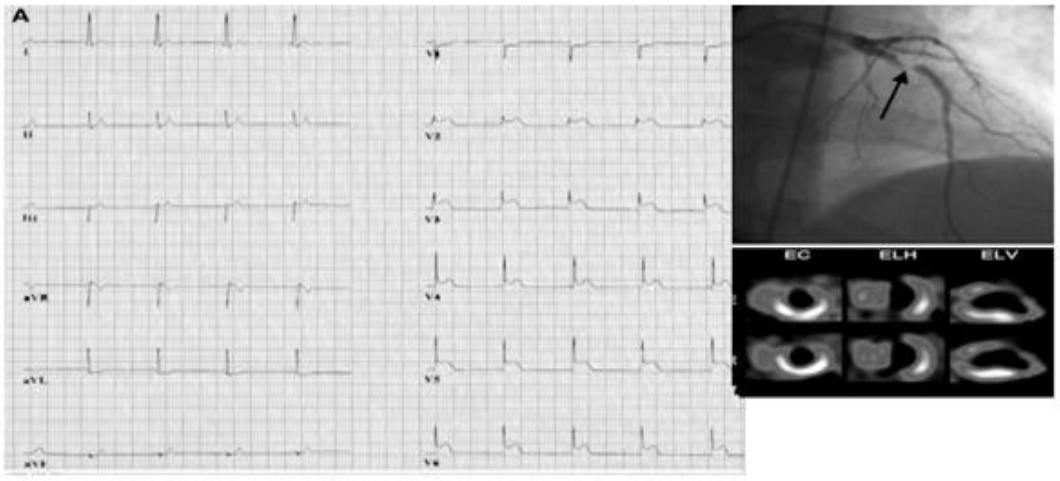

Fig. 7. A Breijo Pattern along with a *Wellens Pattern* can be valued in the image. [10-11]

Table 4. Calculated QTc interval

RR	**0.882352941**	**seg**
QTc (Rautaharju)	390	mseg
QTc (Bazett)	347	mseg
QTc (Framingham)	326	mseg
QTc (Friderica)	339	mseg
QTc (Call)	342	mseg

The electrocardiographic tracing was considered as within acceptable limits and his doctors decided to send him home (Fig. 12).

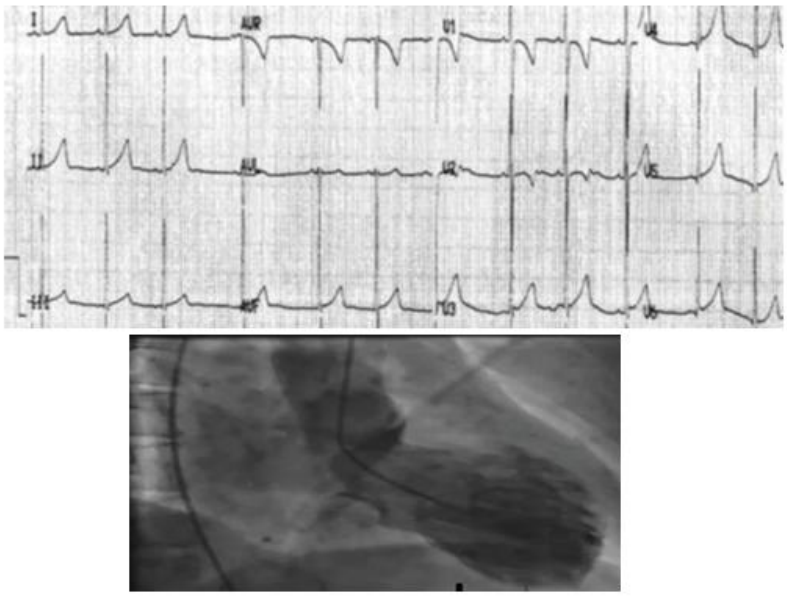

Fig. 8. A Takotsubo disease alongside a Breijo Pattern

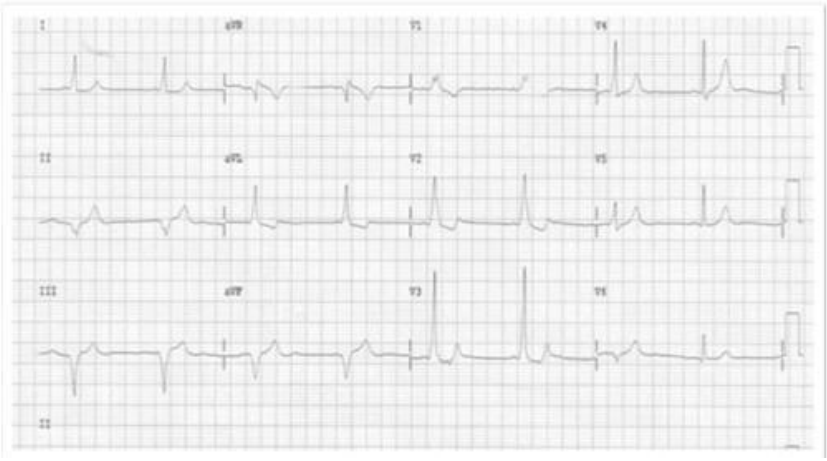

Fig. 9. A *WPW* alongside a Breijo Pattern can be perfectly seen in the image. [14- 15]

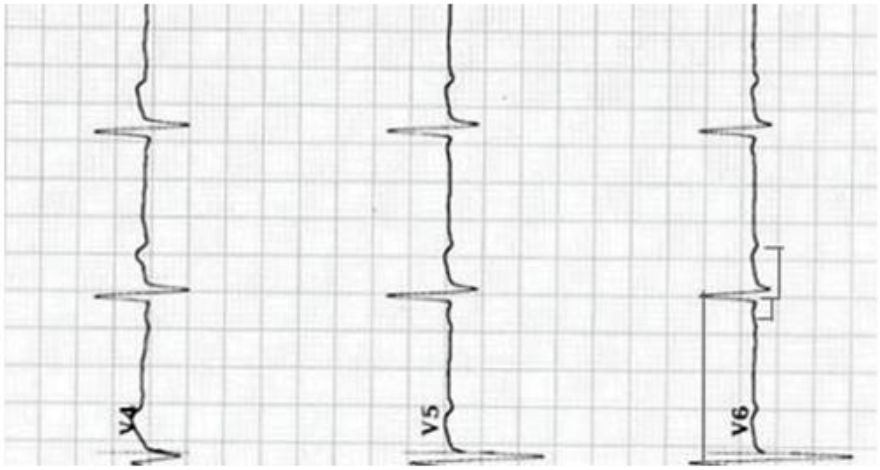

Fig. 10. Detail in precordial left leads

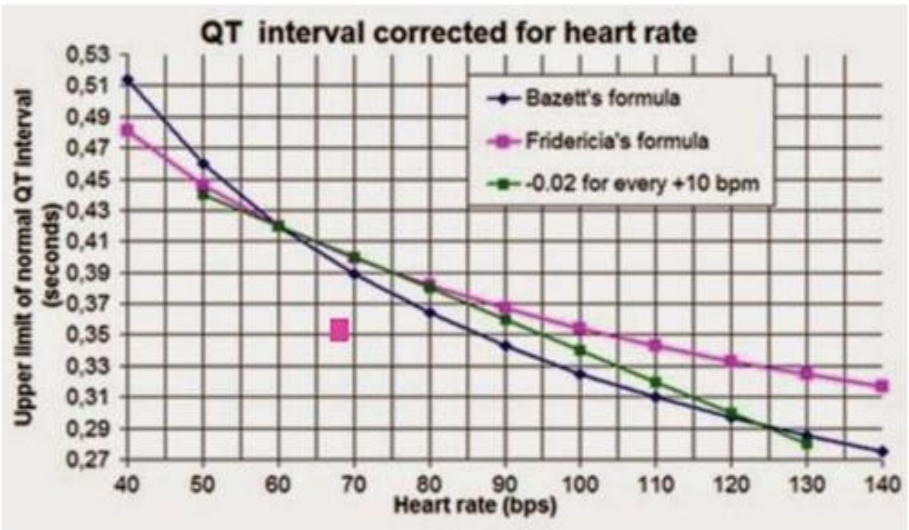

Fig. 11. ** Square in red

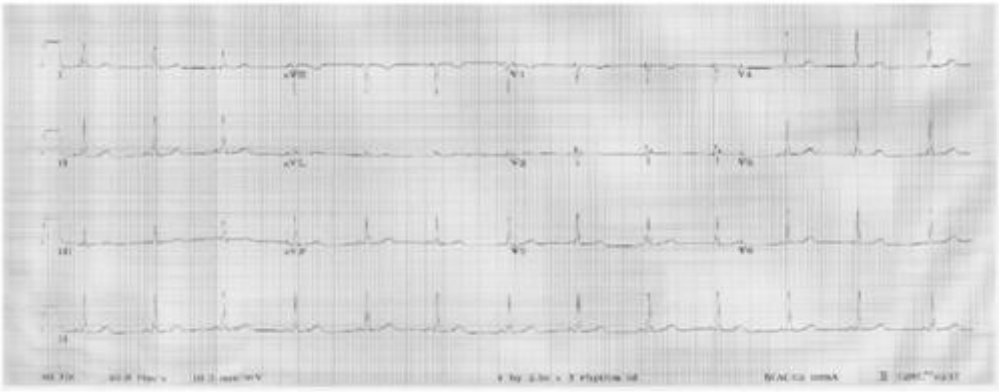

Fig. 12. PR interval value: 0.89 seconds (Very short) Measured QTc value: Between 0.356 and 0.334 seconds Very short)

6. CONCLUSION

In a nutshell, we can say about the Breijo Pattern the following conclusions

1. Although relatively little known so far, it is increasingly being discovered in ECG tracings that at first glance may appear normal.
2. The accurate reading of the ECG tracing must be of mandatory compliance. Despite the fact that symptoms referred by patients may be slight.
3. It is usually characteristic the fact that most of the patients with a Breijo Pattern have suffered in their childhood seizure crisis without being observed any focus of epilepsy in the all the assessed electroencephalography studies.
4. The most harmful consequence of the Breijo Pattern is the sudden cardiac death, which, although fortunately not often occurring, can happen.

SUMMARIZING

* It is imperative to always take into account each and every one of the symptoms that a patient refers, however slight they may seem to us. Especially if they are repetitive.
  Any patient who comes to our hospital with symptoms of nocturnal palpitations (which causes him/her to wake up from normal sleep), especially if they are accompanied by profuse sweating, nausea or throwing up, atypical thoracic discomfort as well as symptoms considered as mild or psychosomatic, especially if they are repetitive, should be evaluated in depth, without leaving any diagnostic elements ignored.
* Any patient with such characteristics must have a thorough examination of his or her background. Especially focused on the existence of syncopes or lost consciousness, as if the patient has suffered from convulsions in childhood, treated with antiepileptics and without focus electroencephalographic epileptogenic that can justify it.
* Carrying out an electrocardiographic study is imperative. Assessing each and every one of its parameters. Making special emphasis on the lengths of the waves, intervals, and segments.
* The presence of a Breijo electrocardiographic pattern makes the heart much more vulnerable to severe arrhythmias and even sudden cardiac death.
* Whenever we find ourselves on an electrocardiogram with a short PR and QTc interval, we must be very alert and careful with the patient.
* Lithium levels in blood must be obligatorily assessed, since all patients with Pattern Breijo have low or very low levels.

COMPETING INTERESTS

Author has declared that no competing interests exist.

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Biography of author(s)

Francisco Ramón Breijo- Márquez
Department of Clinical Cardiology, Boston Hospital, USA.

Research and Academic Experience: He holds a Doctorate in Medicine from the University of Boston and Hartford (MA and CT), where he is also a Professor of Clinical & Experimental Cardiology. He is currently the Director of Doctoral Theses on Clinical and Experimental Cardiology as Research Director at both East Boston and Hartford Universities. He is an advisory board member and an editorial board member for several scientific journals.

Research Area: Clinical & Experimental Cardiology

Number of Published papers: He has published several Textbooks and many research articles on Cardiology.

Special Award: Einthoven Award (1.998)

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Chapter 3
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Uvula in Snoring and Obstructive Sleep Apnea: An Approach to Surgical Intervention

A. M. Elbassiouny[1*]

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ABSTRACT

Objective: Currently, the consideration of the enlarged uvula as a cause of snoring and Obstructive Sleep Apnea (OSA) lacks data for objective interpretation. A uvula was often considered enlarged (i.e., longer, wider) if its length exceeded 15 mm or width exceeded 10 mm. This article focused on some concepts on how we can manage the enlarged uvula in cases of snoring and OSA. The purpose of the present article is to discuss the cost benefits of uvular surgery versus its preservation.
Conclusion: The direct correlation between the uvula and OSA needs to be reevaluated to maintain a balance between reserving its anatomical and physiological functions and surgically manipulating it as a part of palatopharyngeal surgery, yet further objective studies are needed to reach optimal results.

Keywords: Uvula; snoring; obstructive sleep apnea.

1. INTRODUCTION

The palatine uvula, usually referred to as simply the uvula, is that part of the soft palate that has an anatomical structure and serves some functions. Anatomically, the uvula, a conic projection from the back edge of the middle of the soft palate, is composed of connective tissue containing several racemose glands, and some muscular fibers, musculus uvulae muscle; arises from the posterior nasal spine and the palatine aponeurosis and inserts into the mucous membrane of the uvula. It contains many serous glands, which produce thin saliva [1]. Physiologically, the uvula serves several functions.

First during swallowing, the soft palate and the uvula move together to close off the nasopharynx and prevent food from entering the nasal cavity. It's also been suggested that your uvula may help to drain and direct the flow of mucus secreted from your nasal cavities, helping it to flow toward the base of your tongue and down your throat [2].

Second, researchers found that the uvula, after analyzing the frequency and distribution of immune cells in uvula tissue, may be a site for induction of mucosal tolerance to inhaled and ingested antigens [3]. Mucosal tolerance occurs only on mucosal surfaces and results in the suppression of your immune responses to inhaled or ingested antigens. The purpose is to prevent your body from launching an unnecessary immunological attack against harmless substances like pollen or foods [4]. Interestingly, your uvula also has its own protection against potential microbial pathogens, as researchers noted it contains a "subepithelial barrier of macrophages [3]. Third, it has also been proposed that the abundant amount of thin saliva produced by the uvula serves to keep the throat well lubricated [2]. Fourth, it has a function in speech as well. In many languages, the uvula is used to articulate a range of consonant sounds, known as uvular consonants. Due to the large amount of saliva produced from glands in the uvula that are not present in other mammals it has been suggested that the uvula is an accessory speech organ [5]. Lastly, the stimulation of the uvula also causes the gag reflex to be initiated.

[1]*Department of Otolaryngology, Cairo University, Egypt.*
**Corresponding author: E-mail: ahmedeldeen662000@yahoo.com;*

The uvula as a component of the soft palate, from the volumetric soft tissue point of view, was previously linked to snoring and Obstructive Sleep Apnea (OSA) [6]. The uvula of OSA patients, which has a higher percentage of muscle and fat content, may contribute to pharyngeal narrowing and increased pharyngeal resistance during sleep. This is in turn; decreases the retropalatal space leading to snoring and OSA [7]. Most studies identified for inclusion in previous reviews consistently demonstrated a direct relationship between an enlarged uvula and the presence of Sleep-Disordered Breathing (SDB) [8,9].

However, it is not clear whether an enlarged uvula is a causative or a result of SDB. Prior studiesdocumented histological changes in the tissue composition of patients with OSA [10], such as increased intercellular space (indicating edema), plasma cell infiltration, and epithelial hyperplasia. These factors appear indicative of an inflammatory process possibly because of trauma induced by snoring and a reasonable cause of changes that lead to SDB.

Olszewska E. et al in 2021, investigated the hypothesis that individuals with obstructive sleep apnea syndrome (OSAS) demonstrate oxidative stress in the uvular mucosa that correlates with OSAS occurrence and they concluded the Total oxidative status (TOS) and the oxidative stress index (OSI) in the mild, moderate, and severe OSAS were higher than in the non-OSAS group, whereas TAS of the uvular mucosa in the OSAS group was lower compared to the non-OSAS group. In final terms, oxidative stress in the uvular mucosa is associated with the occurrence of OSAS [11].

Although the volumetric soft tissue component has been widely accepted as the cause of upper airway narrowing and collapsibility, evidence has implicated a neurogenic component for the pathophysiology. Because snoring results from the turbulent flow of air vibrating the soft palate, it is possible that long-term vibratory trauma from snoring might result in alteration of neuronal activity of the soft palate, resulting in OSA. Supporting this, habitual snoring often leads to increasing obstructive events and obstructive sleep apnea if left untreated [12-13]. Numerous studies have begun to examine the relationship between neurologic dysfunction of the upper airway and obstructive sleep apnea. These seem to advocate a significant role of neurogenic activity in the multifactorial pathophysiology of OSA.

Duan X. et al in 2021, investigated the possibility of the sensorimotor nerve lesions and their possible relationship with OSA and they concluded that , sensorimotor nerve damage in the upper airway of patients with OSA may be associated closely with the mechanism of OSA [14].

Formal research dedicated to the information related to the measurable characteristics of the uvula still few. The uvular size was described as a grading scale by multiple authors who were classifying the uvula as normal, long, wide, long & wide [15-16]. A uvula was often considered enlarged (i.e., longer, wider) if its length exceeded 15 mm or width exceeded 10 mm [17-18].

Treatment modalities may include surgical resection of the uvula. However, complete removal leaves patients with persistent globus, xerostomia, excessive postnasal discharge and dysphagia [19].

A clearer understanding of the potential role the uvula in the pathogenesis of SDB and how its surgical modification helps improve the outcomes of surgery for specific aspects of SDB such as OSA remains material for further research. The purpose of the present article is to discuss the cost benefits of uvular surgery vs. its preservation.

2. DISCUSSION

The collapsible upper airway is a common cause of obstructive sleep apnea. The exact pathophysiology leading to a more collapsible airway is not well understood. Currently, the relationship between uvula size and sleep-disordered breathing lacks data for objective interpretation. Although the entire pathophysiology of OSA remains incompletely understood, it is becoming increasingly clear that there is more to this problem than simple structural obstruction of the airway due to excessive soft tissues or limited skeletal framework.

Chang et al. [20], in their systemic review, to evaluate the relationship of the uvula and snoring and obstructive sleep apnea, they raised four main findings. First, studies that demonstrateda direct relationship between an enlarged uvula and the presence of SDB cannot conclude whether an enlarged uvula is causative or the result of SDB. Second, if indeed uvula size can predict surgical results, a standardized method of reporting uvula size could be a useful clinical tool to aid in the overall decision-making process for snoring procedure selection. However, until now this is not the case. Third, to make more generalizable statements regarding the specific relationship between elongated uvulas and SDB, a method for objectively evaluating SDB appears necessary. Lastly, more research is needed regarding the significance of the effect of the uvula size on sleep-disordered breathing.

After this review, before deciding on how a surgeon should deal with the uvula in cases of snoring and OSA, some questions and surgical concepts should be answered. First, what is the physical role of uvula as a single anatomical factor in snoring and OSA?

While examining patients referred for possible OSA surgery, ask the patient to produce snoring sound, you will observe that the uvula and the surrounding soft palate will move as one sheet of tissue for this I coined the nomenclature "Uvulopalatal Segment" (Fig. 1). Cutting the continuity between the uvula and the adjacent parts of the soft palate will stop the vibration of this segment. Supporting this, habitual snoring was previously treated using a pharyngeal handpiece with the backstop attached to the articulating arm and the CO2 laser, to creates a full-thickness vertical trench measuring 1.0 cm to 1.5 cm were made on the free edge of the soft palate on either side of the uvula as an outpatients procedure (laser-assisted palatoplasty or uvuloplasty (LAP or LAUP) [21-22].

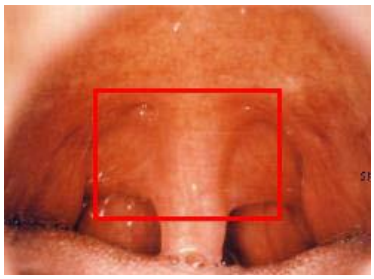

Fig. 1. Uvulopalatal segment

Sometimes the end of the uvula is also removed. With time and scarring, the palate stiffens and elevates. This should give the impression that removal of part or the whole uvula is not well anatomically sound and has no or minimal impact regarding case improvement. So, do not focus on the uvula as a major factor in treating snoring and OSA, The surrounding palatal muscles and soft tissue are the most important. Second, physiologically, you should respect the uvula as it has considerable functions as described earlier besides removal of the uvula has a lot of side effects, even if temporary, it is of considerable effect for most of the patients [23]. Third, pathologically, there is a documented uvular inflammatory reaction and neurophysiological changes, as described earlier, that occurred as a result of the physical trauma induced by vibration of the soft palate and uvula during sleep hours. Whether these changes are reversible or not, nobody can tell as there is no previous studies that have investigated this issue. So, the coast benefit for removal of the uvula depending on these inflammatory and neurophysiological changes is not physiologically sound. Finally, from my surgical point of view, some surgical concepts should be considered. 1) I believe that we should surgically respect the uvula, keeping in mind that if not done, the postoperative palate will become fibrotic and the patient's condition will become worse. The first and most important information is that the uvula is the area of the soft palate separating the two sides of the newly formed palate (Fig. 2). When you have raw area throughout the newly formed soft palate and uvula, the process of fibrosis will start at two angles throughout the soft palate for I coined the nomenclature key angles (Fig. 3).

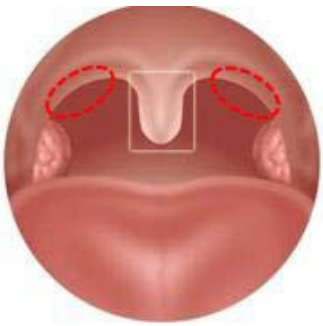

Fig. 2. Uvula is the partition between the two sides of the newly formed soft palate

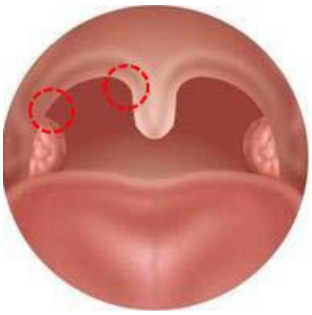

Fig. 3. The key angles of the soft palate

Fibrosis will be in medial and posteromedial directions leading to the increased possibility to have postoperative fibrotic soft palate with a decrease of the postoperative oropharyngeal and nasopharyngeal dimensions and lastly worsening of the patient condition (Fig. 4). 2) Do not leave a raw surface area throughout the newly formed soft palate and uvula. This can be achieved by covering the raw area at the edge of the newly formed palate by normal palatal mucosa as described in my published posterior pillar webbing palatopharyngoplasty techniques [24-26]. The postoperative palate after this palatopharyngoplasty technique that aimed to cover the raw areas of the newly formed soft palate, showed excellent widening and preservation of the transverse diameter soft palate and nasopharyngeal dimensions (Fig. 5). 3) Lastly, in my opinion, there is "No place for total uvulectomy in palatoplasty surgery". With partial uvulectomy, the postoperative palatal fibrosis will pull the newly formed uvula more up and you will have more shortening of the uvula, yet we should not ablate too much uvular tissue and always keep healthy mucosa on both sides of the uvula or use the fish-mouth technique. The uvula will be pulled up by the same distance of the raw area created at the sides of the uvula (Fig. 6).

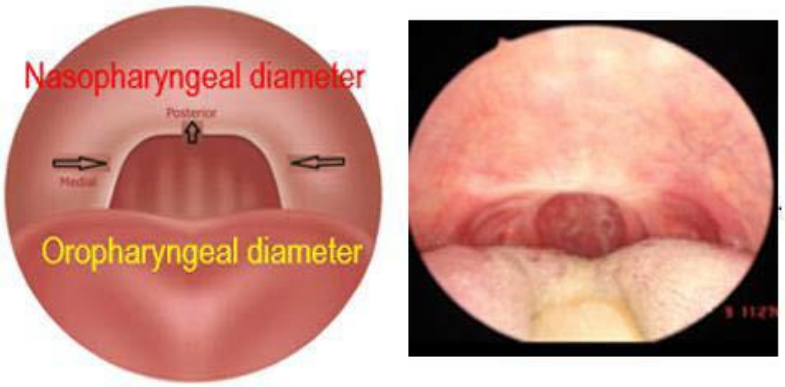

Fig. 4. Postoperative fibrotic soft palate. Fibrosis will be in medial and posteromedial directions

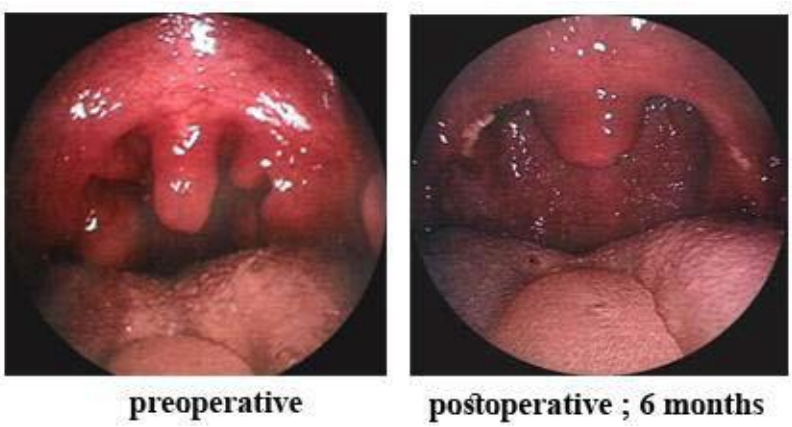

Fig. 5. Preoperative and 6 month postoperative palate after posterior pillar webbing palatopharyngoplasty technique

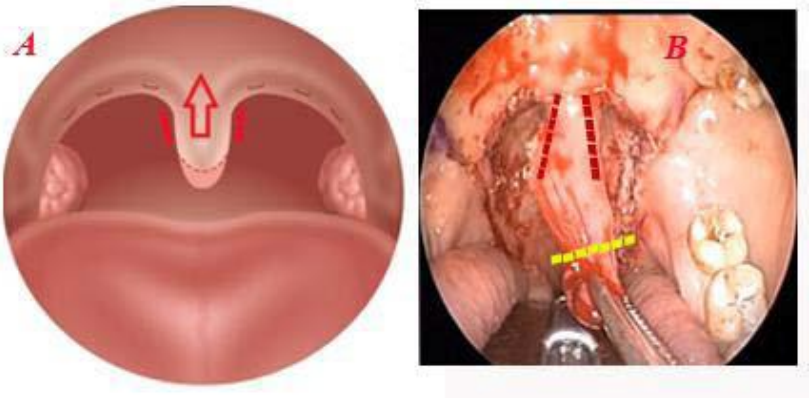

Fig. 6. Partial uvulectomy. Pulling up of the uvula by the same distance of the raw area created at the sides of the uvula; red dotted line in A and B. Observe the presence of healthy mucosa between this raw area and site of cutting of the uvula; yellow dotted line in B.

More studies are needed to improve the level of evidence and consistency. To make the findings more generalizable, the studies should evaluate consecutive patients and use prospective study designs.

3. CONCLUSION

The direct correlation between uvula size and its relationship specifically to snoring and OSA remain as topics for future prospective research. The balance between uvular preservation and the actual need for surgical intervention must be considered for every patient.

COMPETING INTERESTS

Author has declared that no competing interests exist.

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Biography of author(s)

A. M. Elbassiouny
Department of Otolaryngology, Cairo University, Egypt.

Research and Academic Experience: Prof. of Otolaryngology, Cairo University

Research Area: Snoring and Obstructive Sleep Apnea

Number of Published papers: Around 30

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Chapter 4
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A Case Series Describing Management of Chronic Unreduced Anterior Dislocations of the Shoulder

Otman Benabdallah[1*] and Ahmed Khamlichi[1]

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ABSTRACT

Background: Chronic anterior dislocations of the shoulder have been reported on since White's comments in 1741. Anatomopathologically, such cases exhibit modifications of the injured tissues (fibrosis, neo-articulation, muscle contracture, capsular, ligamentous, bone and tendon lesions) because of the longstanding unreduced humeral head. We hypothesize that the clinical status of such cases and the different lesion patterns observed in them obviate the need for conservative treatment or the use of classic open procedures. Treatment decisions also depend on clinical symptoms; patients who do not have much disability are left unreduced.
Methods: The study reports on 53 non-randomized cases showing duration of dislocation of at least 3 weeks in patients aged between 20 and 75 years. The 53 patients were placed in three management categories: conservative, closed reduction and open reduction groups.
Results: The results were evaluated following Rowe's evaluation of results for chronic unreduced dislocation of the shoulder. Among the 49% of patients with open management, the overall score averaged 74 points. In 4 cases, we performed a modified Boytchev procedure, and the overall rating units averaged 81 points.
Conclusion: These results show that the overall prognosis for surgical treatment is improved.
This study is a Level IV case series.

Keywords: Chronic unreduced shoulder dislocation; neglected dislocation; conservative shoulder treatment; open shoulder reduction.

1. INTRODUCTION

A glenohumeral joint that has been dislocated for several days is a chronic dislocation. This condition has been known since 1741 [1] (White, cited by Schulz) and 1825 [2] (Cooper, cited by Rowe). Souchon [3] was the first author to offer a definition: "we will call recent all dislocations no older than a month."

In the medical literature, a number of authors [4-16] have discussed the handling of this condition, many of them adopting an optimistic view [5,6,8-12,16] and offering hope for patients. 1982 saw a landmark, when Rowe [13] presented his rating system for the evaluation of the treatment, and on the base of various different studies [4,10,13] identified three weeks as the criterion for considering a shoulder dislocation as chronic. Chronic unreduced anterior dislocations of the shoulder are rare. Arterial and neurological complications in chronic glenohumeral dislocations are even less frequent [17,18].

Logically, chronic anterior dislocation of the shoulder exhibits the same anatomopathological lesions as acute anterior dislocations: Bankart lesion, ligament lesions, tendon lesions, capsular rupture, glenoid fracture or erosion, Hill Sachs lesion; but these lesions have become longstanding and may have undergone change. Dubousset [19] and Langlais [7] reported cases treated by closed reduction in which Hill Sachs lesions had filled up spontaneously over time; other added modifications of injured

[1]Centres hospitaliers de Tanger, Morocco.
**Corresponding author: E-mail: otman.benabdallah@hotmail.com;*

tissues include fibrosis, neo-articulation, and muscle contracture. The condition thus becomes more complex and shows different lesion patterns, which obviously need different procedures of management.

Treatment of long-term injured structures may be deemed obsolete, so the treatment principle is to look for and treat strategic lesions in order to stabilise the glenohumeral joint after open reduction, which necessitates a variety of complex techniques, each of which is appropriate for a specific pattern of chronic shoulder dislocation. The procedures performed include Bankart, Latarjet, Dutoit, open repair with pinning, humeral head replacement, and resection of the humeral head. In 2002, we began to use the Boytchev procedure. Treatment decisions also depend on clinical symptoms; patients who do not have much disability are left unreduced. The aim of this study is to discuss different treatment options, to evaluate and compare our results using different methods of management, to emphasize the effectiveness of open repair and to shed light on the place of the Boytchev procedure as another possible surgical treatment.

2. MATERIALS AND METHODS

For this study, the patients were selected using the following inclusion criteria: a clinical history of chronic anterior shoulder dislocation as a result of a traumatic event, a time interval between dislocation and treatment of at least three weeks, typical symptoms of anterior dislocation: persistent deformity of the shoulder, discomfort or disability, pain, and possible associated injuries. Pain, motion and function are evaluated using Rowe's evaluation of results for chronic unreduced dislocation of the shoulder. The condition is documented using plain and special radiographs and in some cases computed tomography [20] before and after treatment and follow-up. The patients were placed in the following management categories: conservative treatment, manipulation and closed reduction, and open repair.

The patients who underwent open repair were followed up for at least two years. Some patients with conservative treatment had a follow-up of less than two years because there was no change in their clinical evolution.

Between January 1987 and December 2015, we collected data on 53 non-randomized patients (Table 1) with chronic anterior unreduced dislocations of the shoulder. The age of the patients ranged between 20 and 75 years (average age 44 years). 34 of them were male and 19 were female. 44 patients were affected on the right side, 9 on the left. The causes of injury were in 49 cases a fall, while one case arose from a car accident, one from a sporting accident, one from an accident at work, and in one case the cause was undetermined. The delay before diagnosis ranged between 3 weeks and 156 weeks (Fig. 1), with an average of 18 weeks. The reasons for the absence of treatment were, in 45 cases, a failure to consult, and in 2 cases an unsuccessful reduction; in 2 cases the problem went unrecognized, in one case the problem occurred after an epileptic fit, and in 3 cases the patients had sought treatment by a faith healer. 46 of the patients came from rural areas and 42 were from a low socio-economic category. 28 radiological lesions and anatomical operative findings revealed 17 head lesions (10 compression fractures and Hill Sachs lesions, 5 greater tuberosity fractures, 1 necrosis of the humeral head and a head fracture with avulsion of the supraspinatus), 6 glenoid lesions (5 glenoid rim erosions and 1 fractured rim), 2 diaphyseal fractures, and 3 long biceps lesions (2 dislocations and 1 rupture). No severe vascular or neural injuries were observed. There were 20 cases in which the patients were left untreated. 7 had manipulation and closed reduction under anaesthesia, 3 with pinning, 1 without pinning and 3 cases in which this treatment failed. 26 patients underwent open reduction with preservation of the humeral head. In one case of head necrosis, the patient refused any treatment and was lost in follow-up. 3 patients had complications, which included 2 superficial wound infections and 1 redislocation (1 month after reduction under anaesthesia). The length of follow up, for cases receiving no treatment, ranged between 10 and 120 months, with an average of 19 months; for cases of closed reduction, it ranged between 20 and 41 months, with an average of 28 months; and for open reduction, the follow up ranged between 25 and 130 months, with an average of 34 months. Length of follow up, across all the categories, averaged 27 months. 4 patients were lost in follow up: 3 who refused treatment and 1 whose shoulder was redislocated 4 weeks after manipulation reduction. The evaluation u s i n g

Rowe's grading system for the shoulder before any management shows that initial ratings ranged between 40 and 75 points (75 points for the no treatment category, 40 points for those receiving closed reduction and 44 points for operative management). The patients evaluated by the same system after management were assessed as excellent, good, fair, or poor.

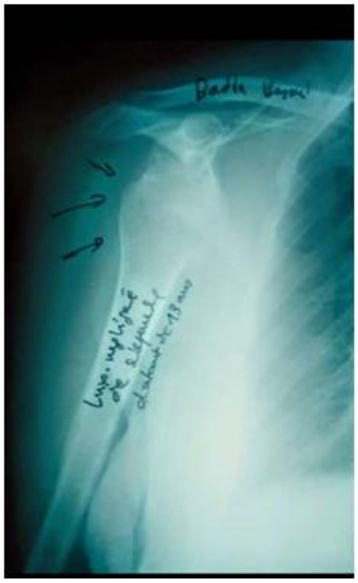

Fig. 1. Patient with one unreduced dislocation of the shoulder after 156 weeks of disability

2.1 Non Operative Management

In some patients closed reduction was performed in supine position under general anaesthesia with total muscle relaxation. We began the maneuver with repetitive gentle rocking of the humerus from internal to external rotation, adding flexion- extension and abduction-adduction movements to liberate the imprisoned humeral head in the neo- articulation; then we made a steady traction along the axis of the arm while applying pressure on the proximal humerus in the axilla to effect reduction.

2.2 Surgical Technique

Under general anaesthesia, the patient is placed in supine position. After antiseptic preparation, the skin incision is made over the classical delto-pectoral groove. The length of incision is generally about 10 cm, but it can be longer if necessary. The approach is made through the deltoid muscle, reclining cephalic vein inside, until the subscapularis appears, covering all the anterior side of the neo-articulation. The subscapularis muscle and capsule are incised near their insertion, preserving some attachment in the lower part. When the neo-articulation is opened, the humeral head is clearly visible below the subscapularis muscle. Adhesions are generally very extensive, and liberation is done step by step without forcing, to avoid any devascularisation or crushing of the humeral head, which is often osteoporotic. Careful liberation with external rotation and lateral traction allows the head to be released from its imprisonment in the neo-articulation. Then with the finger we touch the glenoid fossa (which is strangulated by the contracted deltoid muscles), to see its position, and to have an idea about its new constitution. When the articular surface of the glenoid fossa is well exposed, we excise the soft fibrous tissue with a rongeur, gently, without causing damage, in order to preserve the articular cartilage; this cartilage is then evaluated. We try to preserve soft fibrous tissue around the rim for a good positioning of the head (which must be centralized). Before reducing the humeral head, we maintain an external traction on the highly contracted deltoid muscle for a varying length of time in order to achieve the reduction without damaging the head [21]. All the anatomical structures are repaired and sutured using the usual technique, other accompanying lesions (bone, tendon lesions and other operative findings) are restored when necessary if possible, and the head is stabilized:

- By temporary stabilization with percutaneous pinning, to avoid post-operative instability;
- By definitive stabilization:

Either through capsular and muscular repair carried out via tight sutures, or through a repair using a technique such as that of Bankart, or by using a bony procedure in which the coracoid process is first divided with an osteotome and screwed into the anterior glenoid rim, using the Latarjet technique or another procedure such as that of Dutoit. In some cases we performed the stabilization using a modified Boytchev's technique [22].

Here the incision begins from the level of the coracoid process, extending distally. We expose the horizontal part of the coracoid process with the tendinous origin of the short head of biceps and the coracobrachialis muscle. An anteroposterior drill hole is made from the anterior end of the horizontal part of the coracoid process along its axis. The anterior 2 cm of the coracoid process is divided with an osteotome and mobilized distally. We incise horizontally and liberate the superior border of the subscapularis muscle. On the top of this muscle, we perform the anterior arthrotomy and the opening of the neo-articulation and progressively and carefully liberate the humeral head in order to reduce it. Sometimes we encounter difficulties, so we extend the subscapularis incision into an inversed L incision, but not completely, preserving the muscle tendon's inferior attachment. On the lower border of the subscapularis, we create a tunnel between shoulder capsule and muscle with a curved vascular forceps (taking care not to damage the anterior circumflex humeral vessels), or only under the subscapularis muscle in the room left by the neo-articulation, through which the isolated coracoid process with the conjoined tendons is now passed before being fixed to the predrilled proximal coracoid process with a 3.5 AO screw (Fig. 2). The wound is closed in layers around a suction drain. A well padded dressing is applied. The arm is immobilized at the side of the chest with an elastic bandage.

2.3 Post-operative Management

Once the immobilization is removed, normally after three weeks, shoulder exercises are recommended: passive and active exercises to be increased progressively by the patient himself and with the kinesitherapist for as long as necessary. When the shoulder joint is transfixed with a pin, the arm is maintained in a sling for 2 or 3 weeks. During immobilization, isometric muscular reinforcement is begun. After removal of the pin(s), we continue with manual passive mobilization aimed at amplifying articular mobility and restoring elementary sliding articular movements. After a few days or simultaneously, we begin active physical therapy, which aims at recuperation of strength and motion, and ensures the beneficial effect of early motion on the joint cartilage and muscular reinforcement.

3. RESULTS

The rating scores for our management and the treatment results are summarized in Table 1. In the non-treatment category, which consisted of 20 patients, 3 were untraced, 3 patients were rated excellent (Figs. 3A and 3B), 11 good, 2 fair and 1 poor. The overall score averaged 76, with a pain score of 21 points, a motion score of 26 points, and a function score of 29 points. Out of 7 patients who were reduced by closed manipulation, 4 were rated good, 2 fair and 1 suffered a redislocation after 4 weeks and was lost track of. The overall score averaged 70 points, with an improved pain score of 24 points, motion score of 24 points, and function score of 22 points.

In the open reduction category, which included 26 patients, 1 was graded as excellent, 19 were graded as good, 5 as fair and 1 as poor. With open reduction and pinning (Fig. 4), 5 shoulders showed good results, 4 showed fair results and 1 a poor result. With the Dutoit technique, the 2 results were assessed as good. With the Latarjet technique (Fig. 5A, 5B and 5C), we obtained 5 good results and 1 fair result; with the Bankart technique, there were 4 good results. The 4 shoulders treated using the Boytchev technique (Fig. 6A and 6B) showed 1 excellent result (Fig. 7) and 3 good results. The overall score averaged 74 points, pain score was 24 points, motion score 26 points, and function score 24 points. The result with the Boytchev procedure was 25 to 30 points for pain, 30 points for motion and 15 to 30 points for function. The ratings averaged 81 points.

Table 1. Characteristics of patients, options and results of treatment

	case	age, sex	cause of injury	dislocation side	cause of diagnosis delay and treatment	duration of disability	radiological and/or operative findings	treatment	follow up in months	result	rating units
no treatment	1	60M	fall	R	unsuccessful reduction	3 weeks		abstention	10	good	80
	2	49F	fall	R	consultation of a faith healer unrecognized	3 weeks		refused treatment	lost to follow up		
	3	30M	auto acc	R	shown in assessment	24 weeks	Hill Sachs lesion	abstention	120	good	85
	4	26M	fall	R	no consultation	14 weeks		abstention	17	excellent	95
	5	62F	fall	R	no consultation	3 weeks		abstention	14	fair	65
	6	31M	fall	R	consultation of a faith healer	11 weeks		abstention	10	good	75
	7	53F	fall	R	no consultation	6 weeks		abstention	14	good	85
	8	37M	fall	R	no consultation	48 weeks	deformation of the head and rim erosion	abstention	14	excellent	95
	9	50F	fall	R	no consultation unrecognized	3 weeks		abstention	18	good	70
	10	36M	labour acc	R	shown in assessment	12 weeks	shaft humeral fracture	abstention	28	good	85
	11	28M	fall	R	no consultation	19 weeks		abstention	18	good	80
	12	65F	fall	L	no consultation	36 weeks	great tuberosity fracture	abstention	12	fair	70
	13	33M	fall	L	no consultation	15 weeks		abstention	10	excellent	90
	14	38F	fall	R	no consultation	06 weeks	inferior dislocation	refused treatment	lost to follow up		
	15	70F	unknown	R	consultation of a faith healer	43 weeks	deformation of the head and glenoid fossa	abstention	12	poor	45
	16	35M	fall	R	no consultation	09 weeks		abstention	14	fair	55
	17	75F	fall	R	no consultation	33weeks	necrosis of humeral head and deformation of glenoid fossa	refused treatment	lost to follow up		
	18	66M	fall	R	no consultation	27weeks	Hill sachs lesion	abstention	18	good	70

	case	age, sex	cause of injury	dislocation side	cause of diagnosis delay and treatment	duration of disability	radiological and/or operative findings	treatment	follow up in months	result	rating units
closed reduction	19	58F	fall	R	no consultation	156 weeks	defomation of the head and glenoid fossa	abstention	12	good	75
	20	25M	fall	L	no consultation	50 weeks	deformation of the head and glenoid fossa	abstention	14	good	75
	21	55M	fall	R	no consultation	4weeks		manipulation reduction	10	fair	55
	22	59M	fall	R	no consultation	3 weeks		manipulation reduction	11	good	70
	23	63F	fall	R	no consultation	03 weeks		manipulation reduction	10	good	70
	24	44M	fall	R	after epileptic fit	05 weeks		manipulation reduction	lost to follow up		
	25	49F	fall	L	no consultation	03weeks		manipulation reduction	18	fair	60
	26	27M	fall	L	no consultation	04 weeks	Hill sachs lesion	manipulation reduction manipulation	20	good	80
	27	35F	fall	R	no consultation	03weeks		reduction and pinning	24	good	85
open reduction	28	57F	fall	R	no consultation	8 weeks		open reduction	34	good	80
	29	51F	fall	R	no consultation	5 weeks	great tuberosity fracture	open reduction	21	good	85
	30	61M	fall	R	no consultation	4 weeks	long biceps brachii dislocated	open reduction	26	good	80
	31	31F	fall	L	unsuccessful reduction	3 weeks		open reduction	32	good	80
	32	20M	fall	R	no consultation	11 weeks	great tuberosity fracture	open reduction	130	good	85
	33	35M	fall	R	no consultation	40 weeks	Hill Sachs lesion	open reduction	38	good	85
	34	53M	fall	R	no consultation	4 weeks		open reduction	42	good	80
	35	65F	fall	R	no consultation	9 weeks		open reduction	16	good	70
	36	54F	fall	L	no consultation	8 weeks		open reduction	36	good	70
	37	57M	fall	R	no consultation	4 weeks	rim fracture	open reduction	28	good	75
	38	65F	fall	L	no consultation	3 weeks		open reduction	15	good	70

case	age, sex	cause of injury	dislocation side	cause of diagnosis delay and treatment	duration of disability	radiological and/or operative findings	treatment	follow up in months	result	rating units
39	20M	sport acc	R	no consultation	3 weeks		open reduction	48	good	80
40	33M	fall	R	no consultation	44 weeks	deformation of the head	open reduction	56	fair	75
41	28F	fall	R	no consultation	12 weeks		open reduction	24	poor	45
42	39M	fall	R	no consultation	28 weeks	Hill Sachs lesion	open reduction	11	fair	55
43	59M	fall	R	no consultation	08 weeks	neck and shaft humeral fracture	open reduction	30	fair	55
44	42M	fall	R	no consultation	05weeks	fracture of great tuberosity	open reduction	50	good	85
45	43M	fall	R	no consultation	17weeks		Boytchev	28	good	70
46	38M	fall	L	no consultation	38weeks	deformation of the head	Boytchev	50	excellent	90
47	49M	fall	R	no consultation	20weeks		Boytchev	36	good	85
48	40M	fall	R	no consultation	14weeks	long biceps brachii dislocated	open reduction	24	good	75
49	32M	fall	R	no consultation	08weeks		Boytchev	32	good	80
50	56M	fall	R	no consultation	06weeks		open reduction	14	good	75
51	30M	fall	R	no consultation	36 weeks	rupture of long biceps brachii	open reduction	10	fair	60
52	28F	fall	R	no consultation	32 weeks	deformation of the head	open reduction	34	good	85
53	36M	fall	R	no consultation	24 weeks	neck fracture	open reduction	20	fair	55

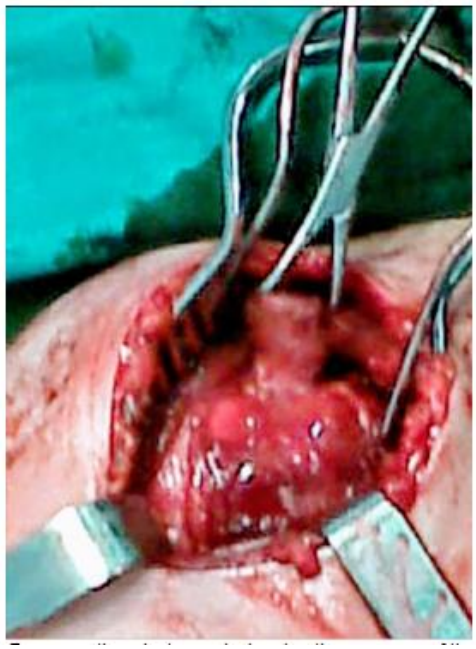

Fig. 2. Per-operative photograph showing the passage of the isolated coracoid process with the conjoint tendon deep under the subscapularis before being refixed in the original site with a screw

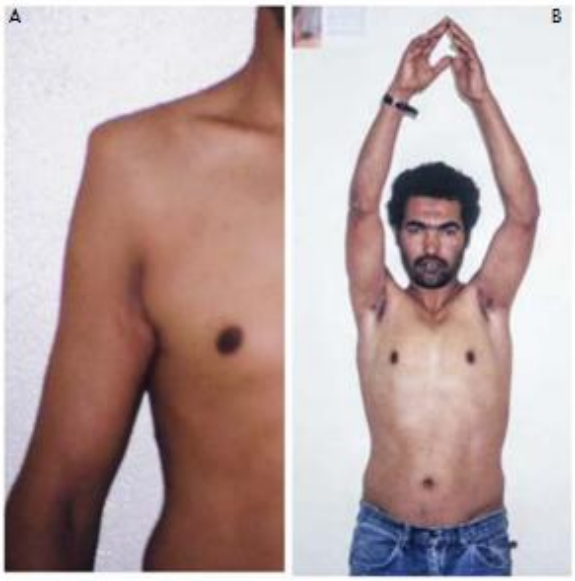

Fig. 3A. Young patient with a chronic anterior dislocation of the shoulder, clinically evident. Fig. 3B. The same patient with a complete range of motion, with no pain or discomfort; he had no treatment. The result is excellent

Comparison of our results: For the no treatment category, at diagnosis 3 were excellent, 9 were good and 5 were fair (3 were lost to follow up). If the patients receiving rehabilitation are included, the score improves from 68 points to 76 points (8 points higher). In the closed reduction category, all the shoulders were initially assessed as poor or fair; the initial score was 58 points and it improved to 70 points (12 points higher). In the open reduction category with preservation of the head, the preoperative evaluation was poor, the initial score was 48 points and the final score was 74 points (26 points higher).

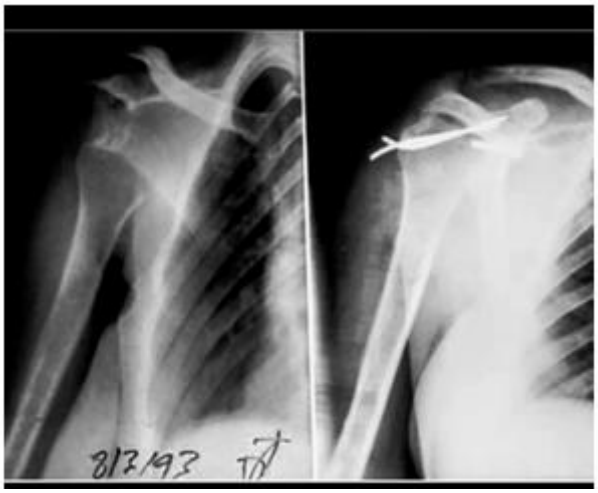

Fig. 4. Patient with anterior chronic dislocation managed by open reduction and pinning

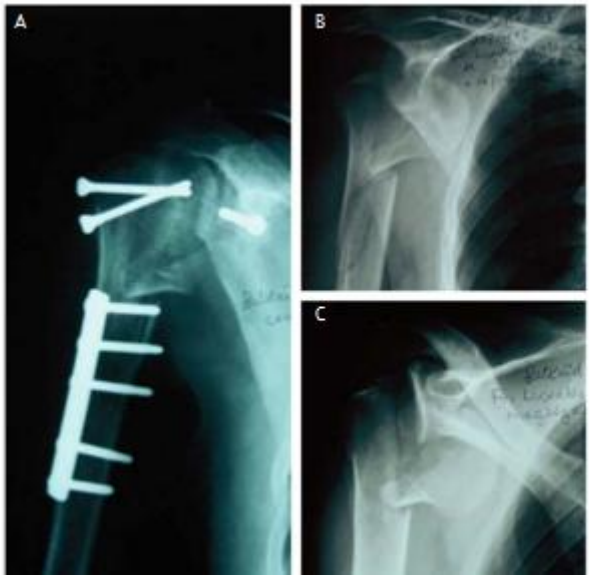

Fig. 5A. Patient with 8 weeks of longstanding dislocation and a humeral head fracture. This patient refused any treatment at first
Fig. 5B. The same patient after a second fall; in addition to the damaged humeral head, he now presented a shaft fracture of the humerus
Fig. 5C. The same patient reduced operatively and stabilized by the Latarjet technique; the head has been screwed and plating performed for the fractured shaft of the humerus

4. DISCUSSION

In comparison with other studies [5,8,12,13] we notice significant differences: this is one large case series, the age of our patients is comparatively young, the delay before diagnosis is long. 42% of the anatomopathological lesions are revealed radiologically and operatively. We must point out that, considering the long duration of disability in our case series, these operative findings are not fresh and have undergone modifications and so cannot be repaired like recent lesions, and this explains why our study contains a relatively low number of open repairs using the Bankart procedure. Also, the Hill Sachs lesion was observed in less than 30% of cases, perhaps because our patients are young, with solid bone constitution, or because the Hill Sachs defect filled up spontaneously, as reported by Dubousset [19] and Langlais [7]. Curiously, we notice 3 long biceps brachii lesions; no other author

[5,8,12,13,16] has reported this lesion.. With regard to conservative treatment, there are two subgroups. Apart from three patients lost at follow-up, three other patients had an excellent result at diagnosis; they did not receive any rehabilitation. In the second subgroup, 14 patients received rehabilitation. Their scores improved by an average of 8 points (from 68 points to 76 points). In this category some patients had a short follow-up, the cause obviously being the good function at diagnosis. In the category of those undergoing closed reduction, the result improved by an average of 12 points (from 58 points to 70 points). This result can be attributed to the relatively short duration of disability. In the category of those undergoing open reduction, the results improved by an average of 26 points (from 48 points to 74 points); this is clearly the best outcome in our case series.

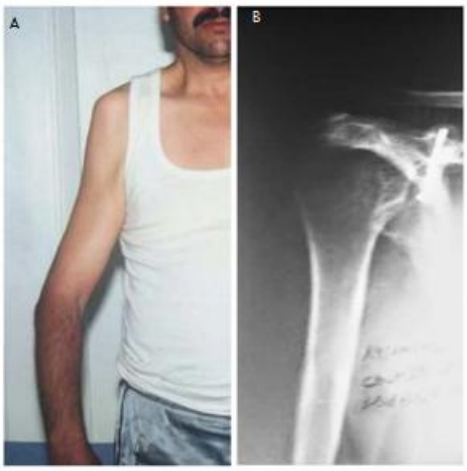

Fig. 6A. A thirty eight year old male patient who has suffered 3 years of disability
Fig. 6B. The same patient after treatment by the Boytchev procedure. Radiograph showing the refixation of the coracoid process with a screw in its original site

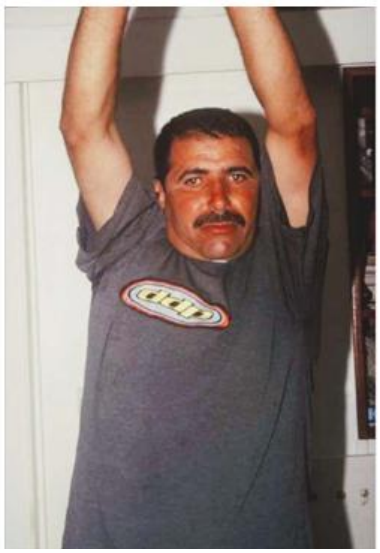

Fig. 7. Six months later, the same patient underwent a Boytchev procedure after 38 weeks of disability. The rating score is 90 points. The result is excellent

In the medical literature, several authors [4-16] have discussed the handling of this condition. Among many reported articles, there is variability of study design (dealing with posterior and/or anterior dislocations, using one or multiple procedures of treatment, different evaluation methods, etc). To sum up, the majority of authors [5-8,12,13] reported improved results when performing open repair, whatever the procedure adopted. With regard to these data, we notice:

- that chronic glenohumeral dislocation is still a current topic;
- that its management is complex;
- that the operative indications are to tackle strategic lesions, but this is often difficult because the lesions are long- established and have undergone modifications due to the longstanding unreduced humeral head;
- that various operative techniques are used to stabilize the reduced head, depending on eventual lesion patterns;
- that the classic techniques like Bankart and Latarjet are effective, while the Boytchev procedure seems promising.

The distinguishing feature of our open management is the introduction of a modified Boytchev technique, which since 2002 has been performed on 4 patients. This is a technique which has several advantages. It allows easy access to the neo-articulation and good visualization because of the existence of sufficient room within the neo articulation for passing the coracoid process. With regard to the biomechanical modes of action, s o m e studies have confirmed the biomechanical effectiveness of this procedure (Shibata [23], Lei Sheng Jiang [24].

The number of cases where this Boytchev procedure [22] has been used remains limited, but the results are very encouraging, and it offers us another alternative for the stabilization of chronic shoulder dislocation.

Generally, with open management, the prognosis is favourable in a majority of patients (77% obtained excellent or good results). Longterm results are important; in most patients, the treatment was carried out between 2 and 20 years ago. For operative treatment, our follow-up averaged 34 months (27 m o n t h s for all categories), whereas Mansat's follow- up averaged 25 months, and that of Rowe 67 months.

Are there clear indications enabling us to select one method rather than another? The answer is clear in the case of patients with mild discomfort or disability, where the best option is to give no treatment. Closed reduction is recommended in early cases. The answer is also clear when the humeral head is seriously damaged, in which case the indication is replacement or resection of the humeral head. But the interesting question is how to select one method versus another in surgery, to determine the effectiveness or superiority of one particular operative treatment. A comparison of our results for each surgical technique shows that open reduction with pinning receives a score of 71 points, the use of the Bankart procedure 73 points, the Latarjet procedure 74 points, the Dutoit procedure 77 points and the Boytchev procedure 81 points. In the literature, Rowe [13] evaluated the results obtained using various types of surgical management: 7 open reductions with preservation of the humeral head, 3 head replacements and 4 resections of the humeral head. The scores for the three procedures averaged 79 points, 75 points and 68 points respectively. Mansat [8] reported the results of 5 patients treated by the reinsertion of the capsulo-labral complex onto the glenoid rim; the scores averaged 75 points. Many other authors [6,8,11,16] report on smaller series and the performance of other open procedures. In fact, then, we see that for open reductions preserving the humeral head, the decisive criterion for choosing one technique over the others depends on the radiological and operative findings and the possibility or otherwise of anatomical repair, depending on their modifications over the long period when the head was unreduced, the ultimate goal being to definitively stabilize the reduced humeral head. Rowe [13] observed (and this is true for the other authors too [25] that 'one should point out that the number of patients in each treatment category was small. Therefore, direct conclusions should not be drawn from comparison of these rating units'. In fact, then, there is as yet no statistically significant difference demonstrating that one surgical treatment is superior to another.

5. CONCLUSION

In this case series, 53 patients were reviewed and evaluated using the rating system of Rowe and Zarins. In reporting the results, we conclude that 38% of patients did not need any treatment because of their insignificant symptoms and level of discomfort. In 13% of the series, we recommended closed reduction for patients with no long standing dislocation. For open reduction (chosen for 49% of our patients), the prognosis is generally favourable. Special attention is paid to the Boytchev's technique,

which obtained a score 7 points higher than our other operative techniques, but the number of patients undergoing this procedure remains small, so we cannot yet conclude that it yields a substantial improvement of the results in comparison with other surgical techniques.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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Chapter 5
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Determination of Electromagnetic Radiations and Liver Fibrosis Influences the “Lifespan” of Bone Marrow Cells

A. I. Bozhkov[1,2*], S. L. Ohiienko[1,2], Eu. G. Ivanov[1,2], A. Yu. Bondar[2] and Yu. H. Kot[3]

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ABSTRACT

Different exogenous and endogenous factors effect on production speed and direction of cells in bone marrow. Earlier, it was shown, that liver fibrosis effects on the morphotypes of bone marrow cells. In this investigation, the bone marrow cells answer to liver fibrosis and electromagnetic radiation was investigated. The research objects were bone marrow cells` of different age rats. The "lifespan" of lymphocytes obtained from old animals (*Wistar* rats, 20 months) in the primary culture exceeded that of young animals (3 months). Fibrosis of the liver, which is induced by the administration of copper sulfate and carbon tetrachloride, reduced the "lifespan" of lymphocytes. Electromagnetic radiation (70 kHz 600 V / m) affects the number of cells in the bone marrow and induces apoptosis. A study of the effect of electromagnetic radiation for 4 hours for 1.3 and 5 days showed a U-shaped dependence of the response at the level of bone marrow cells.

Keywords: Bone marrow cells; fibrosis; electromagnetic radiation; apoptosis; cells "lifespan".

1. INTRODUCTION

As known, the intensity of the natural electric field of the Earth is 120-130 V / m, and the magnetic field is 24-40 A / m [1]. The frequency sector of the Earth's electromagnetic field fluctuates within 7.8 - 8 Hz [2]. Electromagnetic fields, along with temperature conditions, background radiation and illumination, are global factors in the evolution of living systems. All living organisms generate their own electromagnetic oscillations, and they can fluctuate within a fairly wide range from 0.5 to 10 kHz [3]. There is no doubt that the electromagnetic characteristics of biological systems are associated with functional activity in the sense that functions are implemented against the background of electromagnetic fields and changes in radiation can change the functional activity of biological systems. At the same time, the relationship of electromagnetic radiation with functional activity and, first of all, proliferative activity of cells has not been studied enough, and the available data are contradictory [4].

The relevance of such studies has increased enormously in recent years, because the development of modern information technologies has led to a sharp increase in electromagnetic radiation (EMI) [5]. Experts believe that EMI can have both negative and positive effects on the organism [6,7]. However, the mechanism of such various effects is unclear, and a number of authors note the absence of any pronounced biological effects on the body. Solving the problem of the characteristics of the organism's responses to EMI is an important problem both in understanding the evolutionary processes of the development of pathologies and in the potential development of new medical technologies.

[1]Research Institute of Biology, V. N. Karazin Kharkiv National University, Kharkiv, Ukraine.
[2]Department of Molecular Biology and Biotechnology, V. N. Karazin Kharkiv National University, Kharkiv, Ukraine.
[3]Biochemistry Department, V. N. Karazin Kharkiv National University, Kharkiv, Ukraine.
**Corresponding author: E-mail: niibio@karazin.ua;*

We believe that the solution to this topical issue depends to a greater extent on the objects of study, the characteristics of the EMI and on the functional states of the biological object, which is affected by the EMI at the moment of exposure.

The object of investigation of the effect of EMI on biological systems can be the bone marrow, which very quickly and "subtly" reacts to the constantly changing needs of the body for certain types of blood cells and the immune system. The fulfillment of such functions is possible due to the structural organization of this tissue. In the adult organism, the bone marrow mass accounts for up to 4-5% of the organism weight, it is rich in hematopoietic stem cells and blood vessels [8]. Blood vessels in the bone marrow occupy up to 50% of the total bone marrow, and most of the vessels have a sufficiently large diameter (up to 500 microns), which allows the cells formed in the bone marrow to enter the circulatory system.

The proliferation rate and directions of differentiation of pluripotent stem cells are determined by the peculiarities of the microenvironment of bone marrow cells, which can change under the influence of external factors [9].

The study of the cell microenvironment showed that it is provided by: 1. - direct contact between hematopoietic stem cells and stromal cells (fibroblasts, adipocytes and epithelioid cells); 2. - under the influence of various cytokines (colonies of stimulating factors - GM - CSF; G - CSF; M - CSF; interleukins - 3,1,6,7, etc.) [10,11] and other factors present in blood, including cytotoxic ones, which can manifest themselves in various pathologies.

It has been shown that a change in the characteristics of the microenvironment leads to a violation of the speed and direction of differentiation, and the development of certain pathologies, up to oncology.
It can be assumed that the change in the functional characteristics of bone marrow cells in old animals compared to young animals is due to the fact that changes occurring with age in other body systems affect the epigenetic and metabolic characteristics of bone marrow cells, which is the basis of their functional activity. It is known, that highly differentiated bone marrow cells, which include lymphocytes, neutrophils, etc., have a short "lifespan" after entering the bloodstream.

It can be assumed that the "lifespan" of bone marrow cells will depend on the epigenetic and metabolic characteristics of the bone marrow, and they, in turn, will be determined by the microenvironment, which can be influenced by EMI. If the microenvironment for bone marrow cells is different, then it can be expected that the "life span" of cells in the in vitro system obtained from young and old animals will be different. As noted, the response of bone marrow cells can be influenced by the functional state at the time of the action of EMI and other external factors. Our long-term studies have shown that the most pronounced factor in changing functional states is the age of the body and the presence of pathologies. This is also evidenced by the fact that clinical manifestations in COVID-19 depend on the patient's age and the presence of chronic pathologies [11,12]. Liver fibrosis was studied as chronic pathologies, which was induced by repeated sequential administration of copper sulfate [13,14] and carbon tetrachloride [15-17].

It has previously been shown that the induction of liver fibrosis affects the behavior of bone marrow cells. If these assumptions are correct, we can expect that bone marrow cells obtained from young and old healthy (intact) animals and animals with an altered microenvironment, in the case of induction of liver fibrosis in them by various inductors, then in their behavior, in particular, "duration life" in the in vitro system will be different.

To check this, the animals were exposed to a daily four-hour EMI effect for 1.3 and 5 days, as well as in animals of different ages - young (3 months) and old (20 months), and induced liver fibrosis, after which determined the number of bone marrow cells, indicators of early and late apoptosis and "life span" of lymphocytes and segmented neutrophils in primary cell culture.

2. MATERIALS AND METHODS

2.1 Keeping of Animals

A total of 48 mature male Wistar rats were separated into experiments. These rats were obtained from Research Institute of Biology, V.N. Karazin Kharkov National University (Kharkov, Ukraine). They were housed in a temperature-controlled room (20–24°C) and adapted to a 12 hlight/12 h dark cycle. The animals were given free access to food and water before and during the study. All experimental procedures employed were approved by and conducted in accordance with bioethical rules16 and with due consideration to circadian rhythms for the formation of biological responses. For 24 hours preceding isolation of bone marrow cells, animals did not receive any food. Removing animals from the experiment was always carried out from 8 to 10 a.m. local time.

2.2 Experimental Investigation of the Effect on the Organism Electromagnetic Radiation

Investigation of electromagnetic radiation under conditions 1, 3 and 5-day experimental investigation (Fig. 1) was performed on the developed and manufactured original equipment "Potting chamber", which allows you to simultaneously simulate the effects on laboratory animals' various levels of ambient temperature and adhere to the required parameters of electromagnetic radiation (Received a patent for a utility model № 83559 " Potting chamber"). Exposure was performed 5 times a week (for 4 hours daily) in 200-liter seed chamber. Additionally equipped cells for isolated free placement of animals and thermoelectric cooling device of air-air type that provides cooling of the air environment in the temperature range 4°C ± 2°C. A low-frequency signal generator was connected to the camera irradiation system, which is a plane-parallel capacitor formed by two metal plates 35x45 cm. Working frequency in a plane-parallel capacitor - 70 kHz; signal form - continuous sinusoid; voltage of the electrical component electromagnetic field in the working volume of the capacitor - 600 V / m.

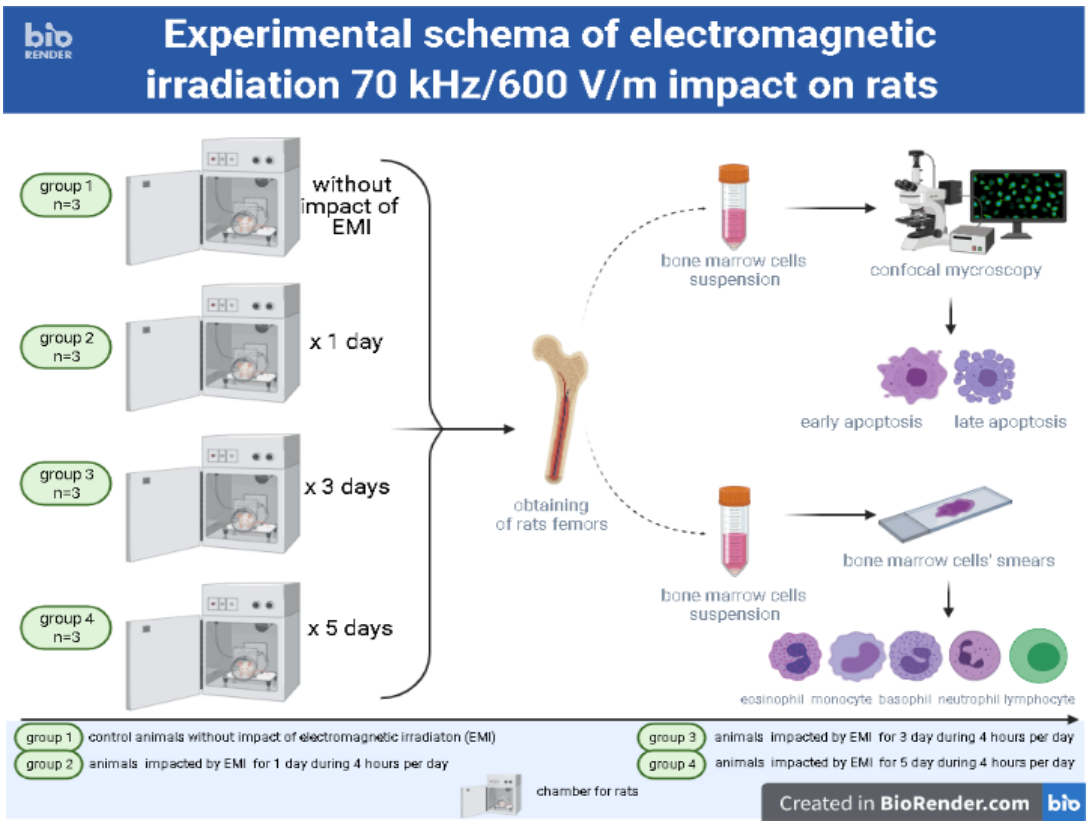

Fig. 1. Experimental schema for electromagnetic irradiation investigations

2.3 Scheme of Liver Fibrosis Induction

The rats were divided into two age groups: 24 young (3-month-old) and 24 old (20-month-old) ones. In each age group, animals were divided into groups: an intact control group (n=8), a group with Cu–induced liver fibrosis (n=8) and a group with CCl_4 – induced liver fibrosis (n=8). Rat models of Cu–induced liver fibrosis was established by multiple intraperitoneally injection of copper sulphate in different doses (1–1, 25 mg/100 g of body weight), as in investigation. CCl_4–induced liver fibrosis was induced by multiple intraperitoneally administration per chloromethane mixed with olive oil at a concentration of 0, 1 mL/100 g of body weight. The induction scheme is justified in the work. Rats injected with saline served as a control group.

2.4 Isolation of BMCs and *in vitro* Culture

The BMCs were obtained from two femoral rat bones following the procedure described in [18] and then they were cultivated in the Eagle's medium with antibiotics (1% gentamycin and 1% streptomycin) and 20% of inactivated fetal bovine serum. Culture was run under the standard conditions: temperature was 37°C in an atmosphere of 5% CO_2.The number of cells and their morphotypes were determined daily from the first to the fourth day of culture. The content of the BMCs at the initial stage of the culture always reached 2 million cells per ml, and it was unchanged during culture.

2.5 Determining the Cell Number and Morphotypes

The cell count and evaluation of BMC viability were carried out as described in [19]. The BMC morphotypes were determined immediately after the suspension preparation, and on the second and fourth days of culture as it was described in [20]. The cytological preparations were stained by the Romanovsky-Giemza method, and then they were analyzed by using 100× Zeiss Primo Star iLED microscope (Germany).

2.6 Cell Counts Using a Semi-Automated Cell Counter Invitrogen Countess

For analysis of the cell samples, firstly a solution containing 20 µl of cell suspension to be counted, and 20 µl of 0,4% trypan blue dye (Molecular Probes, T10282) were prepared. Then, 10 µl of this mixture was transferred to the Countess cell counting chamber slides (Invitrogen, C10228). The chamber was then placed on the counter Invitrogen Countess, the brightness and focus were cells count was performed. The focus of the instrument was calibrated using particle size standards of 5, 10 and 20 µm. A cell-count protocol was customized accounting for sensitivity, circularity, maximum and minimum size, and was applied for readings of both standards and samples. A cell suspension of 1×10^4 cells/100 µl for fluorescence staining was prepared by adjusting the harvested cell suspension concentration with the addition of Live Cell Imaging Solution (Molecular Probes, A14291DJ).

2.7 Cell Staining Protocol, Confocal Microscopy and Postacquisition Image Processing

To 50 µl isolated bone marrow cells suspension was added 5µl 1µM DAPI solution (Abcam, ab228549) for total nuclei label, 20µl Annexin Binding Buffer, 5µl Annexin V-Cy3 solution and 2µl 1µM SYTOX Green solution. Cells were incubated at 37°C in an atmosphere of 5% CO_2 (Eppendorf Galaxy 14S incubator) for 15 min. After incubation 13 µl cells suspension was placed in 2-well of glass imaging slide (X2XER203B#, Thermo Scientific) and covered coverslip (10812ibidi, Germany). Images of cells were acquired using the Olympus FV10i-LIV scanning confocal microscope at 37°C in an atmosphere of 5% CO_2. For postacquisition images processing the Olympus cell Sens Dimension software was used. Fluorophore's excitation was provided by 405 nm (DAPI), 473 nm (SYTOX Green) and 543 nm (Annexin V-Cy3) laser lines, and resulting fluorescence was acquired using 461, 530 and 570 nm emission lines respectively.

2.8 Statistical Analysis of the Results

The mean, standard deviation, standard error of the mean, and sample size were used as the characteristics of the obtained samples. The statistical significance of difference between two data groups was evaluated by the nonparametric Mann-Whitney U-test. The results were statistically processed by the OpenOffice and Origin software packages. The differences between the control and experimental groups were accepted as significant at $p < 0.05$.

3. RESULTS

3.1 "Lifespan" of Lymphocytes in the *in vitro* System during the Cultivation of Bone Marrow Obtained from Young and Old Animals with Liver Fibrosis

The number of lymphocytes in the culture of young intact animals did not change during the culture process (As shown in Fig. 2A). At the same time, the number of lymphocytes obtained from old animals, after transferring them to the in vitro system, to the 96th hour of culturing increased by 72% (As shown in Fig. 2B). It should be noted that the number of lymphocytes in young intact animals was 8.25% of the total number of bone marrow cells, while at the same time, the proportion of lymphocytes in old animals accounted for more than 20% of the total number of bone marrow cells (As shown in Fig. 2B).

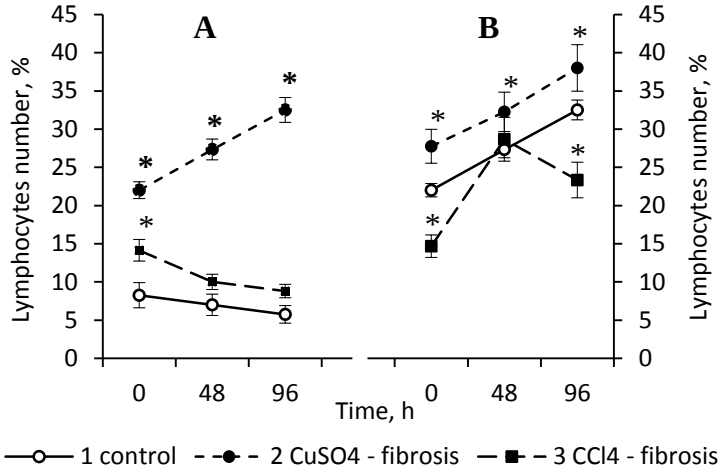

Fig. 2. The number of lymphocytes in the primary culture, obtained from bone marrow of 3-month-old intact animals (1), animals with Cu-induced (2) and CCl_4-induced (3) liver fibrosis (A), and the same for the 20-month-old animals of the same experimental variants (B)

*Data percentage is expressed as mean, in relation to all identified cells, taked to 100%. Data were obtained of three independent experiments ±S.E.M, n=8 per group; p≤0.05 between liver fibrosis and control animals are determined by *, by Mann — Whitney's U-test*

Consequently, lymphocytes obtained from intact old animals are able to fission in culture, unlike lymphocytes of young animals.

Administration of copper sulfate to young animals increased the content of lymphocytes in the bone marrow by 167% compared with the control level, and in older animals by 26%.

It is known, that the number of B - lymphocytes in the bone marrow does not decrease with age [21]. It should be noted that the number of autoantibodies [22] is increased in old animals. An increase in the number of the total lymphocyte population in 20 months rats can reflect the action of factors (inflammatory process, infectious carriers, etc.) in old animals. In favor of the participation of lymphocytes in the adaptive response, there is evidence of an increase in lymphopoiesis in animals with liver fibrosis. However, in old animals that initially had a high level of lymphocytes in the bone

marrow, induced by carbon tetrachloride did not increase their content. Consequently, the adaptive response of the organism to the introduction of the factor depends on the state of the functional system at the time of exposure, and it was different in young and old animals.

Lymphocytes obtained from old animals had a significantly greater proliferative activity in culture in vitro than lymphocytes isolated from the bone marrow of young animals. Lymphocytes are the only blood cell type that is capable of proliferating in peripheral tissues and this ensures their long-term existence and the formation of a long-term immune response [23]. As is well known, the entry of lymphocytes into the cell cycle links extracellular mitogens to membrane receptors [24,25], which stimulate MAP - activation kinase cascade. As a result, several mechanisms can be triggered, in particular, the activation of the Myc genes and the expression of the genes encoding the family of cyclins [26]. An important step in the passage of lymphocytes of the S - period of the cell cycle is the regulation factors E2F [27].

It should be noted that the regulation of lymphocyte proliferation involves not only mitogens and the expression of the corresponding genes that ensure the functioning of regulatory cascades of the proliferative response, but also the state of the epigenetic characteristics of the genome. It has been shown that the dephosphorylating of genes can influence the proliferative activity of cells [28].

The obtained results suggest that with age there was a "change" in the microenvironment of bone marrow cells, which affected the epigenetic and metabolic characteristics of bone marrow cells. Such differences did not affect cell morphotypes, but affected their functional characteristics.

If liver fibrosis was induced in young animals by repeated administration of copper sulfate, the number of lymphocytes in the bone marrow increased compared to intact animals of the same age by 63% (Fig. 2A, point 0). If the bone marrow cells of young animals with Cu - induced liver fibrosis were transferred to culture, the number of lymphocytes increased linearly at 48 and 96 hours of cultivation (Fig. 2A). If liver fibrosis was induced in young animals by the administration of carbon tetrachloride, the number of lymphocytes in them increased slightly compared to their number in young intact animals. However, lymphocytes did not proliferate in culture if they were obtained in young animals with CCl_4-induced liver fibrosis (Fig. 1A).

Therefore, the presence of liver fibrosis in young animals affects the number of lymphocytes in the bone marrow and it depends on the nature of the inducer, features of the microenvironment of bone marrow cells. The nature of the inducer had different effects on the proliferative activity of lymphocytes in the primary culture.

If lymphocytes were isolated in the composition of bone marrow cells in older animals with Cu - IFP, their number increased during cultivation, as well as lymphocytes of young animals (Fig. 2B). Lymphocytes obtained from older animals with CCl_4 – induced liver fibrosis also proliferated, in contrast to the young, and their number increased by 96% by 48 hours of cultivation, and later by 96 hours of cultivation, their number in culture decreased slightly (Fig. 2B). Number of lymphocytes in the bone marrow of old animals after Cu - induced liver fibrosis increased, and after CCl_4 - induced liver fibrosis was decreased in comparison with their intact level (Fig. 2B).

Therefore, the presence of liver fibrosis in young and old animals had different effects on the number of lymphocytes in the bone marrow and their behavior in primary culture in CCl4 - induced liver fibrosis.

The results obtained can be explained by the fact that the induction of liver fibrosis by different antigens changes the factors of the microenvironment of bone marrow cells and the nature of these changes depends on the inducer. An important factor that affects the qualitative and quantitative characteristics of bone marrow cells is the age of animals.

3.2 "Lifespan" of Neutrophils in the *in vitro* System during the Cultivation of Bone Marrow Obtained from Young and Old Animals with Liver Fibrosis

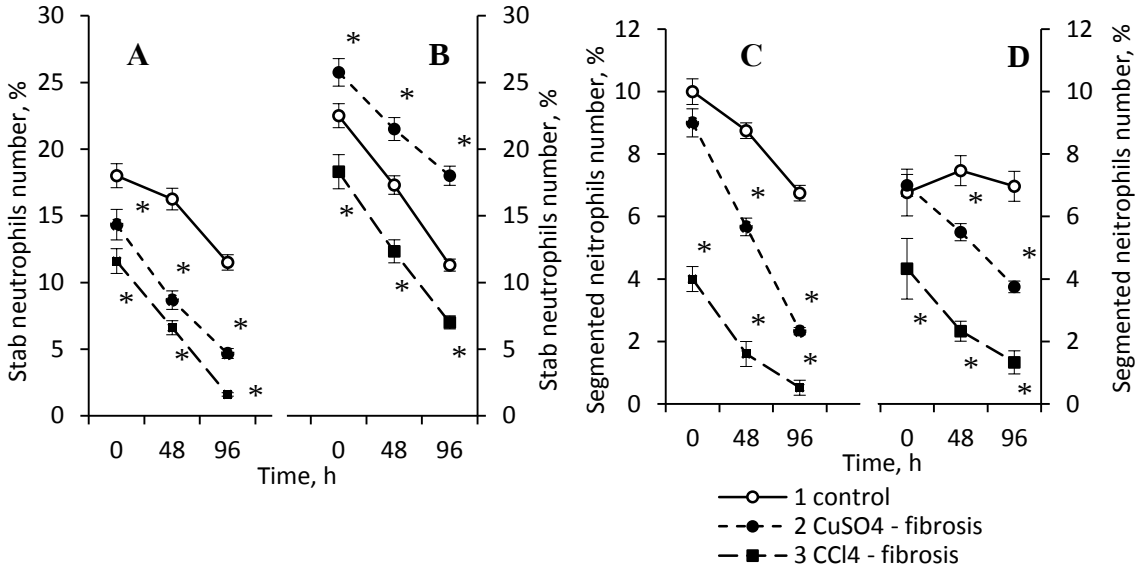

Fig. 3. The number of stab neutrophils in the primary culture, obtained from bone marrow of 3-month-old intact animals (1), animals with Cu-induced (2) and CCl_4-induced (3) liver fibrosis (A), and the same for the 20-month-old animals of the same experimental variants (B), as well as segmented neutrophils of 3-month-old (C) and 20-month-old (D) animals, respectively

*Data percentage is expressed as mean, in relation to all identified cells, taked to 100%. Data were obtained of three independent experiments ±S.E.M, n=8 per group; p≤0.05 between liver fibrosis and control animals are determined by *, by Mann — Whitney's U-test*

As is well known, stab neutrophils are maturing cell types. Their number in the bone marrow of young animals was 18%, and in the bone marrow of old animals – 22,5%. When they were transferred as part of the cell population to a culture isolated from intact young animals, their number did not significantly change. After 48 hours of incubation, their number decreased by 36% to 96 hours of cultivation. At the same time, the lifespan of the stab neutrophils in culture obtained from old animals was shorter compared with those cells isolated from young animals (As shown in Fig. 3A, B).

Such differences in the "lifespan" of stab neutrophils in young and old animals may be related to the rate of their maturation and differentiation into segmented neutrophils. It turned out that the number of segmented neutrophils isolated from the bone marrow of young animals and transferred to culture decreased almost linearly from 0 to 96 hours of cultivation, i.e. these cells had a short lifespan. At the same time, segmented neutrophils isolated from old animals and transferred to culture had a much longer "lifespan" compared with those cells isolated from young animals (As shown in Fig. 3C, D).

The results may indicate a different rate of maturation of neutrophils in both young and old animals. It may also indicate age differences in functional characteristics.

The presence of liver fibrosis in young and old animals was accompanied by a decrease in the lifetime of neutrophils in culture, as evidenced by data on a sharp decrease in the number of cells in the primary culture (As shown in Fig. 3). However, the "lifespan" of neutrophils isolated from young and old animals was different.

If we compare the number of neutrophils of young and old animals that survived to the hour 96 of culturing *in vitro*, we can conclude that the "lifespan" of neutrophils in culture obtained from old animals was significantly longer than that of neutrophils obtained from young animals with the exception of stab neutrophils from intact animals (As shown in Fig. 4).

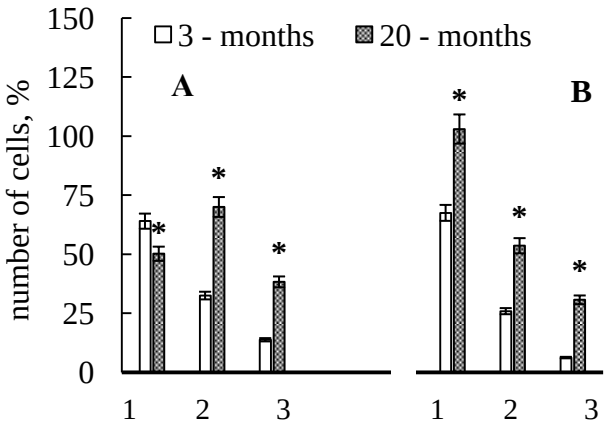

Fig. 4. The number of stab neutrophils (A) and segmented nuclear (B) neutrophils that remained in the culture after 96 hours of culturing *in vitro* for intact animals (1), for animals with Cu – induced liver fibrosis (2) and CCl_4 – induced liver fibrosis (3), which were obtained from young and old animals

*Data percentage is expressed as mean, in relation to all identified cells, taked to 100%. Data were obtained of three independent experiments, n=8 per group; p≤0.05 between liver fibrosis and control animals are determined by *, by Mann — Whitney's U-test*

These results indicate that the lifespan of cells and in particular neutrophils is determined by the integral metabolic characteristics of the organism which are realized at the level of the specific microenvironment of bone marrow cells. We can assume that bone marrow cells are a mirror of the integral characteristics of the whole metabolism in this sense. The characteristics of bone marrow cells will be influenced by a wide range of external factors (in this regard, it is of great interest to study the effect of electromagnetic radiation on bone marrow cells).

3.3 Effect of Electromagnetic Radiation on the Bone Marrow Cells Total Number

The intensive development of information technology and the widespread industrial use of electricity has led to a manifold increase in the general background of electromagnetic radiation. According to experts, at present, EMI has become one of the most powerful environmental factors. Despite intensive studies of the effect of EMI on living objects, at the moment there is no evidence of the negative effect of EMI on the organism, and the available data are very contradictory. This inconsistency can be explained by a number of reasons. First, the EMI frequencies we encounter are in the 10^2-10^{12} range, and the biological effect depends on frequency and power. Secondly, evolution takes place against the background of natural EMI, and the organism has mechanisms of adaptation to the increasing influence of EMI. Thirdly, different organism systems can have different sensitivity and, as a result, different responses are formed. Fourth, there is still no unified methodology for conducting EMI investigations. In this regard, it is extremely important to develop a methodology for investigation the effect of EMI on the organism. In this regard, an important stage is the choice of the object of research and the characteristics of the EMI.

We believe that the bone marrow, which reflects the integral characteristics of metabolism, is a successful model for the investigation of EMI. When choosing the frequency and scheme of the effect of EMI on the organism, we proceeded from the fact that the most powerful and stable, influencing the organism, are production installations that give radiation of 70 kHz / 600 V / m to determine the possible reaction of bone marrow cells of young *Wistar* rats.

It turned out that the number of cells in the bone marrow increased after a threefold consecutive exposure to EMI, after a fivefold exposure, this effect decreased, but remained higher than the control by 46% (Fig. 5).

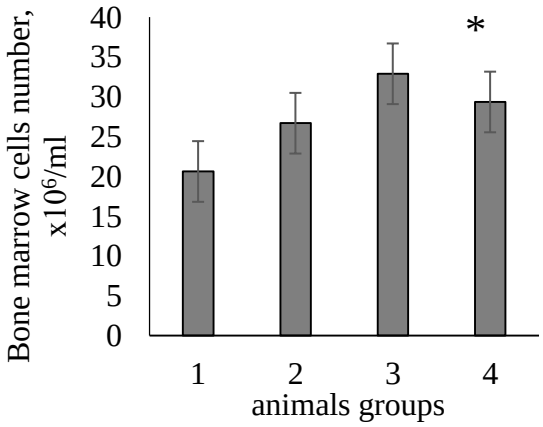

Fig. 5. The bone marrow cells number of 3 months animals, after exposure to electromagnetic radiation (70 kHz / 600 V / m): group 1 - control animals, group 2 - animals that were irradiated for 1 day, group 3 - animals that were irradiated for 3 days and group 4 - animals that were irradiated for 5 days

*Data percentage is expressed as mean. Data were obtained of three independent experiments, n=6 per group; p≤0.05 compared with cells of intact animals are determined by *, by Mann — Whitney's U-test*

As you know, the population of bone marrow cells is very heterogeneous and is represented by both differentiated cells (leukocytes, neutrophils, etc.), stem cells and poorly differentiated ones. Determination of the differentiated cells total number in the bone marrow, which included lymphocytes, basophils, eosinophils, stab and segmented neutrophils, metamyelocytes, myelocytes, monocytes (Fig. 5A), ranged from 50 to 60% of the total number of cells and did not change after 1- 5 times the influence of EMI (Fig. 6B).

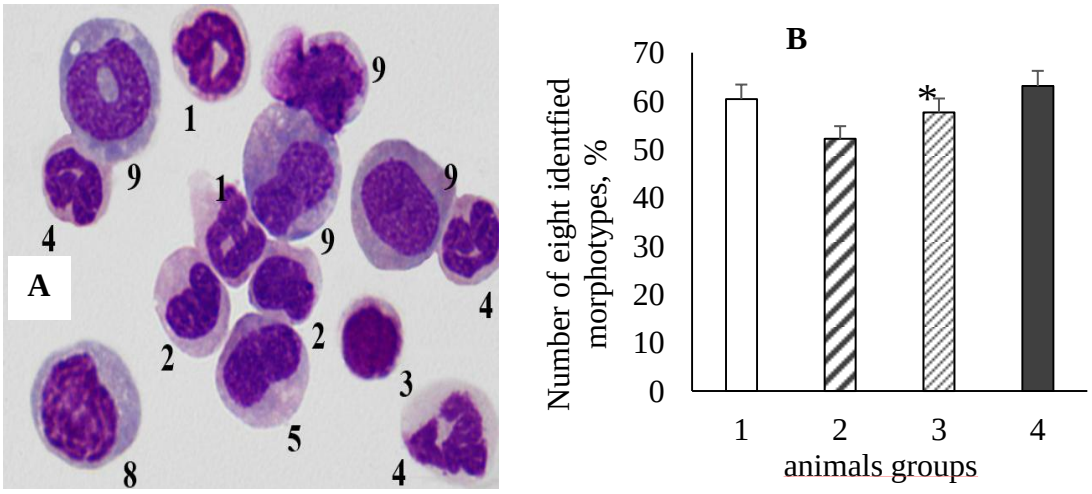

Fig. 6. A – Bone marrow cells types. Romanovsky-Gimza staining, where 1 - rod-shaped neutrophils; 2 - metamyelocytes; 3 - lymphocytes; 4 - segmental neutrophils; 5 - myelocytes; 6 - eosinophils; 7 - basophils; 8 - monocytes; 9 - undifferentiated cell. B – The total number of eight identified morphotypes

*Data percentage is expressed as mean. Data were obtained of three independent experiments, n=6 per group; p≤0.05 compared with cells of intact animals are determined by *, by Mann — Whitney's U-test*

At the same time, after a 1-fold sequential effect of EMI, the total number of eighth cell morphotypes in the bone marrow changed significantly in comparison with the control variants (Fig. 6B).

3.4 EMI Influence on the Apoptosis in Bone Marrow Cells

As known, the duration of the life cycle of bone marrow cells, like other and other types of cells, is different and can vary from several days to several months and years. The investigation of the regulation mechanisms of the cell's life cycle is one of the most important tasks of modern biology. An effective approach to investigation the life cycle and determining the effect of EMI on it is the investigation of apoptosis. It is known that apoptosis plays an important role in the mechanisms of maturation and selection of bone marrow cells, due to apoptosis, cell homeostasis is ensured. It has been shown that the process of apoptosis goes through several stages. The initiation of apoptosis does not mean that the cell will be destroyed, in the early stages it can be reversible. However, after passing through certain stages of its development, apoptosis becomes an irreversible process. Late apoptosis is characterized by fragmentation of nuclear structures and the formation of apoptotic bodies and phagolysosomes.

The composition of bone marrow cells contains up to 70% of non-nuclear cells, cells at the stage of early apoptosis - 7.1%, and at the stages of late apoptosis - 9.6% of all types of bone marrow cells (Fig. 7).

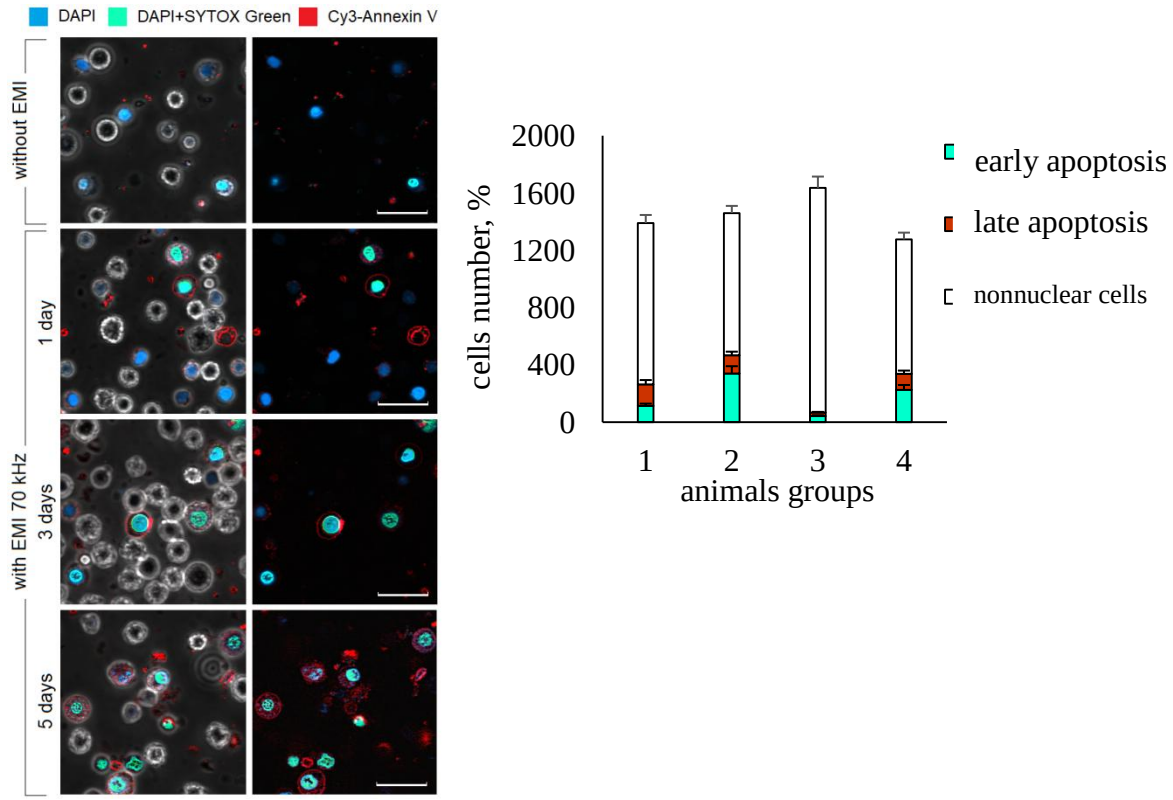

Fig. 7. Fluorescence of DAPI, SYTOX Green and Cy3-Annexin V in bone marrow cells of intact animals (1) and animals irradiated with EMI 70 kHz / 600 V / m for one (2), three (3) and five (4) days. Laser scanning confocal microscopy Olympus FV10i-LIV, x60 (Japan)

If the experimental animals were exposed to a single 4-hour exposure to EMI, then the number of non-nuclear cells decreased and amounted to 20%, and the number of cells at the stage of early apoptosis increased and amounted to 20.5%, while the number of cells with late apoptosis was 7.7% (Fig. 7). These results suggest that a relatively large number of cells undergo reorganization. However, these cells do not enter the late apoptosis stage and they continue to function. These data suggest that EMI induces epigenetic rearrangements and the formation of cells with altered functional characteristics, while the cells retain their morphotypes.

Further repeated irradiation for three days led to even more significant changes in the studied parameters. Thus, the number of nuclear-free cells increased by 24% compared with a single exposure to EMI and was 13% higher than in the control group, while the number of cells at the stage of early apoptosis decreased by an order of magnitude compared with a single irradiation and was 3 times less than the control. values (Fig. 5). In this variant, they were almost not detected at the late stage of apoptosis (there were about 1% of them) (Fig. 7).

A further increase in the number of EMI irradiation up to 5 times led to the fact that the number of non-nuclear cells, cells at the early and late stages of apoptosis did not differ from the control variant (Fig. 7).

The results obtained lead to several conclusions; bone marrow cells - a successful and promising model in the study of the biological effect of EMI; influences the process of maturation and programming of bone marrow cells, it depends on the number of repeated influences, which reflects the formation of induced resistance to the action of EMI.

4. CONCLUSION

Bone marrow cells are a successful model in investigation the response at the cellular level to endogenous (liver fibrosis and other pathologies) and exogenous (electromagnetic and other types of radiation) influences. Responses are manifested in a change in the total number of cells in the bone marrow, a change in the direction of differentiation of stem cells and even a change in the functional characteristics of already differentiated bone marrow cells, in particular, a change in the "life span". It was shown that the "lifespan" of bone marrow cells depends not only on the microenvironment of the cells, but also on the epigenotype. These data are supported by the fact that the same types of bone marrow cells obtained from young and old animals differed in terms of "life expectancy" in the case of cultivation in an in vitro system under the same conditions. The development of liver fibrosis also affects the "lifespan" of bone marrow cells. Since liver fibrosis develops against the background of oxidative stress, changes in the spectrum of cytokines, it can be assumed that changes in the microenvironment of bone marrow cells are accompanied by epigenetic changes in bone marrow cells. In the study [29] was evaluation of the fields of the electromagnetic fields on the concentration of vitamin A, E and antioxidants in plasma. It may indicate the electromagnetic radiation influence on the general regulation system of organism. Electromagnetic radiation effects on the redox system function, which is one of the based system regulation [30]. Further studies of the mechanisms of this phenomenon are important and urgent.

Along with this, it turned out that bone marrow cells are extremely "sensitive" even to small (weak) influences of external factors. Electromagnetic radiation was chosen as such weak effects. It turned out that radiation (70 kHz 600 V / m), which occurs in industrial conditions, affects the change in the number of cells in the bone marrow and the process of apoptosis, which are associated with the "lifespan" of cells.

It should be noted that the action of the EMI has a complex U-shaped character, which indicates the adaptive nature of these changes. Further studies of the response of bone marrow cells to endogenous and exogenous influences are extremely important in the diagnosis of a number of pathologies.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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Biography of author(s)

A. I. Bozhkov
Research Institute of Biology, V. N. Karazin Kharkiv National University, Kharkiv, Ukraine and Department of Molecular Biology and Biotechnology, V. N. Karazin Kharkiv National University, Kharkiv, Ukraine.

He is a Professor, The Head of Research Institute of Biology, V.N. Karazin Kharkiv National University, Ukraine, and specialist of biological systems field. He achieved Doctor of Science in Biology. He is the Vice-president of gerontology and geriatricians of Ukraine. His Main publications are investigation of age-dependent pathologies (liver fibrosis) and mechanisms of animals and plants adaptation to extremal factors of environment (heavy metal ions, temperature, and radiation). He develops the concept of the hormesis effect in increasing longevity. He has published many research articles in the national and international journals.

S. L. Ohiienko
Research Institute of Biology, V. N. Karazin Kharkiv National University, Kharkiv, Ukraine and Department of Molecular Biology and Biotechnology, V. N. Karazin Kharkiv National University, Kharkiv, Ukraine.

She is a Lecturer of Molecular Biology and Biotechnology Department, Research fellow of Research Institute of Biology, V.N. Karazin Kharkiv National University; Biologist in Clinical-diagnostics laboratory, Ukraine. She achieved Doctor of Science in Biology Her Scientific interests are investigation of bone marrow cells characteristics (biochemical, cellular, molecular ones) under influence of organisms pathologies, age and radiation. Her other field of scientific interests is clinical diagnostic of different pathologies by immunofluorescence, cytological and biochemical analysis. She is an Author and coauthor of over 20 scientific publications, published in journals, indexed in Scopus and Web of Science.

Eu. G. Ivanov
Research Institute of Biology, V. N. Karazin Kharkiv National University, Kharkiv, Ukraine and Department of Molecular Biology and Biotechnology, V. N. Karazin Kharkiv National University, Kharkiv, Ukraine.

He is an Associate Professor of Molecular Biology and Biotechnology Department of V.N. Karazin Kharkiv National University , Ukraine. He achieved Doctor of Science in Biology. His main research is devoted to the development of biotechnological methods for processing colostrum and the production of new immunobiotechnological substances and the study of their physiological and biochemical functions (in particular, the effect of colostrum components on the function of bone marrow cells, on the regulation of the pro-antioxidant system in the body). He is a coauthor of over 20 scientific publications.

A. Yu. Bondar
Department of Molecular Biology and Biotechnology, V. N. Karazin Kharkiv National University, Kharkiv, Ukraine.

She is an Assistant Professor of Molecular Biology and Biotechnology Department, Faculty of Biology V.N. Karazin Kharkiv National University, Ukraine. Her Scientific interests are investigation of bone marrow cells characteristics (biochemical, cellular, molecular ones) under influence of organisms pathologies, age and radiation. Her other field of scientific interests is research of the effect of colostrum low molecular weight on the activity of bone marrow cells in culture. She is an Author and coauthor of over 20 scientific publications, published in journals, indexed in Scopus and Web of Science.

Yu. H. Kot
Biochemistry Department, V. N. Karazin Kharkiv National University, Kharkiv, Ukraine.

He is an associate professor in the Biochemistry Department of V. N. Karazin Kharkiv National University and a member of the FEBS. He obtained his PhD in biochemistry at the V. N. Karazin Kharkiv National University. In his PhD thesis, he focused on understanding how connective tissue cells respond to external mechanical stress. Since 2012, he teaches courses and laboratory practices on the methods in cell technology and cell and molecular biochemistry for Master's students in Biochemistry and Bachelor's students in Biomedical Nanotechnology. Since 2018, he is the administrator of the Cell Biochemistry Laboratory, which serves researchers throughout the university. In this role, he oversees the use of cleanrooms for cell culture, analytical flow cytometer, and laser confocal microscope, providing instructions on their use and data analysis, and acts as a consultant on the design and interpretation of experiments. In addition, he manages and assists users in applying post-acquisition confocal data analysis. His main research interests are related to the response of fibroblasts and keratinocytes to external mechanical stress and xenobiotics. In this area of research, he is a co-investigator in a collaborative group of biochemists and cell biologists. His current research focuses on the development and implementation of biologically sustainable, low-toxicity cell therapy regimens for patients suffering from life-threatening burn diseases that can lead to tissue degeneration and trophic ulcers. He is an innovator in the use of mechanically stimulated cells for transplantation to prolong tissue survival after irradiation. He has published many research articles in the national and international journals.

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Chapter 6
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Etiological and Clinical Patterns of Isolated Hepatomegaly at a Tertiary Level Hospital in Bangladesh

Chanchal Kumar Ghosh[1*], Fakhrul Islam[2], Ershaduddin Ahmed[3], Dilip Kumar Ghosh[4], Azizul Haque[5], Q. T. Islam[6] and M. A. Azhar[7]

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ABSTRACT

The aim of the study was to find out the etiology of isolated hepatomegaly in Bangladesh. A prospective cross sectional study was done at medicine department in a tertiary care hospital in Bangladesh. Consecutive one hundred hospital admitted patients having isolated hepatomegaly were included in the study. Hepatomegaly is usually associated with splenomegaly. However, sometimes there may be isolated hepatomegaly without splenomegaly in different pathological conditions. In our study we found that most common cause of isolated hepatomegaly was liver abscess (34.0%) followed by congestive cardiac failure (30.0%), Viral hepatitis (14%), secondaries in the liver (8%) primary hepatocellular carcinoma(6%) and Fatty liver (2%). Among the liver abscess 59% were amebic & 41% were pyogenic abscess.
Keywords: Isolated hepatomegaly; liver abscess.

ABBREVIATIONS

CCF : Congestive cardiac failure;
HCC : Hepatocellular carcinoma;
IHD : Ischemic heart disease;
BCS : Budd-Chiari syndrome.

1. INTRODUCTION

The liver is one of the body's most important organs, as it is involved in synthesis, metabolism, excretion, detoxification, and immunity [1-3]. It obtains 1/4th of total circulating blood despite being only 1/40th of adult body weight.The liver receives two types arterial blood: systematic blood from the hepatic artery and venous blood from the intestines through the portal vein. This enables the liver to interact with toxic elements, food items, and drug metabolites that enter the liver via portal circulation [4,5]. Thus, inflammation, infection, and malignant transformation of the liver can occur as a result of a variety of causes. Hepatomegaly may also be caused by these pathological factors. The common causes of hepatomegaly include hepatitis, liver abscess, congestive cardiac failure (CCF), fatty liver, primary and secondary carcinoma of liver, cystic disease, amyloidosis, tuberculosis, typhoid fever, malaria, constrictive pericarditis and cardiac tamponade, Budd-Chiari syndrome, glycogen storage disease, biliary obstruction, Riedel's lobe, low lying diaphragm and normal variant [6-15]. In many

[1]Department of Gastroenterology, Bangabandhu Sheikh Mujib Medical University, Dhaka, Bangladesh.
[2]Department of Medicine, Holy Family Red Crescent Medical College, Dhaka, Bangladesh.
[3]Department of Gastroenterology, Chittagong Medical College, Chittagong, Bangladesh.
[4]Department of Gastroenterology, Shaheed Suhrawardy Medical College, Dhaka, Bangladesh.
[5]Department of Medicine, Shaheed Ziaur Rahman Medical College, Bogra, Bangladesh.
[6]Department of Medicine, Popular Medical College, Dhaka, Bangladesh.
[7]Department of Medicine, Green life Medical College, Dhaka, Bangladesh.
**Corresponding author: E-mail: dr.chanchalghosh@gmail.com;*

cases of hepatomegaly, inflammation of the liver is associated with that of the spleen. However, isolated hepatomegaly may also occur in various pathological conditions [16,17]. Indeed, almost nothing has been reported about isolated hepatomegaly from Bangladesh.

This study conducted here was accomplished in a tertiary level hospital of Bangladesh. The clinical features of 100 consecutive patients with isolated hepatomegaly were evaluated. In addition, clinical profiles of the patients and demographic data were collected. This study would provide insights about diagnosis and management of this important pathological condition in clinical medicine.

2. MATERIALS AND METHODS

The present prospective study was carried out in different medicine units of a tertiary level hospital in Bangladesh on consecutive 100 admitted patients having isolated hepatomegaly. Isolated hepatomegaly was considered when hepatomegaly was not associated with splenomegaly clinically and sonologically. Hepatomegaly was considered when the total span of the liver in the midclavicular line as determined by clinical examination (palpation and percussion) was >10 cm and >12.5 cm by sonography. After the preliminary selection of patients, detailed clinical history, thorough physical examination and laboratory investigations were done. The details were recorded according to predesigned questionnaire. Investigations, such as complete blood count and erythrocyte sedimentation rate, routine and microscopic examination of stool and urine, serum bilirubin, serum glutamic transferase, serum alkaline phosphatase, ultrasonogram of whole abdomen were performed in all patients. Other investigations like hepatitis B surface antigen, antibody to hepatitis C virus , prothrombin time, alpha fetoprotein, chest X-ray, aspiration of pus for analytic study (physical examination, cytology, staining, culture and sensitivity), CT scan, electrocardiogram, and liver biopsy were done in selected cases. Aspiration of liver abscess was done under sonographic guidance in Nuclear Medicine Department. Aspirated materials were sent for microscopical examination and culture and sensitivity in the Department of Microbiology. Liver biopsy was done in selected patients using Tru-cut needle and the histopathological examinations were done in the Department of Pathology.

3. RESULTS

Total one hundred consecutive patients with isolated hepatomegaly were included in the study. The mean age of the patients was 42.2 years (standard deviation, 3.2 years) with a range of 15 to 80 years. The predominant age group was between 41 and 50 years (29.0%), followed by the age group of 31 to 40 years (24.0%) and 21 to 30 years (20.0%). Among them 76.0% were male and 24.0% were female. Most common cause of isolated hepatomegaly in the study population was liver abscess (34.0%) followed by CCF (30.0%) and viral hepatitis (14.0%), secondary carcinoma of liver (8.0%) and primary HCC (6.0%). Other causes of isolated hepatomegaly were hydatid cyst of liver (2.0%), fatty liver (2.0%), Budd-Chiari syndrome (1.0%), autoimmune hepatitis (1.0%), tuberculosis (1.0%) and typhoid fever (1.0%) (Fig. 1). Out of 34 patients with liver abscesses, 20 had amebic abscesses and 14 were due to pyogenic causes. A single abscess focus was seen in 16 patients, whereas, multiple abscesses were detected in 18 patients.

Among 30 patients with CCF, corpulmonale (40.0%) was the commonest cause of isolated hepatomegaly, followed by valvular heart disease (26.7%), ischemic heart diseases (10.0%) and cardiomyopathy (10.0%). Other causes of CCF in the present study were constrictive pericarditis (6.7%), hypertensive heart disease (3.3%) and congenital heart disease (3.3%). Among the 17 patients with hepatitis, the main cause of hepatitis was hepatitis viruses (82.4%). Hepatitis due to hepatitis A virus (HAV), hepatitis B virus (HBV), HCV and hepatitis E virus (HEV) were 35.3, 23.4, 11.8 and 5.9% respectively. Other causes of hepatitis were dengue virus, tuberculosis, typhoid and autoimmune diseases (5.9% each).

Among 14 patients of malignancy of the liver, six patients (42.86%) were diagnosed as primary hepatocellular carcinoma and eight (57.14%) were secondary carcinoma of liver. Abdominal pain (80.0%), loss of appetite (76.0%), general weakness (60.0%) and fever (54.0%) were the common clinical presentations of these patients. Other clinical presentations were loss of weight (51.0%), pallor

(51.0%), nausea and vomiting (44.0%), jaundice (42.0%) and exertional breathlessness (31.0%). Among the total cases 83.0% patients had tender hepatomegaly and 17.0% did not have any complain of pain. The consistency of the liver was soft in 48.0% patients, firm in 36% patients and hard in 16% patients. Among the primary HCC, 66.7% were associated with HBV infection and 33.3% were anti-HCV positive.

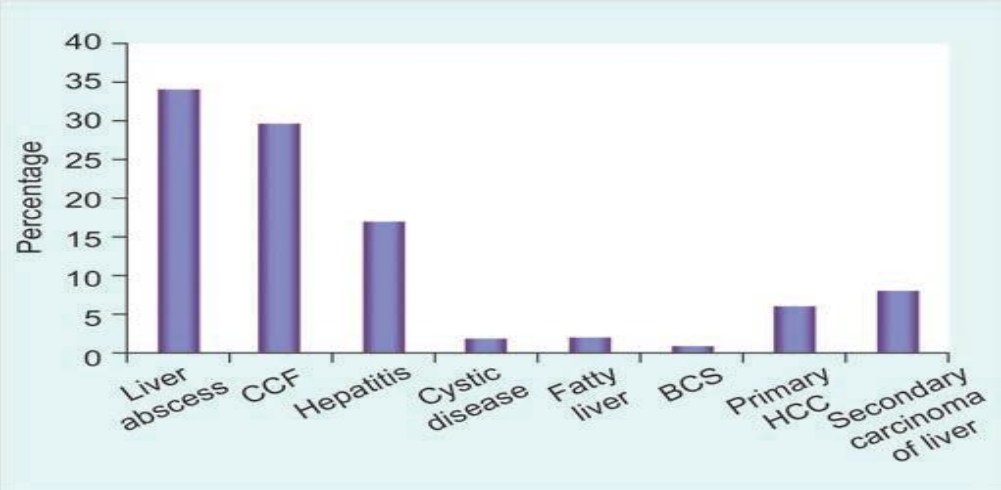

Fig. 1. Causes of isolated hepatomegaly at Bangladesh; CCF, congestive heart failure, BCS, budd-chiari syndrome

Liver span was measured by ultrasound. Liver was markedly enlarged in case of carcinoma of both primary and secondary (64.3%), whereas, the liver was slightly enlarged in most cases with hepatitis (64.7%) (Table 1).

Liver biopsy was done in 25 selected patients. Among them, histopathological findings of eight patients (32%) were consistent with secondary carcinoma of liver, six (24.0%) were primary HCC, three (12.0%) were liver abscess, two (8.0%) fatty liver, one (4.0%) tubercular granuloma and inconclusive findings were seen in five cases (20.0%) (Table 2).

4. DISCUSSION

The aim of this study was to find out the etiology and clinical pattern of isolated hepatomegaly among the patients admitted in different medicine units of a tertiary level hospital in Bangladesh. In this study hepatomegaly was defined when the span of the liver was above the upper limit of normal. There was a male predominance in patients with isolated hepatomegaly, which support Khan et al who reported about male predominance about hepatomegaly [6].

The most common cause of isolated hepatomegaly was liver abscess followed by CCF, hepatitis, secondary carcinoma of liver, primary hepatocellular carcinoma, cystic diseases of liver, fatty change and Budd-Chiari syndrome.

There is no study, so far in our country showing the etiologies and clinical profiles of isolated hepatomegaly and thus a comparative analysis could not be accomplished. But, some findings of our study are comparable with those of other countries [6-15]. Shennak et al have shown CCF (38.5%), carcinoma of liver (19.6%), acute hepatitis (13.5%), cystic diseases (7.88%), fatty change (5.6%), and liver abscesses (2.4%) as major causes of isolated hepatomegaly from a cohort of 800 patients from Jordan7. In the present study, the higher incidence of liver abscesses have been contributed by social and public health factors like; lower socioeconomic condition, malnutrition, poor sanitary condition, contact infection of *E. histolytica*, consumption of local wine prepared by partially fermentation of palm juice. The commonest cause of liver abscess worldwide is amebiasis, but in the developed world pyogenic causes are of increasing importance [1]. In the present study out of 34 patients with liver

abscess, 58.8% were due to amebic abscesses and 41.2% were due to pyogenic abscess. Amoebic infection is the most common cause of liver abscess in south east asia [18]. We found corpulmonale as the commonest cause of CCF, followed by valvular heart disease, ischemic heart disease and cardiomyopathy. The incidence of CCF is on increase due to poorly-developed health care delivery system and late attendance of patients to physicians for social reasons. Hepatitis due to HAV, HBV, HCV, HEV and others etiologies were prevalent among patients with isolated hepatomegaly. Dengue is an emerging and serious public health problem at Bangladesh. Assessment of liver size and their function may help to accomplish early proper diagnosis of dengue. HCC is a primary malignancy of the liver. It is a major complication of hepatitis virus infections in many instances, hepatomegaly is the first subjective symptom of HCC in Bangladesh, as most of the advanced cases of HCC attend physicians. This tremendously restricts treatment option of HCC patients in clinics. Isolated hepatomegaly due to secondaries may give clue to the clinician about primary site which sometimes help in the curative treatment. Colorectal cancer most commonly metastasize to the liver 18.15-25% patient with colorectal cancer have hepatic metastasis during presentation [19].

Table 1. The extent of isolated hepatomegaly in different pathological conditions

Disease	**12.5-15.5**	***Liver span (in cm)*** **15.6-18.5**	**>18.6**	***Total***
Liver abscess	7 (20.6)	15 (44.1)	12 (35.3)	34 (100)
Congestive cardiac failure	18 (60.0)	10 (33.3)	2 (6.7)	30 (100)
Hepatitis	11 (64.7)	6 (35.3)	0 (0.0)	17 (100)
Carcinoma of the liver	0 (0.0)	5 (35.7)	9 (64.3)	14 (100)
Others	1 (20.0)	3 (60.0)	1 (20.0)	05 (100)

Values in the parenthesis indicate percentage

Table 2. Histopathological diagnosis of liver biopsy of patients with isolated hepatomegaly

Histopathological diagnosis	***Frequency***	***Percentage***
Primary HCC	6	24.0
Secondary cancer of liver	8	32.0
Tubercular hepatitis	1	04.0
Consistent with liver abscess	3	12.0
Fatty liver	2	08.0
Normal histology	5	20.0
Total	25	100.0

Liver biopsy was performed in 25 patients with isolated hepatomegaly

In this study two patients with isolated hepatomegaly had fatty change of liver; one was due to alcohol and the other patient had clinical feature suggestive of malnutrition. NAFLD/NASH is now the most common cause of chronic liver disease throughout the world. NASH is mostly diagnosed incidentally with abnormal liver biochemical test or hepatic steatosis on imaging. But some people with NASH can present with right upper quadrant pain or discomfort due to capsular stretching from hepatomegaly [20]. Two patients presented with hydatid cysts which was diagnosed by enlarged right lobe of liver with cystic consistency, sonologic findings and by ELISA test. Hydatid cyst is a zoonotic disease with an endemic regional distribution and the most common localization is the liver (50-70% of all cases). One patient in this study diagnosed as Budd-Chiari syndrome, presented with rapid appearance of huge hepatomegaly, ascites with collateral veins in anterior abdominal wall. Budd chiari syndrome occur in hypercoagulable state and acute budd chiari syndrome typically present with tender hepatomegaly & ascites [21].

Taken together, the present clinical study assessed the causes and clinical factors related to isolated hepatomegaly in Bangladesh. Although Bangladesh is a small country, it possesses variable geographical characteristics and people with different social culture. In this study, analyzes were done in 100 patients with isolated hepatomegaly. If similar studies are done in other centers of the country, data can be accumulated to draw a profile of isolated hepatomegaly in Bangladesh. It is highly tempting to consider that different etiological factors would be prevalent in different parts of Bangladesh on the basis of local social and health care delivery system. Patients presented with

isolated hepatomegaly vary from very benign condition like fatty liver to fatal condition as hepatocellular carcinoma. In the present study it was found that liver abscess is the commonest cause of isolated hepatomegaly followed by congestive cardiac failure.

5. CONCLUSION

From our study we found that etiology of isolated hepatomegaly varies from benign condition like fatty liver to malignant condition like carcinoma of the liver. Multicenter study with large sample size is warranted to develop more insights about diagnosis and management of isolated hepatomegaly.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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Biography of author(s)

Dr. Professor Chanchal Kumar Ghosh
Department of Gastroenterology, Bangabandhu Sheikh Mujib Medical University, Dhaka, Bangladesh.

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Fakhrul Islam
Department of Medicine, Holy Family Red Crescent Medical College, Dhaka, Bangladesh.

He was born in 1967. He is a physician and gastroenterologist of Bangladesh. He completed his S.S.C from Brahmandi Govt School, Narshingdi in 1982. He passed his H.S.C from Dhaka College in 1984. He graduated in medical science (MBBS) in 1992 from Sher-e-Bangla Medical College in Barisal. He obtained his post graduation F.C.P.S degree in medicine from B.C.P.S in 1999. He acquired M.D in gastroenterology from BSSMU, Dhaka. He has several publications in different journals of home and abroad. He was involved in a therapeutic trial for H.pylori induced peptic ulcer disease which is common in Bangladeshi patients. He also worked in a research project regarding discovery of etiology and management of liver abscess in Bangladeshi perspective. He participated in many gastroenterology workshops organized in Bangladesh and outside (i.e- India, South Korea). He presented a poster regarding management of steroid refractory ulcerative colitis in IMKASID 2019 held in Seoul, S.Korea.
He is currently working as a professor of gastroenterology and medicine at Holy Family Red Crescent Medical College & Hospital. He regularly takes part in health related talk shows in different media to raise public awareness about gastrointestinal and liver diseases (i.e- hepatitis, fatty liver, peptic ulcer, IBS) He also acts as a trainer in the field of G.I procedures.

Ershaduddin Ahmed
Department of Gastroenterology, Chittagong Medical College, Chittagong, Bangladesh.

Biography is available in this link: https://www.bookpi.org/wp-content/uploads/2021/10/Ershad-short-CV.pdf

Dilip Kumar Ghosh
Department of Gastroenterology, Shaheed Suhrawardy Medical College, Dhaka, Bangladesh.

Biography is available in this link: https://www.bookpi.org/wp-content/uploads/2021/10/CV_Dr.-DILIP.pdf

Q. T. Islam
Department of Medicine, Popular Medical College, Dhaka, Bangladesh.

Biography is available in this link: https://www.bookpi.org/wp-content/uploads/2021/10/CV-QT-ISLAM.pdf

M. A. Azhar
Department of Medicine, Green life Medical College, Dhaka, Bangladesh.

Research and Academic Experience: 40 years teaching experience.

Research Area: Infectious Disease.

Number of Published Papers: 85.

Any Other Remarkable Point: Ex principle
Sir sallimullah medical college and Green Life Medicle collge.

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Chapter 7
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Bone Scan in Gastric Cancer Patients: A Retrospective Review

Bandar Idrees Ali[1,2*], O. Joo Hyun[1] and Khuloud Omar Bukhari[3]

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ABSTRACT

Background: Bone scan has a very highly sensitive role in detection bone metastases. Bone metastasis from stomach cancer occurs only rarely and it is known to have a very poor prognosis. Bone metastases in Gastric cancer are mainly osteolytic, and disruption of bone integrity and resulting in bone pain and pathological fracture.
In this study, we aimed to review the incidence, clinical characteristics, and related risk factors for bone metastases in patients with a primarily diagnosis of gastric cancer.
Methods: We retrospectively evaluated all patients who diagnosed with primary gastric cancer and underwent initially as staging working up with bone scintigraphy between 2010 and 2014 at Seoul St. Mary's hospital, The Catholic University of Korea. Total numbers of primary diagnosis of gastric cancer patients were 1589/1721 (92.33%) patients received bone scan as initial staging work up. We further analyzed the patients according to eligibility criteria we created and the incidence of and the risk factors for bone metastases were investigated.
Results: Out of 1589 patients analyzed, bone metastases were clearly confirmed only in 15 patients (0.8%). The mean age was 59.0 ± 8.6 years (range 24–90) and a majority of patients were male (60%). Dominant histological type either poorly differentiated type or signet ring cancer type and Bormann's classification type 3 was the majority. In the distribution of the gastric tumor in correlation to the upper, middle and lower third was not clinically variant. The mean tumor size among this group was 3.6 ± 2.3 cm (range 0.6–20 cm). All patients were advanced gastric cancer type clinically and the median follow-up period was 9 months. The incidence of bone metastases was (20%). In (80%) of patients had bone metastases and another site of metastases. Among these, most of the time associated regional lymph node metastases was found. Most patients had multiple bone metastases instead of a single bone lesion. The whole patients of bone metastases were advanced gastric cancers, and the most common metastatic site was the whole skeleton, followed by combined vertebra, rib and scapula.
Bone scintigraphy and PET-CT were mostly used together for diagnosing bone metastasis. The serum alkaline phosphatase at the time of diagnosis had increased in only 5 cases (35.71%) and there were clinical symptoms of bone pain in 8 cases (53.0%). Other variables also were not significantly valued like anemia, tumor markers like carcinoembryonic antigen (CEA) and cancer antigen 19-9 (CA19-9).Treatment was given to 14 cases (93.3%) and it was mostly chemo or concomitant chemoradiotherapy.
Conclusions: The preoperative bone scan was positive in 0.8% for bone metastasis in patients with gastric cancer. Suggesting that whole-body bone scan should not be performed routinely in patients with gastric cancer. For bone scan to be as cost-effective tool, may be needed for selected group of patients i.e. advanced stages of gastric cancer or clinically symptomatic patients. Serum ALK has poor correlation with early bone metastasis detection.

Keywords: Radionuclide imaging; gastric cancer; bone metastasis; ALK.

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[1]*Seoul St. Mary's Hospital, The Catholic university of Korea, Seoul, South Korea.*
[2]*Department of Surgery, Prince Sultan Military Medical City, Riyadh, Saudi Arabia.*
[3]*Ministry of Health, Saudi Arabia.*
**Corresponding author: E-mail: biaa1003@yahoo.com;*

1. INTRODUCTION

Gastric cancer (GC) is the fourth most common cancer diagnosis worldwide in men following lung, prostate and colorectal, and the fifth in women following breast, colorectal, cervical and lung with an expected incidence of 640,000 and 350,000 cases in 2011, respectively [1]. There are only a few studies have been published in the literature focusing on the onset of bone metastases in GC. Approximately 8% of total cases and 10% of annual cancer deaths worldwide are attributed to GC [2]. Bone metastasis is usually associated with disseminated vascular coagulation, hemolytic anemia and other hematological complications, and the prognosis is very poor [3-5]. Bone is considered one of the most common metastatic sites in many types of cancer like breast, prostate, and lung but rarely in gastric cancer. However, gastric cancer generally metastasizes to the peritoneal membrane, liver, lymph nodes, etc., and it may metastasize to the spleen, adrenalin, ovary, lung, brain, and skin. To improve the overall survival among gastric cancer patients, it is very important to find out the presence or absence of bone metastases as well distant metastasis during initial workup. It is usually associated with disseminated vascular coagulation, hemolytic anemia and other hematological complications, and the prognosis is very poor [6].

Some studies have reported that the patients had a relatively younger age, often had a signet ring cell carcinoma, or poorly differentiated adenocarcinoma and exhibited elevated serum alkaline phosphatase (ALP) and/or lactate dehydrogenase levels, but other studies could not identify distinguishing characteristics of BM [7,8,9]. Although no optimal therapeutic strategy has yet been established for Bone metastasis in gastric cancer, many reports needed in this field to conduct the onset of bone metastases and apply standard treatment guideline. Moreover, few international guidelines recommend to routinely evaluate bone metastasis at the time of diagnosis or during follow up or pharmacological treatment. Bone metastases in GC are mainly osteolytic, and disruption of bone integrity and resulting in bone pain and pathological fracture.

Many reports regarding the value of bone scintigraphy in the initial screening work up for curative gastric cancer have not been well focused. The aim of this study was to investigate the incidence of and related clinical risk factors for bone metastases during initial work up after diagnosis of gastric cancer in patients.

We also proposed that the use of elevated alkaline phosphatase (ALP) levels for the detection of bone metastasis could be helpful to correlate with positive bone scintigraphy finding.

2. PATIENTS, SUBJECT AND METHODS

2.1 Patients

From January 2010 till December 2014, all patients visited Seoul St. Mary's Hospital, The Catholic University of Korea, Seoul, Korea, were entered into a database of electronic medical record with the keyword of "Gastric Cancer" were reviewed retrospectively. A total number of 1721 of patients were retrieved and reviewed from the system to this study.

Among these patients, 1706 patients were excluded from this study because many reasons. Although 1045 patients have baseline bone scintigraphy for the initial staging, were excluded because they have a clear feature of a benign lesion on bone scan, 473 with concomitant or history of cancer other than gastric cancer, 132 patients had no bone scan during initial staging and 56 patients with insufficient data.

Finally, a total of 15 patients were enrolled in our study (Table 1). Data were collected for demographic characteristics and clinicopathologic findings. Approval by the Institutional Review Board of the study was taken

Table 1. Patients detailed data and results of procedures

No	Age/Sex	Histology type	Borman's type	Gastric ca location	pTNM	AJCC 7th stage	Surgery (Yes/No)	Metastases site	Anemia (Hb> 10g/dl)	ALP (44-147IU/L)	CEA (<5ng/ml)	CA19-9 (>37ng/ml)	
Patient.1	55 M	Signet Ring ca.	III	Antrum	T4aN2M1	IV	No	Peritoneal carcinomatosis; perigastric, gastrohepatic ligament, left paraaortic space LAP; left clavicle; right scapula	12.9	43	51.29	2630	--
Patient.2	61 M	Intestinal type	Not available	Fundus	T4aN3M1	IV	No	hepatogastric ligament, splenic hilum, periportal, retroperitoneum, left common iliac, and left supraclavicular area, rib cage, left scapula, both pelvic bones, sacrum, and T-L vertebrae, subcutaneous layer of left lower back and left lateral chest wall	11.3	99	38.38	101.59	
Patient.3	65 M	Signet ring Ca.	III	Cardia	T3N0M1	IV	Yes	left 7th rib	13.9	74	0.05	5.59	
Patient.4	41/M	Signet ring ca.	III	Antrum	T3N3M1	IV	No	Metastatic LNs in bilateral supraclavicular areas, mediastinum & left pulmonary hilum, left internal mammary chain, cardiophrenic angle, left anterior peridiaphragmatic area, bilateral retrocrural regions, gastrohepatic ligament area, porta hepatis, peripancreatic area, retroperitoneum, bilateral iliac chains & obturator regions and left inguinal area, omentum & mesentery and rectovesical pouch, liver (S7)) perihepatic space, adjacent to hepatic segment 3, bilateral adrenal glands, axial & appendicular skeletons	11.0	88	203.11	834.6	
Patient.5	48/M	Intestinal type	IV	Antrum to pyloric ring	T3N3M1	IV	Yes	metastatic LNs in perigastric area, hepatogastric ligament, celiac axix, SMA, splenic hilum, portocaval, pericaval, aortocaval, left paraaortic areas, T vertebrae, right hepatic lobe	10.8	52	1.06	52.26	---
Patient.6	73/F	Intestinal type	II	Antrum, lesser curvature	sT3N2M0	IIIa	Yes	LAPs in perigastric and portocaval regions	11.8	56	2.47	56.85	
Patient.7	73/M	Intestinal type	Not available	Cardia	T4aN3M1	IV	No	LNs in perigastric, retroperitoneum, both common iliac chains, both adrenal glands, both rib cage, sacrum, left iliac crest, T vertebrae	9.4	64	3.74	-Not done--	---

No	Age/Sex	Histology type	Borman's type	Gastric ca location	pTNM	AJCC 7th stage	Surgery (Yes/No)	Metastases site	Anemia (Hb> 10g/dl)	ALP (44-147IU/L)	CEA (<5ng/ml)	CA19-9 (>37ng/ml)
Patient.8	54/M	Intestinal type	Not available	Antrum	T4aN3M1	IV	No	Metastatic LNs in gastrohepatic ligament & perigastric areas, porta hepatis, periportal, portocaval, retrocaval, aortocaval & left paraaortic areas, and possible, Lt. supraclavicular area & posterior cervical space, Carcinomatosis peritonei, liver with tumor thrombi in portal vein and SMV, C-T-L vertebrae, sacrum, sternum, both rib cages, both pelvic bones, and Lt. femur	9.0	141	451.84	16.15
Patient.9	50/F	Signet ring ca.	Not available	Antrum	M1	IV	No	Skull, scapula, ribs, humerus, CTLS spines, pelvis, BM,	8.9	1675	12.6	98.69
Patient.10	63/F	Signet ring ca	IV	Body	cT3N2M0	IIIa	No	Left paraaortic region, aortocaval region, C-T-L spine, both iliac bones, right pubic bone, right 1st, 3rd and left 3rd & 5th ribs	13.1	65	13.5	1.01
Patient.11	69/F	Signet ring ca	IIb+IIc	Body	T3N3M1	IV	No	Lt. posterior cervical space, both supraclavicular areas, Rt. internal mammary chain (1st ICS).Lt. highest mediastinum, Rt. retrotracheal area, both perivascular, paratracheal areas, subcarina, Rt. inferior pulmonary ligament, both hila and peri bronchial areas. subphrenic area, perigastric, peri splenic, paraaortic, retrocaval, aortocaval areas, porta hepatis, presacral area. Skull (including facial bone), sternum, bilateral ribs, scapulae, clavicles, C-T-L-S vertebrae, bilateral pelvic bones, humeri and femurs	8.9	276	375.46	Not done---
Patient.12	32/F	Intestinal type	IIb+IIc	Body	T1N0M0	IV	Yes	none	12.8	47	0.53	8.27

Table 2. Patients detailed data and results of procedures

No	Age/Sex	Bone lesion type	Number of bone Metastases	Site of bone metastases	Diagnostic modality	Bone pain	Modality of bone Mets. treatment				
Patient.1	55/M	MIXED	2	Left clavicle; right scapula	PET/CT	No	CTx.	---	---		---
Patient.2	61/M	MIXED	multiple	Rib cage, left scapula, both pelvic bones, sacrum, and T-L vertebrae	Bone scan, MRI, PET/CT	Yes	Palliative RTx/ CTx		---		
Patient.3	65/M	MIXED	1	7th rib	Bones can, PET/CT	No	Surgery	,	-----		
Patient.4	41/M	OSTEBLASTIC	multiple	bilateral 4th ribs, humeri, femurs, acetabula, iliac and ischial bones	Bone scan, PET/CT	Yes	CTx, RTx		---		
Patient.5	48/M	OSTEOLYTIC	1	T vertebrae	Bone scan, PET/CT	No	CTx	---	---		---
Patient.6	73/F	MIXED	0	none	Bone scan PET/CT	No	None	---	---		
Patient.7	73/M	OSTEBLASTIC	multiple	both rib cage, sacrum, left iliac crest, T vertebrae	Bone scan, PET/CT	Yes	None	---	---	---	---
Patient.8	54/M	MIXED	multiple	C-T-L vertebrae, sacrum, sternum, both rib cages, both pelvic bones, and Lt. femur	Bone scan, PET/CT MRI	Yes	CTx	---	---		
Patient.9	50/F	MIXED	multiple	skull scapula ribs humerus CTLS spines pelvis, whole skeleton	Bone scan PET/CT	Yes	CTx	---	---	---	
Patient.10	63/F	MIXED	multiple	C-T-L spine, both iliac bones, right pubic bone, right 1st, 3rd and left 3rd & 5th ribs	Bone scan PET/CT MRI	Yes	CTx	---	---	---	
Patient.11	69/F	OSTEBLASTIC	multiple	skull (including facial bone), sternum, bilateral ribs, scapulae, clavicles, C-T-L-S vertebrae, bilateral pelvic bones, humeri and femurs	Bone scan PET/CT MRI	Yes	CTx		---	---	
Patient.12	32/F	OSTEOLYTIC	none	none	Bone scan PET/CT	x	none	---	---		

2.2 Bone Scintigraphy

The patients were injected IV with approximately 740 MBq of Tc-99m hydroxymethylene diphosphonate (HDP). Hydration and frequent urination were encouraged. Two to three hours after the IV injection, anterior and posterior whole-body images were obtained (Siemens E.CAM, Siemens Healthcare Solutions USA, Inc., Deerfield, IL, USA). Two to four pairs of anterior and posterior spot images were additionally obtained. Pinhole images were not routinely performed.

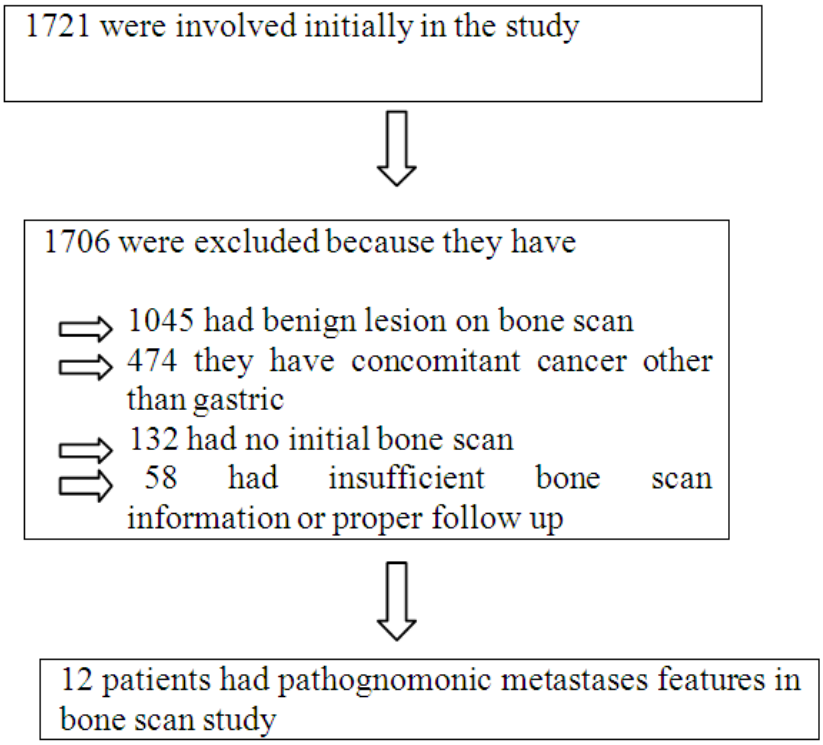

Fig. 1. Enrollment and outcomes

2.3 Image Interpretation and Review

The images and reports of all patients involved in this study were agreed for bone scintigraphy scoring as follow:

0: negative
1: bone metastasis - less likely
2. Either benign or bone metastasis.
3. Bone metastasis - more likely

clinicians have independently reviewed all the data and they were blind to each other score. Then, all results were submitted to a senior radiologist for final scoring. Cases with equivocal findings were submitted to a second radiologist for review and PET–CT or MRI used to validate the bone scan finding if labeled as a score of a final interpretation was achieved by consensus.

3. RESULTS

The patient and tumor clinicopathological feature of gastric cancer cases giving rise to bone metastasis of the study population are shown in Table 1. The mean age was 59.0 ± 8.6 years (range 24–90) and the majority of patients were male (60%). Dominant histological type either poorly differentiated type or signet ring cancer type. And Broman's classification type 3 was the majority.

Distribution of gastric tumor in correlation to upper, middle, and lower third was not differently observed. The mean tumor size among this group was 3.6 ± 2.3 cm (range 0.6–20 cm). All patients were advanced gastric cancer type clinically and the median follow-up period was 9 months.

The incidence of bone metastases was 20%. In 80% of the patients, they had combined bone metastases and another site of metastases. Among these, most of the time associated regional lymph node metastases was found. 4 patients 28.57% had palliative surgery due to bleeding or obstruction complication. Other variables were not significantly valued like anemia, alkaline phosphatase (ALK), tumor markers like carcinoembryonic antigen (CEA) and cancer antigen 19-9 (CA19-9).

Table 2 showed the clinicopathological characteristics of bone metastasis. In bone lesion type, the mixed type was mostly predominantly found (53.3%). The number of bone metastases at time of diagnosis was multiple sites in most of the patients by (73.3%). Although the site of bone metastases was not clearly remarkable, combined vertebra, rib and scapula or whole skeleton was observed at most of the time. In (60.0%) of patients, the diagnostic modality was one scan + PET-CT. Regarding clinical symptoms of presence or absence of bone pain was not different among patients.

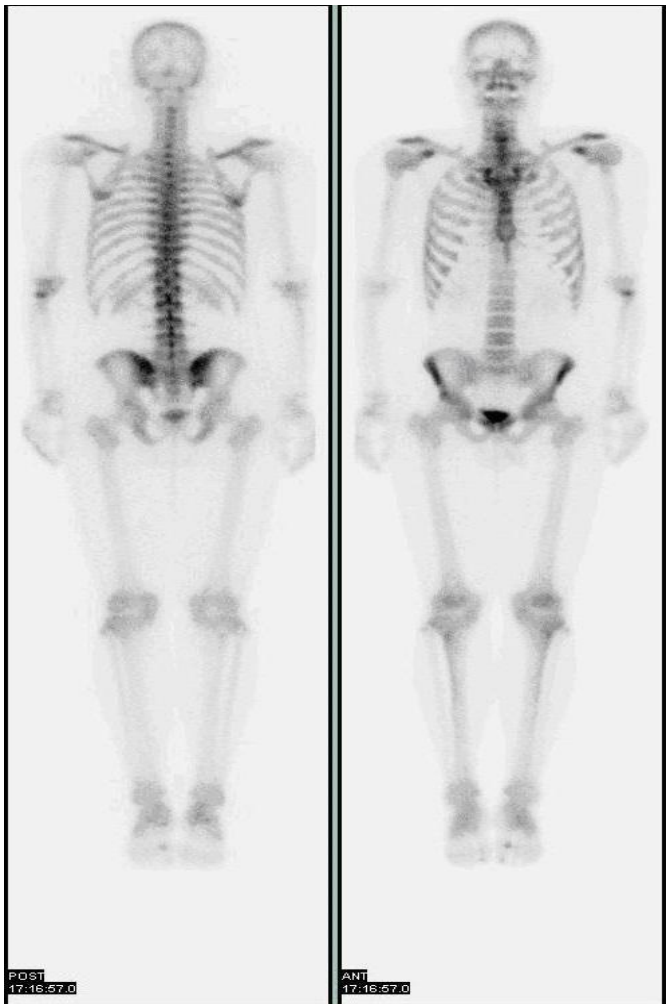

Fig. 2. Bone scintigraphy (gamma scan) or bone scan for the whole body

3.1 Bone Scan

A bone scan was performed using ^{99m}Tc-hydroxymethylenediphosphonic acid (HDP, Mallinckrodt, and St. Louis, MO, USA). The dose of ^{99m}Tc-HDP was 1295 MBq (= 35 mCi). Whole body bone scan

images were acquired using a dual-head gamma camera (Forte, ADAC-Philips, Holt, MO, USA) equipped with low energy high resolution collimator 3 hours post ^{99m}Tc-HDP injection.

A whole-body bone scan should not be done routinely if the cost effectiveness is considered in all patients with gastric cancer. A clinical assessment for symptomatic patients i.e. bone pain as well the stage of gastric cancer, simple x-ray as well the biochemical markers i.e. serum ALK can help to detect the bone metastases.

The bone scan is considering part of the nuclear medicine which is a specialized area of radiology that uses very small amounts of radioactive materials, or radiopharmaceuticals, to examine organ function and structure. Technetium-99m (commonly Tc-99m-methylene diphosphonate (MDP) will be used as the active agent. The study has three phases which follow intravenous injection of the tracer. Sometimes a fourth (delayed/delayed) phase is performed. It allows visualization of bone metabolism or bone remodeling. In which the other imaging techniques (such as X-ray computed tomography, CT) cannot. The Bone scintigraphy competes with positron emission tomography (PET) for imaging of abnormal metabolism in bones, but is considerably less expensive.

3.2 Pet Bone Imaging

Bone scintigraphy refers to gamma camera imaging of ^{99m}Tc radiopharmaceuticals, imaging with positron emission tomography (PET) scanners is also possible, using fluorine-18 sodium fluoride ([^{18}F]NaF).

For quantitative measurements, ^{99m}Tc-MDP has some advantages over [^{18}F]NaF. MDP renal clearance is not affected by urine flow rate and simplified data analysis can be employed which assumes steady state conditions. It has negligible tracer uptake in red blood cells, therefore correction for plasma to whole blood ratios is not required unlike [^{18}F]NaF. However, disadvantages include higher rates of protein binding (from 25% immediately after injection to 70% after 12 hours leading to the measurement of freely available MDP over time), and less diffusibility due to higher molecular weight than [^{18}F]NaF, leading to lower capillary permeability [10].

3.3 Interpretation of Bone PET and Bone Scan

A positive finding for BM was defined as the presence of an abnormally high bony uptake, which is not associated with typical degenerative, traumatic or peri-articular lesions [11]. Diagnostic accuracies were also analyzed using the receiver operating characteristic (ROC) curve analysis by utilizing a 4-point grading system; definite, probable, less likely, and no evidence of BM. The Consensus was reached by two nuclear medicine physicians in order to call a lesion BM.

A gold standard for BM was either the presence of typical findings compatible with BM in at least 2 imaging studies among MRI, ^{18}F-FDG PET/CT or ^{131}I whole body scan, or the presence of a clinical progression causing a change of treatment plan during at least a one-year follow-up. Those who had at least one proven BM lesion were considered BM positive patients regardless of the presence of any false positive BM findings

Note.-PET = positron emission tomography

There are many advantages of the PET technique in comparing for the bone scan imaging, which are including improved spatial resolution and more developed attenuation correction techniques.

Patient experience is improved as imaging can be started much more quickly following radiopharmaceutical injection (30-45 minutes, compared to 2-3 hour. PET/CT is shown to be more specific for metastatic disease than bone scan. PET/CT also has a higher specificity and can be used to monitor response to therapy.

While the bone scan is more reliable to detect the osteoblastic lesions, the osteolytic lesions are more likely to be detected by PET/CT scan.

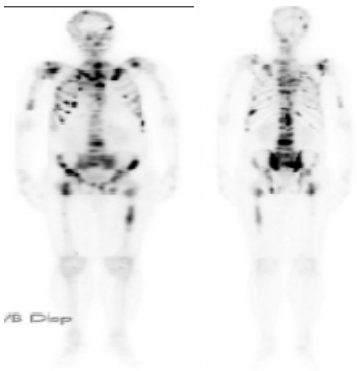

Fig. 3. Anterior and posterior view of bone scintigraphy shows increased uptake in multifocal area which indicated the bone metastases

There are many factors affecting the prognosis of bone metastases in gastric cancer (GC). The diagnosis time, the axis of distribution in the axial or vertical of the skeleton. The pattern of the metastases (mixed, osteolytic, osteoblastic). Concurrent extra-bone metastatic sites, single or multiple bone sites involve.

Although the bone metastases in gastric cancer patient is not common, other concomitant cancer should be kept in mind. Any cancer liable to spread to the bones i.e., common ones to spread are prostate cancer, breast cancer, lung cancer, kidney cancer, thyroid cancer and multiple myeloma.

Once the bone metastases found in the bone scan, bone biopsy if feasible is advisable for confirmatory purposes. The symptoms of bone metastases vary according to location, size, and number of lesions. Symptoms at the most of time is pain, which is expected to be diffuse, vague, and continuous. Easy breaks in the bones (pathological fracture) with or without simple trauma. Altered level of consciousness, lethargy, confusion, and constipation secondary to increase level of serum calcium.

Bone scan is not only used for primary or seconday bone cancers. It has a wide clinical application from benign to malignant bone pathology. It can be used for bone fractures, assessment of bone density (osteoporosis), joint inflammation (Arthritis), Avascular necrosis, bone infection (osteomyelitis) Fibrous dysplasia, Osteomalacia and Paget's disease, a disorder affecting normal bone remodeling.

There are many advantages for the bone scan, the exposure for the radiation is very low in compared to the computed tomography (CT). The injected active material has a short half-life 4-6 h and can be cleared from the body within 24h-36h.

Some precautions should be taking for the patients with heart diseases, pregnant or breast lactating women. Allergic reactions to the injected active material are very rare, pain at the injection site, chest tightness, increase heart rate and change in the taste might encountered.

4. DISCUSSION

The first aim of the present study was to assess the incidence of bone metastases among gastric cancer patients at the first time of diagnosis. In our reviewed data, we showed that the bone metastases found rarely and only in about 0.8 % of patients who initially worked up for gastric cancer.

In the present study, we showed that Bone metastases occurred at a significantly higher incidence in the patients with a multiple numbers of regional metastatic lymph nodes and advanced tumor stage and depth. As with most other tumors, bone metastases developed in multiple sites rather than the solitary site, and even in the more aggressive form of involvement of the whole-body skeleton.

Bone metastasis is a rare condition in GC and is clinically underestimated. Mori et al. investigated 719 cases of malignant tumors among 2240 consecutive autopsies [12] in Tokyo Medical and Dental University. These included 176 cases of GC of which 28 cases (15.9%) exhibited metastasis in bone, the third-most common site of GC metastases. The metastatic rate in the liver and lungs was 34.7% and 31.3%, respectively. Consistent with these findings, Yoshikawa and Kitaoka and Yamamura et al., showed that the metastatic rate in bone from curatively resected GC cases was high among autopsy cases, but was comparatively low in clinical practice at a rate of 1.2-1.4% [13,14]. Also, in support of these findings, Maeyama et al. reported the rate of bone metastasis in clinical practice and autopsy as 0.7% and 17.6%, respectively. Choi et al., evaluated bone metastasis from GC by bone scintigraphy [15]. They investigated 234 bone scans from a total of 1776 GC patients. The 234 patients were classified according to their original clinical stage rather than by standard stage and were identified as having advanced stage disease. Of these cases, 106 (45.3%) had metastatic bone lesions. The findings discussed above suggest that asymptomatic bone metastasis is underestimated as examination by bone scintigraphy is not a routine clinical practice. It is also possible that peritoneal dissemination or liver metastasis masks the clinical manifestation of bone metastasis. For these reasons, the rate of bone metastasis in clinical cases may be higher than expected.

Many theories reported regarding the mechanisms of bone metastases in gastric cancer patients. Maehara et al., investigated bone micro metastasis using a monoclonal anti-cytokeratin antibody and they found that 9 (20%) of 45 EGC cases examined had cytokeratin-positive cells in the bone marrow at the time of primary surgery [16]. These findings suggested that the presence of micro metastatic cells in the bone marrow was closely related to angiogenesis in the primary tumor. Macadam et al., showed in multivariate analysis of risk factors revealed that bone marrow cytology was a significant factor for recurrence and death. While investigations on bone marrow micro metastasis by immunocytochemistry may play a role in predicting disease recurrence, at present it cannot be clinically applied to detect bone metastasis [17].

Lehnert et al. used light and transmission electron microscopy to investigate lymph and blood capillaries of human gastric mucosa and found that the upper and middle levels of the lamina propria of the gastric mucosa contained no lymph capillaries. The mucosa has a rich supply of blood capillaries, many of which are adjacent to the basal lamina of gastric glands and surface epithelium. He suggests that the low incidence of lymph node metastases in the early mucosal GC might be explained by the rarity of lymph vessels in the mucosa, and that blood-borne metastases in recurrent EGC might be related to the rich vascularity of the gastric mucosa [18]. Rino et al., reported five EGC cases without vascular invasion (v0) resulting in bone metastasis and concluded that high risk factors for bone metastasis were accompanying ulceration, lymph node metastasis, and distant metastasis to other organs [19]. GC might metastasize to the bones through the vertebral vein system as suggested by Batson [20]. Yamamura et al. reported that GC with bone metastasis resulted in the invasion of the lymphatic vessels more frequently than the invasion of venules [21]. They also speculated that GC might metastasize to the bone through the thoracic duct. The latter is supported by the higher rate of lymph node metastasis in those cases with bone metastasis compared to that for EGC as a whole. For advanced cancer that has invaded adjacent organs, cancer cells may invade a fairly large vein, such as those found in the vertebral vein system outlined by Seto et al. [22]. Most cases of bone metastasis do not show liver metastasis, most venous drainage from the stomach is via the portal vein, and many cases of bone metastasis are associated with lymph node metastasis, suggesting that the mechanisms underlying bone metastasis involve lymphatic channel into the systemic circulation.

Ell reviewed skeletal imaging of metastatic diseases and identified MRI as sensitive in detecting bone marrow involvement [23]. Despite this finding, bone scintigraphy continues to be the modality of choice in view of its simplicity, low cost, and ability to screen the entire body [23]. Choi et al., evaluated bone metastasis from GC by Tc-99m MDP imaging [15]. They investigated 234 bone scans of GC patients. One hundred and six patients (45.3%) had bone scan abnormalities that qualified as metastatic bone lesions.

The most frequent metastatic sites were the spine (66%), the ribs (59%), pelvis (43%), femur (30%), and skull (22%) [15]. The least frequent metastatic sites were the shoulder girdle (17%), such as the scapula and clavicle, sacroiliac joint (7.2%), humerus (6.0%), sternum (4.2%), and tibia (3.0%) [15].

On the other hand, roentgenographic evaluation of bone metastases has limited value because symptoms caused by bone metastases frequently occur before abnormal imaging. Bone metastases were diagnosed by bone scintigraphy in 13 out of 24 cases described in our review. Laboratory data may be helpful in the diagnosis of bone metastasis. Although tumor markers do not play an important role, many cases with bone metastasis show elevated serum alkaline phosphatase (ALP). In the study by Choi et al., ALP was elevated in 64% of 106 patients with bone metastasis [15]. Seto et al., also found that ALP was significantly elevated in GC cases with bone metastasis compared to those without [22]. Once cancer disseminates to the bone marrow, disseminated intravascular coagulation (DIC) may occur. Clinically, patients show a tendency to bleed that is confirmed on a combination of laboratory data for platelet count and fibrin degradation products. Several chemotherapy regimens for bone metastasis of GC have been reported as case reports. Kobayashi et al., and Hironaka et al., demonstrated that sequential methotrexate and 5-fluorouracil chemotherapy resulted in a high rate of alleviation (80% and 89%, respectively) of DIC caused by bone metastasis from GC [24,1].

Limitations of this study include its retrospective design, a small number of patients although the initial study group was large, and the highly positive bone metastases cases in bone scan were not confirmed with pathologic diagnosis although additional radiological tool was used to confirm the finding in bone scintigraphy study. In this study, we assessed initially bone metastases as a screening modality by a sensitive examination tool, i.e. technetium bone scintigraphy. We showed above in during this report the strategy and grading system for bone scintigraphy reading. When the findings highly suspicious for bone metastases in technetium bone scintigraphy, additional radiological tools were used, either CT, MRI, PET-CT to differentiate inflammatory bone lesions from bone metastasis and finalize the findings which were not conclusive.

5. CONCLUSION

Although it had a very poor prognosis, the incidence of bone metastasis from GC is very rare. It seems that the higher stage and poorly differentiated type of gastric cancer, the higher chance for bone metastases. It is not well understood the mechanisms of underlying bone metastasis arising from gastric cancer, but in most likely involve the lymphatic channels. Some cases give rise to late bone metastasis after surgery. It can be considered that bone metastasis arising from EGC is a rare condition and as a result adjuvant chemotherapy is not recommended. In the post-operative follow-up of EGC cases that are histologically less differentiated and display involvement of lymph nodes, recurrences as bone metastasis should be considered. Elevation of ALP can be used to detect bone metastasis and bone scintigraphy is recommended for its diagnosis.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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Biography of author(s)

Dr. Bandar Idrees Ali, MD
Seoul St. Mary's Hospital, The Catholic university of Korea, Seoul, South Korea.
Department of Surgery, Prince Sultan Military Medical City, Riyadh, Saudi Arabia.

He was born in Medina, Saudi Arabia. He is a graduate of King Saud University in 2007 with medical degree. His residency was at King Khalid University hospital in Riyadh and its affiliated Hospital, and he completed his training with fellowships at the Catholic University of Korea in Seoul in 2016 in the upper GI oncology and robotic Surgery. He is very active in the field of medical education. He is teaching in cataract and corneal surgery. He contributed too many medical articles, researches and conferences.

Dr. O. Joo Hyun, MD
Seoul St. Mary's Hospital, The Catholic university of Korea, Seoul, South Korea.

She is a Clinical Assistant Professor, Seoul St. Mary's hospital at the catholic university hospital, Seoul, South Korea. She did her internship at the Seoul St. Mary's hospital from Mar 2004 – Feb 2005 Employment Duration for 1 year. Then she did her residency for four years from March 2005 till February 2009. She did her clinical fellowship for 3 years from March 2009 till February 2012. She joined John Hopkins University as nuclear medicine research fellow from March 2012 till August 2013. She is interested in Oversee and interprets nuclear medicine studies, with emphasis on oncology and cardiology. She Participate as investigator in pre-clinical experiments and clinical trials. She has published many research articles in the national and international journals.

Dr. Khuloud Omar Bukhari, MD
Ministry of Health, Saudi Arabia.

She had her bachelor's degree in medicine and Surgery (M.B.B.S), Second Semester 2007-2008AD, Faculty of Medicine, Umm Al-Qura. She had her KSU Fellowship in Diabetology , Riyadh, Saudi Arabia. She was appointed as a training completion certificate of Saudi Commission for health Specialties in the Family Medicine program, Riyadh, Saudi Arabia. She is an Arab Board of Health Specialization in FAMILY MEDICINE, July 2016. She is holding a Jordanian Medical Council certification for Family Medicine Board, February 2017. She is a trainer in the joint family medicine program of ministry of health, member in assessment committee in the program. She had attended course of (TOT), February 2017. She is a Member of Saudi Hypertension Management Society (SHMS), Member of Riyadh Diabetes club. In her career object, she intends to keep up-to-date with new technologies and treatments in her field. She is seeing herself as fully motivated, initiative, team worker, good in interpersonal communications, looking for best and bright future, holding high certificate in her interest not to be just an account number but distinguished person in her career. She has published many research articles in the national and international journals.

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Chapter 8
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Study on the Role of Rebiopsy in Relapsed Non-Small Cell Lung Cancer for Directing Oncology Treatments

Antti P. Jekunen[1*]

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ABSTRACT

Background: Currently few rebiopsies are performed in relapses of advanced non small cell lung cancer. They not customary in clinical practice.of lung cancer. However, it is not possible to properly target treatments in cases of relapse without knowing the nature of new lesions.
Objectives: Clarify current status and explore needs of rebiopsy.
Design: This paper comprehensively summarizes the available literature about rebiopsy and broadly discusses the importance of rebiopsy in advanced non small cell lung cancer.
Results: Altogether 560 abstracts were used as material for further analysis. Of which 19 articles were about clinical rebiopsy in lung cancer and were reviewed in detailed manner.
Conclusions: This review shows that rebiopsy is feasible in non small cell lung cancer, and success rates can be high if accompanied by adequate evaluation before biopsy. Its use may resolve the difficulties in sampling bias and detecting changes in cancer characteristics. In cases where treatment was selected based on tissue characteristics that then change, the treatment selection process must be repeated while considering new characteristics of the tumor. However, before performing rebiopsy adequate evaluation of risks for complications should be performed including anatomic and technical aspects of accessing tumor. Rebiopsy may be used to predict therapeutic resistance and consequently redirect targeted therapies. Such knowledge may resolve the difficulties in sampling bias, and also in selecting pre-existing clones or formulating drug-resistant ones. Rebiopsy should be performed more often in non small cell lung cancer. Rebiopsy is done after the initial biopsy that provided the diagnosis.

Keywords: Rebiopsy; repeated biopsy; lung cancer; tumor characterization.

ABBREVIATIONS

CT : Computer Tomography
PET : Positron Emission Tomography
VATS : Video-Assisted Tomography
EBUS : Endobronchial Untrasound
EMN : Electromagnetic Navigation
NSCLC : Non Small Cell Lung Cancer
DNA : Deoxyribonucleic Acid
RNA : Ribonucleic Acid
EGFR : Epidermal Growth Factor Receptor
ALK : Anaplastic Lymphoma Kinase Gene
TP53 : tumor Protein p53
KRAS : Kirsten rat Sarcoma Viral Oncogen Homolog
STK1 : Serological Thymidine Kinase 1
TKI : Tyrosine Kinase Inhibitor

[1]Clinical Cancer Research Center, Vaasa Oncology Clinic, Turku University, Hietalahdenkatu 2-4, Vaasa 65100, Finland.
**Corresponding author: E-mail: antti.jekunen@vshp.fi;*

PubMed: An Internet Site for Biomedical Literature
TK : Tyrosine Kinase
T790M : a Gatekeeper Mutation in EGFR
ERCCI : DNA Excision Repair Protein
RPMI : Disease Resistance Protein
PFS : Progression Free Survival
TS : Thymidylate Sythetase
SCLC : Small Cell Lung Cancer
MET : Mesenchymal-Epithelial Transition Factor
PIK3CA: Phosphatidylinositol-4,5-biphosphate 3-kinase, Catalytic Subunit Alpha
AKT1 : RAC-alpha serine/threonine-protein kinase
BRAF : v-Raf murine sarcoma viral oncogene homolog B
NRAS : Neuroblastoma RAS Viral Oncogene Homolog
HER2 : Human Epidermal Growth Factor Receptor 2
LNA : Locked Nucleic-Acid
PCR : Polymerase Chain Reaction
QALY : Quality Adjusted Life Year
ICER : Incremental Cost-Effectiveness Ratio
CK : Cytokeratin
NOS : Not-Otherwise Specified
PSA : Prostate Specific Antigen
ER : Estrogen Receptor
PR : Progesterone Receptor,
PD-L1 : Programmed Death-Ligand 1
T-cell : Lymphocytes Maturing In Thymus
ESMO : European Society of Medical Oncology

1. INTRODUCTION

This study was originally published 2015 [1], at which time gene driven cancer drugs started entering to clinical practice. However, all findings and concerns in the study are still relevant, even though much more articles about rebiopsy have been published since then [2,3]. Based on large 713 043 primary malignancies diagnosed 1985 and 1995 overall 10 year survival was 7%, however early stage I, 5 year survival was more than 50% [4], underlining the importance of histological early biopsy. In addition, data from the Veteran's Health Administration between 2006 and 2012 found increased use of hospice care and increase in aggressive care at the end of life [5]. Maybe rebiopsy could give more guidance to direct therapies more carefully and spare patients from too aggressive therapies.

1.1 Imaging

Lung cancer is usually suspected in individuals who have an abnormal chest radiograph results or symptoms caused by either local or systemic tumor effects [6]. An initial diagnosis relies on imaging examinations when patients seek help for symptoms. Today, more tumor lesions are found secondarily in routine checkups. Chest X-ray and computer tomography (CT) scans are widely used. Positron emission tomography (PET) is a golden standard for staging of lung cancer. Additionally, it is used when doctors require more information about metabolic activity in certain lesions or when seeking lymph nodes or lesions for biopsy, in case of relapses and metastases.

1.2 Methods of Tumor Biopsy

In cases of peripheral tumor, ultrasound- or CT-guided percutaneous fine-needle aspiration or core biopsy is performed (Table 1). Video-assisted thoracoscopy (VATS) is used for wedge excisions and needle aspirations. A thoracotomy is usually an option when a lobectomy is being considered. Central tumors, often with symptoms such as repeated pneumonias and hemoptysis, can be diagnosed by sputum cytology. Bronchoscopy provides better samples with brush, fine-needle biopsy, or core biopsy. Percutaneous-core needle biopsies, when it is possible to perform them, give larger samples of tissue material for further studies. However, a thoracotomy would be the best option when tissue

sample size is important. Based on recent meta-analysis, endobronchial ultrasound (EBUS) and electromagnetic navigation (EMN) bronchoscopy have the potential to increase the diagnostic yield of peripheral lung tumors [6]. A thoracoscopic biopsy of the pleura had the highest yield for diagnosing metastatic pleural effusion in a patient with lung cancer. When stereotactic high dose radiotherapy is considered tissue samples need to taken before radiation, because afterwards there are nothing to be biopsied for. Acquiring adequate tissue samples for histological and molecular characterization of non small cell lung cancer (NSCLC) is considered paramount.

Biopsy is used to characterize tumors. Here in this study, rebiopsy means biopsy after cancer progression on initial therapy and repeated biopsy is used for conditions where an initial biopsy was not adequate for diagnosis and a new biopsy is performed. Basic staining and immunohistochemistry are routine in pathological diagnosis and also useful in rebiopsy. Table 1 lists various means of obtaining tissue and gives estimation of tissue yields.

Table 1. Techniques for obtaining tissue

Method	Nature of sample	Size	Suitable for
Sputum	Cytology	50 mg	Limited immunohistology
Bronchoscopy Brushing	Cytology	50 mg	Limited immunohistology
Fine needle biopsy	Cytology	100 mg	Immunohistology, PCR
Core needle biopsy	Histology	200-400 mg	Plus gene mutation testing, FISH, DNA tests
Resection	Histology	> 1g	Plus exome tests, large immunohistology panels, RNA tests (-70C)

1.3 Risks of Biopsy

Taking sputum samples is without safety issues, while all others have some risk for complications. As needle size increases, risk level increases also for biopsy complications. Clearly, it is of importance to determine what are risks coming from the location of biopsy target. The most serious complications include pneumothorax and bleeding. Of course, in resections overall risk of general anesthesia needs to be calculated before operation.

1.4 Molecular Pathology

Molecular methods are becoming more common in the pathological diagnosis (Table 2). Molecular biology techniques, particularly gene-expression microarrays, proteomics, and next-generation sequencing, have recently been developed to facilitate molecular classification [7]. Proteomics can further characterize tissue with two-dimensional gels. Third-generation immunoassays and protein pathway circuit arrays are also being used experimentally. DNA is quite stable and can be genotyped by different oligonucleotide arrays, based on PCR or sequencing. RNA is more difficult to extract, as it is rapidly destroyed by ribonucleases if samples are not quickly frozen to -70°C after biopsy. RNA provides opportunities to measure gene expression by complementary DNA microarrays or microRNAs by sequencing. Many analyses are already part of a standard care (Table 2). Protein analysis by immunohistochemistry is routine and widely available. Gene testing is becoming a regular practice, and preparations for sending adequate tissue samples with sufficient numbers of malignant cells to central laboratories are becoming common practice in all clinical pathology laboratories. This process depends upon determining gene changes that are related to drug activity.

Table 2. Information from rebiopsy

Standard of care	Experimental
Histologic	Proteomics
Immunohistochemistry	RNAsequencing
Molecular information	Exome analysis
EGFR/KRas/ALK	

1.5 Changing Therapies on Genetic Mutations

Consequently, measurements are needed to direct therapies, thus justifying collecting biopsy samples. In non small cell lung cancer (NSCLC)-type adenocancer, two mutations are widely used to direct treatments: an epidermal growth factor receptor (EGFR)-activating mutation indicating use of gefitinib and erlotinib [8], and an ALK (anaplastic lymphoma kinase) gene rearrangement, indicating use of critsonitib [9,10].

1.6 Mutations

In NSCLC, there are many variations and mutations in DNA, and it is only a matter of time and successful research before there are more predictive mutations available to clinical practice. The most frequent mutations in adenocarcinomas are in TP53, KRAS, and STK11 and EGFR genes. ALK mutations are measured in 3% to 5% of all lung adenocarcinomas. Genomic pathology provides an opportunity to stratify patients, based on genomic predictive features after successful rebiopsy, and consider changing treatment.

A common clonal origin indicates intrapulmonary multifocal metastases in almost two-thirds of cases, while 36% of multifocal NSCLC display unique molecular profiles, which suggests separate primary tumors. Divergent KRAS and/or EGFR mutations have been observed in 8% of cases [11]. The same research studied the clonal relationship of multifocal NSCLC with indistinguishable histomorphology in 78 patients by polymorphic short tandem repeat markers and mutation testing of KRAS and EGFR [11]. This could provide remarkably increased response rates and better treatment outcomes, compared to ordinary histopathology-based stratification. This increased response rate is already the case with tyrosine kinase inhibitors (TKIs) and ALK inhibitors.

1.7 Histology

Diagnosis of lung cancer is challenging. Resected tumors provide histological tissue, and diagnosis can almost always be obtained. However, there are a lot of situations were obtaining adequate material for diagnosis is challenging in initial biopsies, and a lot of tumors are not operated at all. An additional challenge is presented by known intratumor heterogeneity, which must be considered, especially when histological material is limited and not representative of the entire tumor. However, there can be small lesions or a situation that does not require an operation. In those clinical cases with small lesions requiring biopsies, histological tumor sampling remains difficult, and obtaining biopsy samples for thorough pathological assessments is difficult. Often, molecular pathology is simply not done. In some cases, only cytology is available, and further sampling is not possible because of the lesion location or the patient's low lung function. Treatment will begin, based on fine-needle biopsy, or even sputum sample, but there must be evidence of cancer. At the very least, lesions should behave like lung cancer. Different lung cancer types and different NSCLC cell clones behave differently and require different treatments.

There must be clinical confirmation of cancer, since oncology treatments generate so many side effects that clinical indication is required for their use. For a proper diagnosis, adequate histological or cytological material is required for morphological assessments, immunohistochemistry, and gene testing in cases of adenocancer. General feasibility of performing needle biopsies varies, and especially advanced techniques including immediate freezing, capture microdissection, and subsequent sophistigated gene expression analysis at RNA levels is limited, because of the required infrastructure [11]. Here rebiopsy means biopsy after cancer progression on initial therapy and its role will be comprehensively summarized and broadly discussed in lung cancer.

2. MATERIALS AND METHODS

This review is based on a PubMed search for the terms *rebiopsy* and *lung cancer* (Table 3). Publications in languages other than English and trials involving non human subjects were excluded. Fourteen publications were reviewed, and a classification was performed with the predetermined variables listed in Table 3. The number of publications and trial protocols cited are as researched in

March 2014. Additionally, *recurrent lung cancer* and *relapsed lung cancer* search terms were used resulting 5225 and 1182 hits, but no additional articles were found by combining them with a *rebiopsy* search term. In order to check other articles and validate the search procedure, a repeat search was performed with the terms *repeated biopsy*, *lung cancer*, and *clinical*. It produced 544 hits from year 1975 to date. All abstracts were reviewed, and adequate articles that focused on rebiopsy were selected and included in this literature review. Two articles and two letters to the editor were evaluated for additional adequate information, and were subsequently incorporated into the analysis as additional articles.

3. RESULTS

A PubMed search of the term *cancer diagnosis* produced almost 2 million hits. With the term *clinical biopsy*, there were 152,197 hits. This number dramatically decreased when the search was conducted for both *cancer diagnosis* and *clinical biopsy* or with lung cancer terms (see Table 3). Combining the term *rebiopsy* with *colon cancer*, *lung cancer*, *breast cancer*, and *prostate cancer* produced two, 16, 23, and 14 hits respectively (Table 3). Of the articles with all indications, abstract analysis revealed that DNA and mutations were central to 12 and 11 articles respectively, while histology was discussed in 235 of 309 articles with the term *rebiopsy*. No review articles were found in the area of rebiopsy in lung cancer by the study at 20.

Eighteen articles with the search terms *rebiopsy* and *lung cancer* were targeted for further analysis (Table 3). These articles were used to find more suitable works, which were then referenced. Four articles dealt with other cancers and were excluded from further analysis. The remaining 14 articles focused on NSCLC. Details of major findings are given for each article. Four were case reports. One was about the pharmaco-economic aspects of rebiopsy, and ten were original articles. Of these ten articles, one was a prospective clinical trial report, and one reported on extensive mutation genotyping. Two articles focused on a specific gene expression, while the remaining six focused on tyrosine kinase (TK) resistance and mainly discussed the most frequent secondary mutation T790M.

Table 3. PubMed literature search for rebiopsy

Rebiopsy	309
+ colon cancer	2
+ lung cancer	16
+ breast cancer	23
+ prostate cancer	104
Rebiopsy histology	235
Rebiopsy DNA	12
Rebiopsy mutations	11

3.1 Chemotherapy and Gene Expression

The prospective study assessed if chemotherapy selection based on *in situ* excision repair cross-completion group1 (ERCC1) and ribonucleotide reductase M1 (RRM1) protein levels would improve survival in patients with advanced NSCLC [12]. A total of 275 eligible patients were randomly assigned to the control arm with gemcitabine/carboplatin or the trial's experimental arm. Chemotherapy therapy was given based on protein levels at repeated biopsy: if RRM1 and ERCC1 were low gemcitabine/carboplatin were given, and if RRM1 was high and ERCC1 was low then docetaxel/carboplatin, if RRM1 was low and ERCC1 was high then gemcitabine/docetaxel, and if both were high then docetaxel/vinorelbine. While no statistically significant differences were observed between the experimental and control arms in PFS (progression free survival) (6.1 months versus 6.9 months) or overall survival (11 months versus 11.3 months), a subset analysis revealed that patients with low levels of both proteins who received the same treatment in both treatment arms had a statistically better PFS ($P = 0.02$) in the control arm (8.1 months) than in the experimental arm (five months). This study was in newly diagnosed patients with advanced-stage NSCLC. However, a repeated tumor biopsy without complications was needed in 17% of cases to ensure enough material

for protein-level measurements [12]. This study gives a prospective setup for repeated biopsies, and even in the chemotherapy context may warrant conducting proper justification and direct chemotherapy.

Jakobsen et al. published two studies about specific gene expressions at the protein level obtained using immunohistochemistry. They discovered that thymidylate synthase (TS), which was a potential predictive marker for treatment efficacy with pemetrexed, did not significantly change in rebiopsied lung tumors compared to primary tumors in 65 NSCLC patients taking after preoperative carboplatin and paclitaxel [13]. In another study, 65 NSCLC patients taking preoperative carboplatin and paclitaxel, and a group of 53 NSCLC patients treated with surgery alone showed no statistically significant change between primary and rebiopsy material of lung tumors in class-III-beta-tubulin expression, which may be a potential predictive factor for microtubule interfering cytotoxic drug treatment [14]. In these situations, the biomarker was not valid and thus rebiopsies were not justified. However, there was intratumoral heterogeneity in both studies, which highlighted the need for sufficient representative material for diagnosis.

3.2 Tyrosine Kinase Inhibitors and Resistance

Understandably, the main area for rebiopsies is among TKIs in adenocancers of NSCLC. All patients with EGFR-mutant lung cancers eventually develop acquired resistance to EGFR TKIs. This is associated with second-site mutations in the EGFR kinase gene (e.g., T790M), amplification of alternative kinases (e.g., mesenchymal-epithelial transition factor, MET), histologic transformation to small cell lung cancer (SCLC), and epithelial to mesenchymal transition. Various mechanisms have been identified to account for resistance, and many methods have been proposed to overcome resistance, especially caused by T790M [9,15,16]. The EGFR mutation T790M is reported in approximately half of adenocancers with acquired resistance to EGFR inhibitors and is a potential prognostic, predictive biomarker. Patients with EGFR-mutant lung adenocarcinoma develop acquired resistance to EGFR TKIs after a median of 10 to 16 months. In half of these cases, a second EGFR mutation, T790M, underlies acquired resistance. However, rebiopsy to confirm T790M status can be challenging due to limited tissue availability and procedural feasibility. Furthermore, little is known of the differences among patients with or without T790M mutation. Here, various rebiopsy studies reporting the frequency of T790M, reporting analysis for EGFR/ALK mutations and reporting responses to EGFR TKI are described. When there is a mechanism of resistance found, that is potentially actionable, new drug development could be initiated. So that for T790 mutations found, a T790M mutant specific inhibitors could be developed, and for MET amplification, a MET inhibitor could be tested.

A mutation genotype was investigated in a large, 155-patient study reported by Yu et al. [17]. Adequate tumor samples from rebiopsies for molecular analysis were obtained in lung adenocarcinoma tumors with acquired resistance to erlotinib or gefitinib. Sample material included fine needle aspirations, core biopsies, surgical samples and cytology from malignant effusions. There was one recorded complication of pneumothorax requiring a catheter placement. Futhermore, sites of rebiopsies included lung tumor (82), pleural effusions (14), bone (9), liver (13), lymph nosed (9), peritoneal fluids (1) and central nervous fluid (1) and other organs (9). The tissue samples were obtained via operational procedures in 17 cases, including 10 brain resections, 5 lymph node excisions and 3 adrenalectomies and two autopsies. Of these 155 patients, 98 had second-site EGFR T790M mutations (63%; 95% confidence interval [CI], 55%–70%). Four samples had small-cell transformation. MET amplification was seen in four of 75 samples, and HER2 amplification was seen in three of 24 samples. No acquired mutations were observed in PIK3CA, AKT1, BRAF, HER2, KRAS, MEK1, or NRAS genes (0 of 88). The study identified EGFR T790M as the most common mechanism of acquired resistance, whereas MET amplification, HER2 amplification, and small-cell histologic transformation occurred less frequently. The authors concluded that more rebiopsy studies were needed to characterize molecular alterations in situations of acquired resistance to EGFR TKIs [17].

Using a highly sensitive, locked nucleic-acid (LNA) PCR/sequencing assay with an analytical sensitivity of approximately 0.1%, T790M was detected in as many as 68% of patients with acquired

resistance presenting either relapses or metastases. Tumor samples (153 samples in 121 patients) included the samples from clinically required procedures in 84 cases (e.g. 11 VATS biopsies, 6 lung resections, 3 image guided lung biopsies and 2 fine needle biopsies and 26 pleural effusions). In addition, the samples were obtained from other organs than lung in resections (14), biopsies (12) and fluid aspirations (8). The samples were studied for sensitizing EGFR mutations [18]. A total of 121 patients were rebiopsied and samples underwent tissue sampling. Of these, 104 (86%) samples were successfully analyzed for sensitizing EGFR mutations. Most failures were related to low tumor cell content. All patients (61) with matched pretreatment and resistance specimens showed susceptibility to the original sensitizing EGFR mutation. Standard T790M mutation analysis of 99 patients detected 51(51%) mutations. Retesting of 30 EGFR-negative patients by the LNA-based method detected 11 additional mutations, for an estimated prevalence of 68%. MET was amplified in 11% of cases (4/37). The authors concluded that rebiopsy of lung cancer patients with acquired resistance was feasible and provided sufficient material for mutation analysis in most patients [18].

Of 126 patients referred for rebiopsy with NSCLC that was resistant to conventional chemotherapy or EGFR TKIs, 94 patients were selected for rebiopsy [19]. CT chest images excluded 32 patients. Percutaneous trans-thoracic lung biopsy was performed with a CT-guided, C-arm cone-beam, which had a technical success rate of 100%. In 75 (80%) of the 94 patients, specimens were adequate for mutational analysis. Thirty-five specimens were tested for EGFR mutation, 34 for ALK rearrangement, and six for both. The results were positive for EGFR-sensitizing mutation (exon 19 or 21) in 20 patients, EGFR T790M mutation in five, and ALK rearrangement in 11. Rebiopsy complications occurred in 13 (14%) patients. The study concluded that rebiopsies are feasible and safe when applying rigorous CT criteria, and provide adequate material for gene analysis [19].

A study of 93 patients with EGFR-mutant lung cancer and acquired resistance to EGFR TKIs, compared T790M status in terms of post-progression survival and characteristics of disease progression [20]. Mutation of T790M was observed in the initial rebiopsy specimens from 58 patients (62%, 95% CI: 52–72). T790M was more common in biopsies of lung/pleura tissue and lymph nodes than in other sites and they were more likely to progress in an existing site of disease than in new sites. Patients with T790M had a significantly longer post-progression survival time than patients without. Additionally, patients without T790M more often progressed to tumors in new, uninvolved organs and had a poorer performance status at time of progression. This study suggested that T790M serves a prognostic value that can be found by rebiopsy. Among patients with acquired resistance to EGFR TKIs, the presence of T790M defines a clinical subset with a relatively favorable prognosis and slower progression. The authors concluded that knowing T790M status was essential for clinical treatment decision making and understanding results of clinical trials after TKI use [20].

A study investigated 78 EGFR-mutant patients who underwent rebiopsy after TKI failure [21]. A sensitive, peptide nucleic acid-LNA polymerase chain-reaction clamp method was used in EGFR mutational analyses. The study found that patients with T790M after TKI failure had better prognoses than those without T790M. The T790M mutation was only identified rarely in four (17%) of 24 central nervous-system lesions and 22 (41%) of 54 other lesions ($P = 0.0417$). Median PFS was 31.4 months in 26 patients with T790M, and 11.4 months in 52 patients without T790M ($P = 0.0017$). In the multivariate analysis, statistically significant factors for longer PFS included positive for T790M, good performance status, and no carcinomatous meningitis [21].

Post-progression tumor specimens were prospectively collected for T790M mutation analysis in 70 NSCLC patients with acquired resistance to initial EGFR TKIs [22]. Thirty-six patients (51%) had T790M mutation in the rebiopsy specimen. There was no difference between the pattern of disease progression, PFS for initial TKIs (12.8 and 11.3 months), post-progression survival (14.7 and 14.1 months), or overall survival (43.5 and 36.8 months) in patients with and without T790M. After rebiopsy, 34 patients received afatinib treatment. The response rate was 18%, and the median PFS with afatinib was 3.7 months for the entire group, and 3.2 and 4.6 months, respectively, for the subgroups with and without T790M. This means that there might be benefits for directing subsequent TKI therapies according to T790M status. Although T790M had no prognostic or predictive role in this study, identifying T790M as an acquired resistance mechanism was clinically feasible. Further

research was felt to be necessary to identify patients with T790M-mutant tumors who might benefit from new T790M-specific TKIs currently in development [22].

3.3 Pharmaco-economic Study

One report evaluated rebiopsy in NSCLC by cost-benefit modeling [23]. A decision-analysis model compared the costs and effects of platinum combination chemotherapy (carboplatin and paclitaxel; carboplatin and pemetrexed; and carboplatin, pemetrexed, and bevacizumab) with erlotinib therapy in patients with EGFR mutation-positive tumors. Compared with a combined carboplatin paclitaxel regimen, targeted therapy based on testing available tissue yielded an incremental cost-effectiveness ratio (ICER) of $110,644 per quality-adjusted life year (QALY). The rebiopsy strategy yielded an ICER of $122,219 per QALY. With a willingness to pay of $100,000 per QALY, the testing strategy was cost effective 58% of the time, and the rebiopsy strategy was cost effective 54% of the time. Compared with carboplatin, pemetrexed, and bevacizumab, ICERs were $25,547 per QALY for the testing strategy and $44,036 per QALY for the rebiopsy strategy. Personalized therapy with an EGFR-TKI was more favorable when the nontargeted chemotherapy regimen was more expensive. The authors concluded that cost-effectiveness analysis supports testing for EGFR mutations in patients with Stage IV or recurrent lung adenocarcinomas, performing rebiopsy if insufficient tissue is available for testing, and treating patients with EGFR mutations with erlotinib as a first-line therapy. However, this study assumed that erlotinib offered a PFS benefit, and total costs were greatly depended on costs of nontargeted chemotherapy, which could also depend on the health care system. QALY costs were much higher in the erlotinib group, and rebiopsy increased costs. In practice, patients tend to receive both targeted therapy and chemotherapy as the cancer evolves, so crossover is evident, and it is difficult to extract a single therapy element.

3.4 Case Reports

Four case reports were identified. Two of the reports dealt about rebiopsies on cancer progression, and two additional ones about insufficient initial biopsy and necessity to perform repeated biopsy to obtain sufficient arterial for a proper diagnosis. The first case highlighted acquired EGFR-TKI resistance through transformation to the high-grade neuroendocrine carcinoma spectrum and that such transformation might not be evident at time of progression on TKI therapy [24]. A case of relapsed, EGFR exon-19 deletion, lung adenocarcinoma was treated with erlotinib and cisplatin-pemetrexed after resistance. Liver rebiopsy on progression identified an afatinib-resistant cancer with combined SCLC and NSCLC within neuroendocrine morphology, retaining the EGFR exon-19 deletion. Several acquired resistance mechanisms of EGFR-mutant lung adenocarcinoma to EGFR-TKI therapy were described, the most recent being transformation to SCLC [24].

The second case report demonstrated repeated responses to EGFR TKIs in a woman with adenocarcinoma and no history of smoking [25]. After six cycles of gemcitabine and cisplatin, the patient was treated by gefitinib for four months until progression. Following six cycles of third-line pemetrexed, gefitinib re-treatment was initiated, with partial response for six months. After progression, the patient was recruited for an irreversible EGFR inhibitor trial. Time to progression was 11 months. Although EGFR direct sequencing on the initial diagnostic specimen revealed a wild-type (non mutated), rebiopsy of a progressed subcarinal node was performed at the end of the trial. Analysis showed an EGFR of mutation of L858R/L861Q [25].

The third study addressed the problem of tumor heterogeneity encountered in small bronchoscopic biopsies and the difficulties of evaluating the histological subtype in poorly differentiated carcinomas [26]. Initial diagnosis of squamous cell cancer (SCC) of the lung obtained by bronchoscopic biopsy was based on immunohistochemical staining only by positive results for cytokeratin (CK) 5/6 and p63 because morphological diagnosis was not possible. However, bronchoscopic repeated biopsy showed a mixed squamous/glandular immunophenotype with nests of undifferentiated tumor cells. There was weak immunoreactivity of some tumor cells for CK5/6 and p63, and no positivity of some tumor cells for thyroid transcription factor-1. In addition, an EGFR mutation was found in exon 21 (L858R). This was missed on initial biopsy. The patient achieved TKI and prolonged clinical benefit from treatment. The authors concluded that initial bronchoscopy should be performed by an experienced

pulmonologist to obtain sufficient material from different areas of the tumor. In the era of targeted therapy, a patient having a history of remote smoking in cases of not-otherwise-specified (NOS) NSCLC that favors SCC should also provoke EGFR mutation testing [26]. Similarly, the fourth study also addressed the importance of adequate material for pathological evaluation in a report of five cases of regenerative, atypical squamous metaplasia at the site of a previous bronchial biopsy that was unnecessarily resected based on erroneous diagnosis of squamous cell carcinoma on repeated biopsy [27].

3.5 Additional Articles

In order to check for other articles and validate the search procedure, the search terms *repeated biopsy*, *lung cancer*, and *clinical* were entered, generating 544 hits. All abstracts were reviewed, and four additional articles were selected for this review: one case report about rebiopsy, and three others dealing with repeated biopsy: two original articles and one letters to the editor.

A case report in a letter discussed an 80-year-old male with relapsed EGFR exon-19 deletion lung adenocarcinoma treated with EGFR-TKI. There was poor response and rapid increase of serum neuron-specific enolase [28]. Rebiopsy characterized transformation from NSCLS adenocancer to SCLC, and the EGFR mutation remained.

Three additional articles were about repeated biopsy rather than rebiopsy. Welker et al. [23] studied 118 patients with a solitary lung nodule (4 cm or smaller) who underwent transbronchial biopsy, percutaneous needle aspiration, clinical observation, repeat CT scans, and repeated biopsies. The mean follow-up was four years. The incidence of malignancy was 61%, and the positive predictive value, negative predictive value, sensitivity, specificity, and accuracy were all 100%. Moreover, this procedure reduced the incidence of unnecessary surgical excision of benign nodules from 60% to 5% [29]. Another letter to the editor stated that repeat needle biopsies were recognized to be safe and accurate in the management of a solitary pulmonary nodule [30]. The second original article was a retrospective study of 836 cases. Ninety-five cases with fine-needle aspiration +/- core biopsies over a five-year period were identified initially as nonmalignant [31]. Of these, 21 were confirmed later benign, and the remaining 74 including 53 initially benign and 21 nondiagnostic. Seven of 53 benign (13%) and six of 21 nondiagnostic specimens (29%) were malignant at excisional biopsy during radiologic follow-up. Sixteen of 95 cases (17%) had post-procedural pneumothorax that required a chest tube [31]. Therefore, repeat biopsy or resection is necessary for benign nonspecific and non-diagnostic biopsy results due to an unacceptably high rate of malignancy.

3.6 Safety

Serious complications in rebiopsy are rare. As there is already initial diagnosis available, additional biopsies are carefully considered. Patients with lung cancer tend to develop metastases and especially liver and lymph node lesions highly accessible for a biopsy. Probably a selection of biopsy sites have impact on low number of reported complications. In this review, one serious complication among 155 rebiopsies patients (12), and 13 minor complications in 94 patients (14) were reported in articles of this review. Additionally, no complications in 47 biopsied patients were reported by Bepler et al. in their repeated biopsy article (7). In conclusion, rebiopsy appears to be safe when biopsy sites are carefully selected and the risk evaluation made before rebiopsy.

4. DISCUSSION

PubMed results reflect a lack of activity in rebiopsy for many indications, such as colon and lung cancers. Only 14 articles were found about rebiopsy in lung cancer by the search terms *rebiopsy* and *lung cancer* (Table 3). Prostate cancer had more hits (104) on the term *rebiopsy*. This reflects the attitude among urologists of actively performing repeated biopsies in follow-up and rebiopsies on relapses on prostate cancer patients. Of course, in first place it needs to recognize that the multiple biopsies are easier to do in prostate cancer than in lung cancer because of anatomical accessibility, lesion location, and minimal risk of complications. The situation with breast cancer is similar. The

location of tumor relapse in breast tissue is usually accessible, but enlarged lymph nodes may be situated in places where performing a rebiopsy would pose too great a risk.

This study was originally published in 2015, 6 years ago (1). During recent 6 years growing number of articles are being found under a term “rebiopsy and lung cancer” in PubMed. Number of articles has increased over 20 fold from 14 to 336 (July 2021). Apparently, as gene tests have become part of the routine practice and a need for rebiopsies has increased dramatically, which reflects on publication activity. Rebiopsy has become necessity, however, its nature has remained challenging. On the other hand, liquid biopsy has also entered in clinical use, but it is has remained as an additional tool not replacing paraffin sample testing (33). In addition, it seems that getting new gene driven cancer drugs in use access to gene tests is far more complicated than just having rebiopsy done and tissue sample available (34). There are many obstacles to a new cancer drug before it becomes available for prescriptions by clinicians, like the existence of a new drug with efficacy relating to gene testing, availability of appropriate gene tests and furthermore the new drug needs to be affordable to patients (33). In fact, in our recent case study about situation in our clinic in Finland, we found that a reimbursement was a major factor that actually influenced the use of gene tests and finally also use of gene-driven cancer drugs. This is mainly because if there was no reimbursement, no testing was organized for the particular gene, and no prescription was written to a patient (34). In our experience, rebiopsy for tissue samples was challenging, partially because only restricted personnel could take the biopsies but also because sampling was technically difficult or had excess risks. While blood samples for liquid biopsy were easier to take, reliability of tests were often a problem, and only a few clinically recognized reliable tests were available, e.g. tests of EGFR and its resistance mutation T790M.

Solid tumors have a heterogeneous histological background, which make it impossible to cover all metastases, even with only one highly targeted agent, which can only block one cell clone at a time. In tumor growth and spread, cancer clones are probably randomly selected to survive, some of which may be resistant to given therapies, having a edge over other cell clones [32]. Furthermore, metastasizing involves one cell type and originates from one cell clone. New therapies block certain cell clones, but miss others that develop based on other mutations [33,34]. Therefore therapy fails, and redirection is needed.

In the optimal situation, therapeutic effect should be constantly monitored by repeating the histological examination, as the primary tumor can change. One or two clones may become resistant to a given therapy and dominate. In the metastasizing process, a limited number of cells fix themselves on remote places in the body. Some of these cells can avoid immunoreaction and start forming metastases. So a metastasis of a solid tumor can be very different from its parent tumor. Rebiopsy of lung cancer patients with acquired resistance is feasible and could provide sufficient material for mutation analysis in most patients [12]. Using a highly sensitivity method, a LNA PCR/sequencing assay, T790M was detected in up to 68% of these patients, which was 12% more than with ordinary analysis methods.

Rebiopsies are widely used in cancers other than those in the lung. In prostate cancer, repeated biopsies and rebiopsies are readily performed, when prostate specific antigen (PSA) is increased, because doing so is easy, as there are no vital organs in the neighborhood of the prostate [35]. Similarly, rebiopsies are often performed in breast cancer to confirm cancer relapse and provide characteristics of a new breast cancer lesion. This will direct treatments, such as hormonal treatment in hormone receptor-positive cases. It will also confirm if the mutation in the HER2 oncogene and the elevated levels of HER2 protein are present, which triggers use of targeted therapies [36,37]. It is difficult to access bone lesions and to retrieve good histological samples, and consequently bone lesions are normally not biopsied. The metastatic lesions were rebiopsied by core needle aspiration, or CT- or ultrasound- guided biopsy with no major complications. Additionally, rebiopsies may show a second malignancy [37,38].

In neuroendocrine lung cancers, rebiopsy is widely used to pick up transformations to more aggressive types of cancer, such as small-cell cancers. Transformation is also highly important to uncover in cases of suspected lymphoma relapse e.g. in thoracic area. There is also increased risk of

secondary cancer in areas that have been radiated in Hodgkin's disease. The risk increases remarkable after decades from given radiotherapy. In certain cases rebiopsy is not recommendable.

Schneider et al. [39] recommended omitting rebiopsy from clinical practice in esophageal cancer for objective response evaluation, based on his prospective study of 80 patients [39]. Table 5 summarizes the general reasons for not performing rebiopsy. The common reasons being for not doing rebiopsy are that it is not routine practice, the anatomical location for the target tumor may make the operation too risky, and general perceptions of there being high risk involved.

One clear benefit from rebiopsy in treatment of NSCLC is that it provides an updated look at tumor characteristics, which can be used to redirect treatments [9]. This was demonstrated in the case reports addressing individualized approaches to lung cancer treatment. There was tumor heterogeneity in small bronchoscopic biopsies and challenges in histological subtyping of poorly differentiated carcinomas, repeated responses to EGFR TKIs based on EGFR mutation (in spite of initial wild-type characterization), and acquired EGFR-TKI resistance through transformation to the high-grade neuroendocrine carcinoma spectrum. All these cases highlight a need for rebiopsy.

Fig. 1 summarizes the potential benefits of rebiopsy in reassessing treatment options, Table 5 lists main reasons not to perform rebiopsy, while Table 6 gives recommendation for rebiopsy in management of NSCLC. It can be important in treatment control when tumor behavior changes, as happens in a transformation into a more aggressive cancer type. It is important to get a look at changed tumor characteristics to determine the proper action. For example, neuroendocrine lung cancer can switch to SCLC type, which could be detected by rebiopsy. Tumor characteristics are important in directing treatments. Old targets can validate the choice to use existing and previous therapies. Moreover, material from rebiopsy makes it possible to explore a new target and to conduct clinical trials on new molecules [40]. When a TKI is used in NSCLC, there is a resistance tendency that becomes evident within two years. Some patients develop treatment resistance quicker than others, and rebiopsy is needed to confirm progression and look at new molecules that are being developed to overcome resistance. Without doing a rebiopsy to investigate the type of resistance and new targets, it would not be possible to use therapies against resistance in a controlled manner. One obstacle to drug development is that second-line patients with adequate tumor re-characterization to indicate gene alteration are difficult to find because rebiopsies are not customarily performed. However, nearly all clinical study protocols in relapsed adenocancer NSCLC now require a rebiopsy option to gather histological samples.

Novel immunotherapy strategy is pending on histological definition of targets in tumor; and those targets can change in time [41,42]. So it is essential to search, e.g. PD-L1 positivity for confirming reactivity of lung cancer on nivolumab before initiating treatment [43,44]. PD-1 is expressed by activated T cells and down modulates T-cell effector functions on antigen-presenting cells; and in cancer patients, its expression on tumor-infiltrating lymphocytes and its interaction with the ligands on tumor and immune cells in the tumor microenvironment undermine antitumor immunity [45]. As PD-L1 measurement is done regularly in clinical trials that will be used in registration purposes, future treatment instructions after approval by drug agencies will include PD-L1 check before starting nivolumab. PD-L1 can be measured at protein level by immunohistochemistry, but there are only centralized measurements available at this early stage. This makes it necessary to send samples first for EGFR and ALK testing and then if negative send them to different central laboratory for PD-L1 testing, and less than 40% of samples will turn negative. This adds time for treatment decision making and may slow down remarkably clinical trials. In addition, in case of relapses patient cannot be entered into the trial if there is no rebiopsies done at relapse, as is usually the case. At the end, there will be no possibility to include patients in the treatment after the drug is approved by drug agencies, if fresh rebiopsy is missing.

Rather than performing rebiopsies, some researchers have proposed analyzing serum for tumor DNA. Recent studies show that genomic alterations in solid cancers can be characterized by massively parallel sequencing of circulating, cell-free tumor DNA released from cancer cells into plasma. This represents a noninvasive liquid biopsy [46,47,48]. Cell-free DNA fragments from multiple lesions in the same individual all mix together in the peripheral blood. Therefore, serum tumor DNA is likely to

contain a wider representation of the genomes from multiple metastatic sites, whereas mutations present in a single biopsy or minor subclone may be missed [49]. Furthermore, intratumor heterogeneity in renal cell cancer makes it difficult to fully characterize primary tumor material and metastases that may be derived from a subclone missed in the primary tumor biopsy [50]. Similar situations were found in breast cancer [51], and probably in other solid cancers, including lung cancer [52]. When this new technology is clinically available, it will revolutionize NSCLC treatment with TKIs, as the development of resistance could be followed frequently and without rebiopsy restrictions, and further treatments could be properly redirected. Exceptions may occur where this approach may not work, as in immunotherapy. However, more research is needed, along with development of a methodology to suit clinical practice, which will certainly take many years. Meanwhile, it is important to use available methods in all clinical practices and to bridge new methodology with the old data, which will require rebiopsy material.

Table 4. Rebiopsy and lung cancer

	Number of articles	**Number of patients**	**Content**	**Reference**
Case reports	4	8		[17, 18, 19, 20]
Pharmaco-economic analysis	1			[16]
Original articles	9			
		53	TS expression	[9]
		65	Beta tubulin	[8]
		70	T790 mutation	[15]
		78	T790 mutation	[14]
		93	T790 mutation	[13]
		94	EGFR mutations ALK rearrangement	[12]
		121	T790 mutation	[11]
		155	Mutation genotyping	[10]
		331	ECCI and RRMI proteins	[7]

Table 5. Why rebiopsy is not done in NSCLC

- Not part of clinical routine
- Anatomical location is difficult for biopsy
- Sense of risk involved in rebiopsy
- Limited number of drugs that can be directed by rebiopsy
- Only a few reports available in the literature.

Table 6. Recommendation for rebiopsy in NSCLC

When rebiopsy should not be performed:

- Too difficult a location for safe biopsy
- Result will not change treatment

When rebiopsy should be performed:

- If the prior specimen is too small for adequate tumor characterization, including genetic testing for predictive alterations
- If relapse happens a long time (six months) after CR treatment result
- If the new tumor behaves in a different way than expected from the primary tumor
- If new molecules entering clinical trials in the near future is foreseeable, such as adenocancer relapses

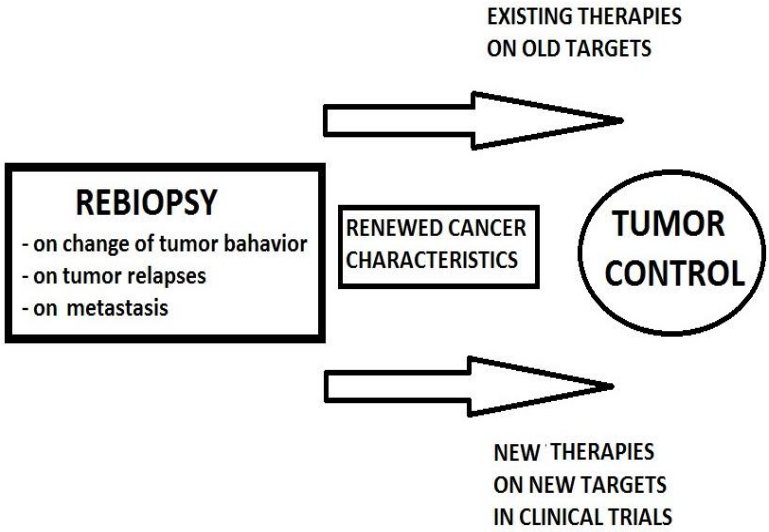

Fig. 1. Role of rebiopsy in NSCLC treatment selection. Rebiopsy will renew tumor characteristics and give opportunity to act on changes of tumor behavior. Rebiopsy can confirm old existing targets when current therapies are allowed, or it can find new targets that need to be treated with new drug molecules in clinical trials. Thus changes in treatment facilitate better tumor control

5. CONCLUSIONS

This review from 2015 shows that rebiopsy is feasible in NSCLC, and success rates can be high if accompanied by adequate evaluation before biopsy. As rebiopsy can be valuable method in clinical practice to help in selecting more efficient therapies for NSCLC patients, it should be performed more often (Table 6). However, before performing rebiopsy adequate evaluation of risks for complications should be performed including anatomic and technical aspects of accessing tumor. A patient overall condition should also be taken in account. In situations, where no possibility for active oncological interventions can be considered, rebiopsy is not indicated. Use of rebiopsy may resolve the difficulties in sampling bias and selecting pre-existing or forming new drug-resistant clones. In cases where treatment was selected based on tissue characteristics that change, the treatment selection process must be repeated while considering new characteristics of the tumor. In the near future, rebiopsy will be used to predict therapeutic resistance and consequently redirect targeted therapies. Rebiopsy is done after the initial biopsy that provided the diagnosis. It is important to remember that metastases may behave differently, so have distinctive histological content. Primary tumors can develop in such a way that the original histological content will change. This can be enhanced by efficient cancer therapy that usually influences nearly all cells. However, those cancer cells that do not die can develop into resistant clones. It would be critical to know when this development occurs. Even with the development of promising, new noninvasive methods for following cancer characteristics in serum samples, rebiopsy material will be urgently needed to identify and ensure those characteristics. Rebiopsies should be performed on lung lesions that were inadequately sampled by an initial biopsy or when new metastatic lesions or relapses occur, in order to confirm the nature of the lesions and select the optimal targeted therapy.

Accordingly, some clinical practice guidelines already include this recommendation. For example, the ESMO 2012 guideline of advanced NSCLC states that obtaining adequate tissue material for histological diagnosis and molecular testing is important to individual treatment decisions, and that rebiopsy at disease progression should be considered [52]. Clinical treatment will benefit from accurate histological diagnosis, and patients will be offered more focused therapies. Fig. 1 addresses the importance of rebiopsy in NSCLC, in which treatment control can be received by recharacterization of tumor and selecting proper treatment on defined targets. If there is no tumor tissue available from a relapsed or progressed primary tumor, changed tumor behavior and cancer

transformation are missed, and the molecularly guided stratification of patients into redirected treatments fails to happen.

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COMPETING INTERESTS

Author has declared that no competing interests exist.

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Biography of author(s)

Prof. Antti P. Jekunen
Clinical Cancer Research Center, Vaasa Oncology Clinic, Turku University, Hietalahdenkatu 2-4, Vaasa 65100, Finland.

He is a professor of Clinical Oncology at Turku University and a Chief Physician of Oncology Clinic, at Vaasa Central Hospital, Finland. He graduated as a Doctor of Medicine in 1986, and he earned the degree of Doctor in Medical Science in 1988, at the University Oulu. He did his post-doctoral fellowship at UCSD at San Diego, USA. He received the Board Certification in Medical Oncology and Radiotherapy at the University of Helsinki in 1996. During 1996-2008, he worked as a director, chief-medical-officer, and vice-president in International Pharmaceutical Industry in Europe and USA in the field of drug development. He has received senior lectureships within Experimental Oncology, Applied Oncology and Clinical Drug Research at Helsinki and Turku Universities. He is a contributor of over 200 articles, of which over 90 are listed in MEDLINE. The main principle of his research operations has been to develop better cancer treatments for cancer patients as well as improve existing ones. He is also the permanent expert of National Supervisory Authority for Welfare and Health and the expert physician in the Finnish Patient Insurance Centre and Patient Claim Centre. He has been a member of a planning group for the first biobank in Finland, Auria BioBank, and for the West-Coast Cancer Center. He is a board member in the Finnish National Cancer Center and Cancer Foundations in Finland.

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Chapter 9
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An Unusual Case of Leaf-like Traumatic Fibroma in a Dentate Patient

Luiz H. A. Nascimento[1*], Tiago N. Pinheiro[2], Joel M. Junior[1], Gustavo C. Albuquerque[1], Marcelo V. de Oliveira[1] and Valber B. Martins[1]

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ABSTRACT

The leaf-like traumatic fibroma (LTF) is a benign fibrous lesion of the connective tissue. Its clinical characteristics feature a flattened pedicle aspect, adapting well to the palate, resembling a leaf. Its color is usually pink, similar to the adjacent epithelium. The growth is slow. The lesion may be larger than 2 cm in diameter. It may be present in the oral cavity for a long time. The LTF is commonly associated with poorly adapted removable complete or partial prostheses which cause a flattening of the lesion against the palate. This report presents an unusual case of an LTF in a dentate patient who did not use any type of prosthesis. Instead, the lesion was related to an injury caused by food trauma, which is an unusual etiology for this type of lesion. We reported the mechanical pressure of the tongue and the action of negative intraoral pressure, as possible modifying agents in causing the characteristic shape of the lesion. Treatment consists of conservative surgical removal since its recurrence is rare.

Keywords: Leaf-like fibroma; palate; wounds and injuries.

1. INTRODUCTION

Traumatic fibromas are the most common benign tumors of the oral cavity, with a prevalence of 1–2% in the general population [1]. They develop from a hyperplastic tissue reaction, usually related to traumatic stimuli that are responsible for triggering inflammatory connective tissue reactions [2]. These lesions have numerous forms, the most common being the nodular-shaped type. Leaf- like traumatic fibroma (LTF) is so named because of its flattened pediculate aspect, presenting a leaf-like shape, which can interfere with chewing and speech, causing discomfort to the patient [3]. Geriatric dentistry or gerodontics is the delivery of dental care to older adults involving the diagnosis, prevention and treatment of problems associated with normal aging and age-related diseases as part of an interdisciplinary team with other health care professionals [4]. Lingual varices, oral squamous cell carcinoma, fbroma and denture induced infammatory fbrous hyperplasia were more commonly associated with the geriatric patients. The oral lesions (fbroma and lichen planus) were strongly associated with women while leukoplakia was strongly associated with men. Ageing is an important factor that can infuence the occurrence of mucosal lesions and with age the oral mucosa becomes more permeable to noxious substances and more vulnerable to external carcinogens [5].

The traumatic fibroma is traditionally asymptomatic. The lesion is usually pedunculated, with an average size of 7–8 mm in diameter but may be larger than 1–2 cm [6]. The surface of the mass is usually papillary, and the lesion may be clinically confused with a papilloma. The most commonly affected site is the oral mucosa along the line of occlusion. However, it can occur anywhere in the oral cavity including the labial mucosa and the tongue [3,7].

[1]Department of Cirurgia e Traumatologia Bucomaxilofacial, Universidade do Estado do Amazonas–UEA, Manaus, Amazonas, Brazil.
[2]Department of Patologia Bucal, Universidade do Estado do Amazonas, Estomatologia e Cirurgia, Manaus, Amazonas, Brazil.
**Corresponding author: E-mail: luisnasc2010@hotmail.com;*

The treatment of choice for fibromas is surgical excision of the lesion, with rare recurrence reported so far [8]. Treatment alternatives include cryosurgery, which uses liquid nitrogen, and the strangulation technique. Cryosurgery is used in cases where patients are allergic to anesthetics or at high surgical risk. Other techniques used are electric and conventional scalpels. Some authors have proposed the application of penicillin G to the lesion, which they reported as having sclerosing effects on the lesion, thereby detaching it, and preventing recurrence [9,10].

2. CASE DESCRIPTION

A 38-year-old female melanoderma patient sought care complaining of “growth in the roof of the mouth”. She did not present any systemic problems, denied being a smoker, and had no relevant family history. The patient related this growth to a food trauma injury at the site. On intraoral physical examination, a flat, pink- colored, pedunculated lesion was observed. It was well-delimited, asymptomatic, resilient to palpation, and rough in texture. It has been present in the mouth for approximately 1 year, according to the patient’s report. The lesion is located on the palatine mucosa, near teeth 26 and 27, and approximately 3 cm in diameter (Fig. 1).

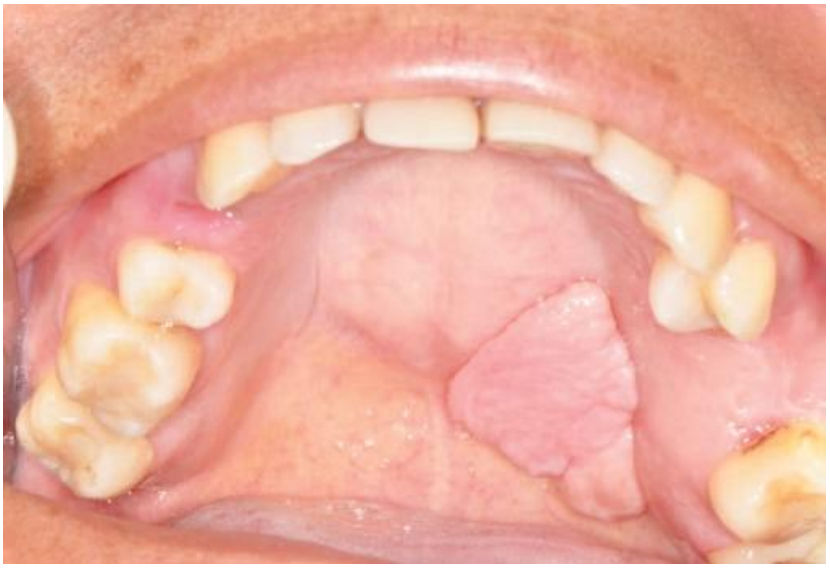

Fig. 1. Clinical aspect, showing mucosal-like, malleable lesion of a flattened shape, measuring about 3 cm in diameter

The patient did not use any dental prosthesis. A periapical X-ray of the region was exposed, which showed no localized alterations. Given the clinical findings, our presumptive diagnosis was traumatic fibroma or inflammatory fibrous hyperplasia (IFH).

Excisional biopsy of the lesion under local anesthesia was performed for the patient. At the beginning of the surgical procedure, a 4-0 silk suture was placed into the lesion to better visualize the base of the lesion. Using a number 15 scalpel blade, the incision was applied to the base of the lesion, and the margin extended 2 mm into healthy tissues. The wound was sutured with a 4-0 suture (Fig. 2). The hyperplasia tissue removed was referred for histopathological analysis. The histopathology findings reported that a fragment of the buccal mucosa was covered by stratified parakeratinized squamous epithelium with pseudoepitheliomatous hyperplasia and the underlying connective tissue was loose, unmodeled, well-collagenized, rich in large nucleated fibroblasts which were sometimes stellar or fusiform, and eventually binucleated or trinucleated. A diagnosis of giant cell fibroma was confirmed (Fig. 3). The patient was monitored at 7 and 30 days postoperatively. There was evidence of scar healing, with no signs of recurrence.

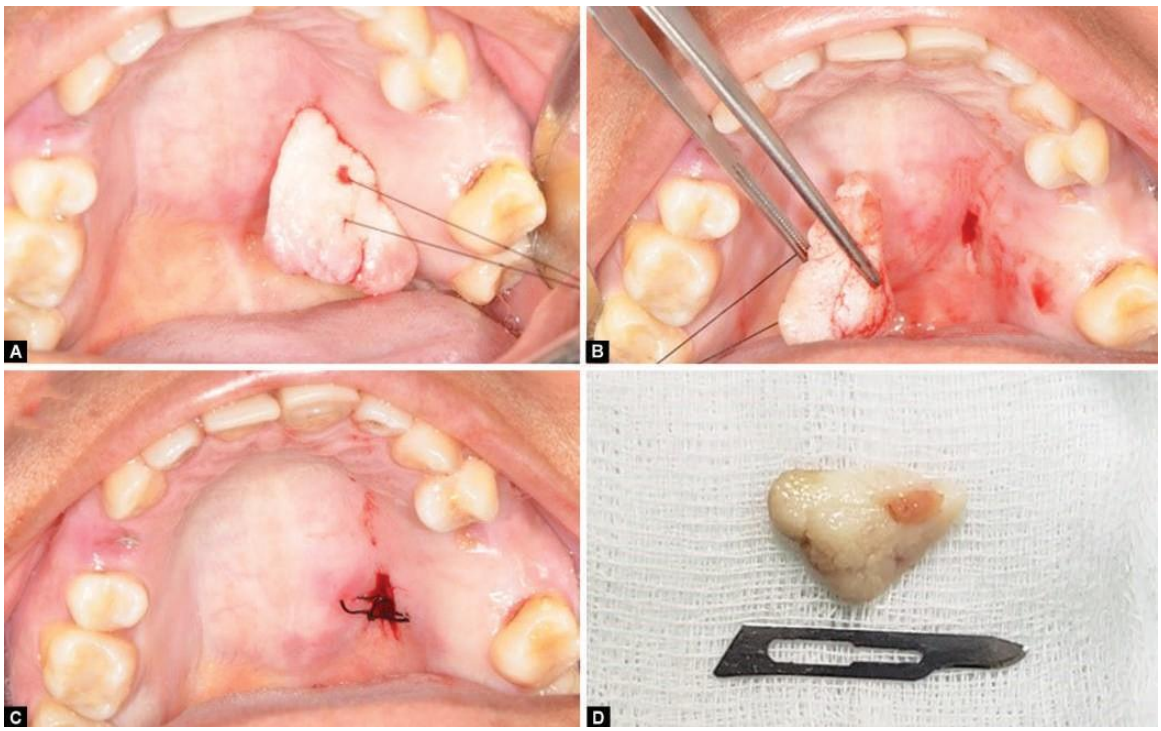

Fig. 2A to D. Raphea performed in order to better expose the base of the lesion for subsequent removal, followed by suture, and image of the removed surgical specimen

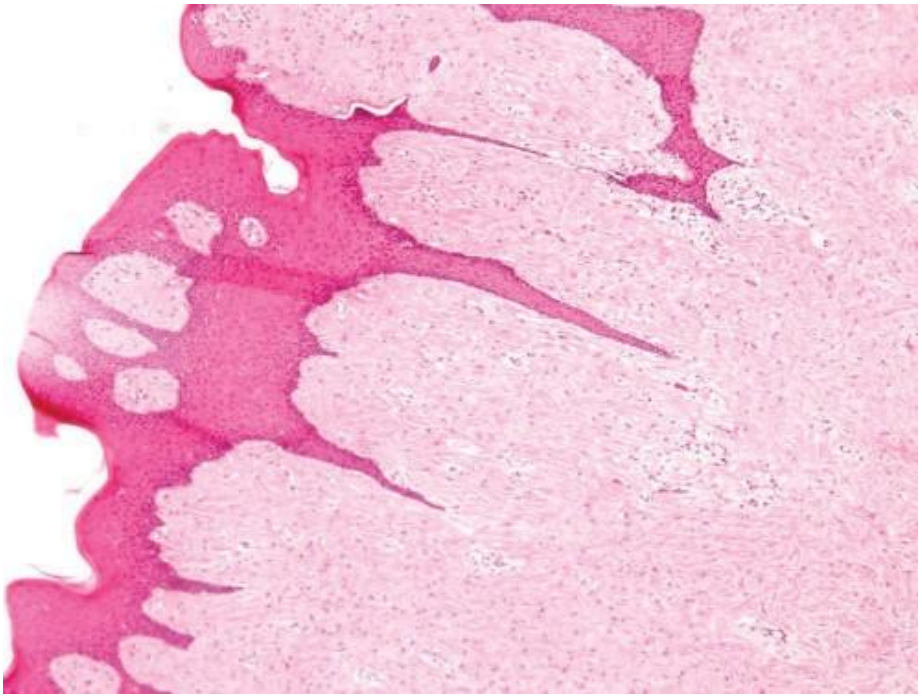

Fig. 3. Histological image. Microscopic sections revealed a fragment of oral mucosa coated with parakeratinized pavement stratified epithelium, showing pseudoepitheliomatous hyperplasia. The underlying unmodeled, well-bonded connective tissue was rich in voluminous core fibroblasts, sometimes star-shaped or fusiform, and eventually binucleated or trinucleated. A diagnosis of giant cell fibroma was confirmed

3. DISCUSSION

The fibroma is a conjunctival tumor, originating from the proliferation of fibroblasts. It is a hypovascularized lesion coated by the oral mucosa, characterized by the presence of collagen and connective tissue [6]. Its development takes place in response to local irritations or trauma, presenting as a nodular mass, with several morphological variations, one of which is the LTF, characterized by its flattened pedicle [3].

These lesions have a higher prevalence in females between the second and the fifth decade of life [11,12]. The case presented corroborates with the occurrence described in the literature. We can observe that the gender and age of the patient fit within the epidemiological pattern. Leaf-like traumatic fibroma usually arises from a local irritation, caused mostly by poorly adapted removable complete or partial prostheses, where a reactive growth of the tissue that develops under the prosthesis occurs [13]. In the present very atypical case, the patient did not use any prosthesis and related the lesion to a fishbone that injured the site. It can be assumed that there was a traumatic stimulus in this reported case, resulting in tissue hyperplasia, to which the patient's tongue may have caused a negative compression of the lesion against the palate, thereby generating a flattened pedicle shape to the lesion. This case demonstrates that other factors can trigger the development of an LTF, such as oblique forces resulting from occlusal maladjustments and deleterious habits [14,15].

The mechanical stimulus that causes tissue trauma can develop different types of pathological lesions, such as pyogenic granuloma, IFH, traumatic fibroma, and fibroepithelial polyp; and the differentiation of these lesions is related to the biological responses of the tissues, where cell maturation may be analyzed [3,15]. The LTF is a lesion that can be clinically confused with IFH. When we observe the lesions microscopically, the IFH is distinguished by a large number of collagen fibers and fibroblasts, in addition to an intense chronic inflammatory infiltrate present, [13] while the traumatic fibroma is characterized by a rich dense hypovascularized collagen below the epithelial surface, with low fibroblast mitotic activity, and the presence of minimal inflammation except in dispersed submucosal areas [6]. These observations were reported in the histopathological examination of the reported case. The reported lesion was differentiated by the presence of multiple large stellar-shaped nuclei and multinucleated fibroblasts. Thus, the GCF reported in the case is an intermediate lesion between IFH and fibroma. It presents a well-collagenized connective tissue rich in fibroblasts with epithelial projections toward the connective tissues, without a significant inflammatory process.

The treatment of choice for fibromas is surgical excision [16]. The technique used should be individualized according to the characteristics and location of each fibroma. The usual anesthesia technique used for excision is a local infiltration around the lesion. In planning the excision, it is mandatory to remove the body and the base of the entire lesion. In addition, the exposed regions of the connective tissues at the surgical area require coverage and protection. This can be done by coating the tissues with surgical cement, or when the exposed tissues are muscular, as in cases of lingual and labial fibromas, the limits of the incision should be sutured. Some authors choose to let the surgical area heal by second intention. Removal of the fibrous hyperplastic areas can also be performed using electrosurgery, which is an option that provides the advantage of concurrent tissue excision and coagulation, thereby controlling bleeding at the same time [9]. There are other treatment alternatives for these types of lesions, such as cryosurgery which uses liquid nitrogen and is very useful in cases where patients are at high risks for surgery or allergic to anesthetics. However, with cryotherapy, not being able to perform the histopathological studies of small samples is a limitation [9].

The strangulation technique is proposed for resection of lesions 5 mm or less in ammeter, in the oral mucosa of the lips, cheeks, and the back of the tongue [8,9]. It consists of using a surgical suture to surround the lesion with a knot, then applying firm pressure in such a way that the vascular supply reduces the lesion to a pale coloration, and the base of the lesion is incised by removing it from adjacent tissue using an $N^0$15 scalpel blade. The tissue bed appears slightly retracted without bleeding, so the procedure ends, and no suture is required [9]. Some authors have proposed

infiltration of the lesion with penicillin G, reporting that it produces sclerosis of the lesion, thereby detaching it, and avoiding recurrence [9,17].

The reported case was managed by means of excisional biopsy of the lesion, using a conventional scalpel. This technique was chosen because the lesion was easily accessible. Excision was performed using an elliptical incision with a perimeter of 2 mm of normal tissues. The therapeutic option selected for the reported case was based on the clinical characteristics of the lesion and the available conditions [9,10,16]. This technique was effective for this case [9,10,16].

4. FINAL CONSIDERATIONS

The present case is an atypical LTF in a patient who did not use any type of removable prosthesis. Being very rare, there are few reports in the literature. Its location, clinical appearance, and size were unusual. We reported the mechanical pressure of the tongue and the action of negative intraoral pressure, as possible modifying agents in causing the characteristic shape of the lesion.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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Chapter 10
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Determination of Susceptibility Determination of Pattern of Fosfomycin against Drug Resistant Bacteria Obtained from Non - Urinary Clinical Samples in a Tertiary Care Hospital, South India

Premalatha Ethirajulu[1], Jeyakumari Duraipandian[2*], Kandasamy Sankararaman[1], Sony Mary Paul[1], Priestly Vivekkumar[3] and Sukumar Rathnamgiri[1]

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ABSTRACT

Background: Multi drug resistance (MDR) in bacterial infections has been an ever growing problem worldwide. To combat this some of the old drugs like fosfomycin used in the past are revived. The aim of our study was to know the susceptibility of fosfomycin for the drug resistance isolates from non - urinary samples. Most of the studies showed less susceptibility of fosfomycin to Pseudomonas aeruginosa. The objectives of the study were (i) To determine the susceptibility of fosfomycin against Methicillin sensitive and Resistant *Staphylococcus aureus*, ESBL producing *Escherichia coli*, *Klebsiella species* and Metallo Beta Lactamase producing *Pseudomonas aeruginosa* isolated from the specimens other than urine and to evaluate the agreement between the two methods, disk diffusion and agar dilution methods performed as per CLSI guidelines.
Methods: First isolate of each species per patient (n=250) were tested for susceptibility to fosfomycin concomitantly by the disk diffusion and agar dilution methods described by CLSI guidelines and comparison of the two methods were studied.
Results: *Staphylococcus aureus* and ESBL *E. coli* were showing 100% susceptibility, Whereas ESBL producing *Klebsiella species* showed 88% susceptibility to fosfomycin 200 µg/disc and 80% and 72% by agar dilution method as per CLSI and EUCAST criteria. For MBL producing *Pseudomonas aeruginosa*, 90% isolates were susceptible to fosfomycin 200 µg/disc and in agar dilution (60%) (≤32 µg/ml) were susceptible as per EUCAST criteria. Disk diffusion method showed good agreement for *S. aureus* and *E. coli* whereas moderate agreement for *Klebsiella species* and very poor for *Pseudomonas aeruginosa*.
Conclusion: Fosfomycin can be considered as an alternate drug to treat infections with multi drug resistant bacteria, not only for the UTI but for systemic infections also. This is achievable with establishment of breakpoint values and zone diameter for all common isolates.

Keywords: Fosfomycin; MRSA; ESBL E. coli; ESBL Klebsiella; MBL Pseudomonas aeruginosa; disc – diffusion; agar dilution.

1. INTRODUCTION

Multi drug resistance (MDR) in bacterial infections has been an ever growing problem worldwide and emergence of resistance to antimicrobial drugs have been increasing in the organisms like

[1]Department of Microbiology, Tagore Medical College & Hospital, Affiliated to Tamilnadu DR MGR Medical University, Chennai, India.
[2]Department of Microbiology, Jawaharlal Institute of Postgraduate Medical Education and Research, Karaikal, Pondicherry, India.
[3]Department of Pharmacology, Tagore Medical College & Hospital, Affiliated to Tamilnadu DR MGR Medical University, Chennai, India.
**Corresponding author: E-mail: karailabscuddalore@yahoo.co.in;*

Staphylococcus aureus, Enterococcus, Enterobactericeae group of organisms, *Pseudomonas aeruginosa* and *Acinetobacter baumannii* [1]. The isolates of MDR organisms reduce the number of active drugs used to treat these infections [2,3]. Infections by gram negative bacteria is becoming an immense challenge due to incipient of drug resistance among these pathogens, render even the broad spectrum and newer antibiotics resistant [1]. Extended spectrum of Beta lactamases (ESBL), Amp C beta lactamases, carbapenemases producing gram negative bacteria and MRSA, VRE among gram positive bacteria have emerged as significant therapeutic challenge. The organisms called as "ESKAPE" ie) *Enterococcus faecium*, *S. aureus*, *Klebsiella pneumoniae*, *A. baumanii*, *P. aeruginosa* and *Enterobacter* species are the greatest threat in the present scenario, as they easily evade from the action of antibiotics [4].

Nonetheless, drugs with considerable antimicrobial activity are dearth of in clinical practice since one decade and escalating progression of drug resistance coupled with a diminished antibiotic pipeline has led some to claim that a post-antibiotic era is eminent [5]. To battle this problem some of the old drugs used in the past like colistin, polymyxin, tenocillin for gram negative bacteria and Fosfomycin for both gram positive and gram negative bacteria is revived. Out of these fosfomycin is phosphonic acid compound discovered in 1969 in Spain [6]. It inhibits cell wall synthesis by inactivating phosphophenol pyruvate transferase enzyme. It is a broad spectrum and bactericidal antibiotic showing 90% or greater susceptibility to ESBL producing Enterobacteriaceae, CPE and also to MDR *P. aeruginosa*, MRSA and VRE [7,8,9]. *A. baumanni* is intrinsically resistant to fosfomycin [8]. There are few studies showing the action of fosfomycin against these commonly encountered bacteria isolated from the specimens other than urine [10]. Hence the aim of our study was to determine the susceptibility of fosfomycin against *S. aureus* (both MSSA & MRSA), ESBL producing *Escherichia coli*, *Klebsiella species* and MBL producing *Pseudomonas aeruginosa* isolated from the specimens other than urine and to evaluate the agreement between the two methods, disk diffusion and agar dilution method performed as per CLSI guidelines [11].

2. MATERIALS AND METHODS

2.1 Sample Collection and Identification

First isolates of each species from a patient were included and total isolates tested were (n=250), of which fifty isolates of each species included were Methicillin sensitive *S. aureus* (MSSA), Methicillin Resistant *S. aureus* (MRSA), ESBL producing *E. coli* & *Klebsiella species* and Metallo beta lactamase producing (MBL) *P. aeruginosa* were tested. These bacterial species were isolated from various clinical specimens from 2015 to 2016 as follows. MSSA (n= 50 from Pus -40, Blood -8 and Peritoneal fluid -2), MRSA (n=50 from Pus -43, Blood-6, Synovial fluid-1), ESBL *E. coli* (n=50 from Pus-28, Blood -18, CSF-1, Synovial fluid -1), ESBL *Klebsiella species* (n=50 from Pus-14, Blood -15,CSF-2, Asicitic fluid - 1, Sputum -18) and MBL *Pseudomonas aeruginosa* (n=50 from Pus - 48 and Sputum - 2). Urinary isolates were not tested in our study. All the isolates were identified by conventional methods. MRSA was detected by using Cefoxitin 30 mg disc, ESBL by double disc synergy test and MBL by Imipenem - EDTA disk method were carried out [12,13].

2.2 Antimicrobial Testing

The antimicrobial susceptibility testing to fosfomycin was determined concomitantly by the disk diffusion and agar dilution methods described by CLSI guidelines [11]. For disk diffusion method, Mueller hinton agar with fosfomycin disk 200mg containing 50mg of G6PO4 was used. For agar dilution method, Mueller Hinton agar (Himedia) supplemented with 25 mg/ml of Glucose - 6 PO4 was used. The MIC is defined as the lowest concentration of drug that inhibits visible growth of the organism. Control strains were included viz; S.aureus ATCC 29213, *E. coli* ATCC 25922 and P.aeruginosa ATCC 27853. (Himedia) Interpretative criteria for disk diffusion was based on CLSI breakpoints given for urinary isolates of *E. coli* and Enterococcus faecalis, whereas for agar dilution method both CLSI and EUCAST criteria were followed. EUCAST criteria are given for Enterobactericeae, Pseudomonas and Staphylococcus species (Table 1) [14].

Table 1. Interpretive criterion of fosfomycin recommended by CLSI [11] & EUCAST [14]

Standard and Organism	MIC (µg)			Zone diameter breakpoint (mm) for the following interpretive criteria		
	S	I	R	S	I	R
CLSI-(Urinary tract Isolate only) *Escherichia coli* *Enterococcus fecalis*	≤ 64	128	≥ 256	≥ 16	13-15	≤ 12
	≤ 64	128	≥ 256	≥ 16	13-15	≤ 12
EUCAST Enterobacteriaceae i.v[a]	≤32	-	>32	NA[b]	NA	NA
Enterobacteriaceae **(Fosfomycin trometamol, Uncomplicated UTI only)**	≤ 32	-	>32	NA	NA	NA
Pseudomonas species i.v[d]	≤32	-	>32	NA	NA	NA
Staphylococcus species	≤ 32	-	> 32	NA	NA	

[a] i.v., intravenous, [b] NA, not available, [c] UTI, urinary tract infection; [d] Intravenous fosfomycin may be used in combination with other antibiotics to treat P. aeruginosa infections. S– Sensitive, I– Intermediate & R – Resistant

2.3 Data Analysis

All the findings were recorded and a susceptibility pattern of the disk diffusion method was compared with agar dilution method as the reference method. The results were analysed statistically using SPSS version 20. Agreement and discrepancies between the evaluated and reference methods are classified as Very major errors (VME), Major errors (ME), and minor errors (Mi). Value for the kappa coefficient, which gives measure of the percentage of agreement between the categorical results of susceptibility testing methods, were interpreted according to the classification by Landis and Koch. (Table 2) [15].

Table 2. Kappa coefficient – by Landis & Koch [15]

Kappa value	Strength of agreement
<0.2	Poor
>0.2 -< 0.4	Fair
>0.4 - <0.6	Moderate
>0.6 - <0.8	Good
>0.8 – < 1	Very good

3. RESULTS

The susceptibility pattern of fosfomycin to the different isolates Viz: *Staphylococcus aureus* (both MSSA & MRSA), ESBL producing *Escherichia coli*, *Klebsiella species* and MBL producing *Pseudomonas aeruginosa* are given in the (Table 3).

Susceptibility Pattern of Fosfomycin against *S. aureus*: Both MSSA 50/50 (100%) and MRSA 50/50 (100%) were showing susceptibility to fosfomycin 200 µg/disc and by agar dilution method. As zone diameter breakpoint (mm) is not available for *S. aureus*, the results were interpreted as per CLSI criteria given for *E. coli* and *Enterococcus fecalis*. The MIC value was interpreted according to the EUCAST criteria. All the isolates were having MIC value ≤ 32 µg/ml (Table 3).

3.1 Susceptibility Pattern for Gram Negative Bacteria

ESBL producing *E. coli*: All the isolates 50/50 (100%) were susceptible to both the fosfomycin 200µg/disc and agar dilution method as per CLSI criteria (Table 3).

ESBL Producing *Klebsiella Species*: The isolates 44/50 (88%) were susceptible to fosfomycin 200 µg/disc and in agar dilution, 40/50 (80%) were sensitive with MIC value ≤ 64 µg/ ml and 10/50 (20%) were resistant (>64µg/ml) as per CLSI guidelines and following EUCAST criteria 36/50 (72%) isolates were susceptible and 14/50 (28%) were resistant (≥32µg/ml) (Table 3).

MBL Producing *Pseudomonas Aeruginosa*: The isolates 45/50 (90%) were susceptible and 5/50 (10%) resistant to fosfomycin 200 µg/disc and in agar dilution 30/50 (60%) (≤32 µg/ml) were susceptible and 20/50 (40%) resistant as per EUCAST criteria. But according to CLSI guidelines given for *E. coli*, the susceptibility of 70%, (≤ 64 µg/ml), 10% Intermediate (128 µg/ml) and resistant 20% (≥ 256) was observed.

Comparison of Susceptibility Tests: The comparison of disk diffusion with gold standard agar dilution method was carried out. The categorical agreement was 100% for *Staphylococcus aureus* (MSSA & MRSA) and ESBL producing *E. coli*. No errors were found. Whereas for ESBL producing Klebsiella, the VME of 40% and 39.2% according to CLSI and EUCAST criteria respectively was perceived. No major and minor errors were observed. The kappa value was 0.70 and 0.51 as per CLSI and EUCAST guidelines which indicate good and moderate agreement respectively (Tables 4 & 5).

In case of MBL producing *Pseudomonas aeruginosa*, the very major error of 50% and 75% was observed following CLSI and EUCAST criteria correspondingly. No major error was found. Minor error (10%) was observed only by CLSI criteria. The kappa value was 0.412 and 0.286 according to CLSI and EUCAST which indicates moderate and fair agreement respectively (Tables 4 & 5).

Table 3. Interpretation of Fosfomycin MIC by CLSI and EUCAST criteria

Organism	Disk Diffusion			Agar dilution-CLSI			Agar dilution - EUCAST	
Tested	S 16	I (13-16)	R 12	64	128	256	32	>32
MRSA (n=50)	50/50(100%)	0	0	50/50 (100%)	0	0	50/50 (100%)	0
MSSA (n=50)	50/50(100%)	0	0	50/50 (100%)	0	0	50/50 (100%)	0
ESBL *E. coli* (n=50)	50/50(100%)	0	0	50/50 (100%)	0	0	50/50 (100%)	0
ESBL *Klebsiella* spp (n=50)	44/50 (88%)	0	6/50 (12%)	40/50 (80%)	0	10/50 (20%)	36/50 (72%)	14/50 (28%)
MBL *P. aeruginosa* (n=50)	45/50 (90%)	0	5/50 (10%)	36/50 (70%)	5/50 (10%)	10/50 (20%)	30/50 (60%)	20/50 (40%)

Table 4. Correlation of Disk Diffusion method with reference Agar dilution method

	CLSI (%)			EUCAST (%)		
	VM[a]	M[b]	Mi[c]	VM[a]	M[b]	Mi[c]
ESBL *Klebsiella* spp	4/10(40%)	0	0	8/14 (39.2%)	0	0
MBL- *Pseudomonas aeruginosa*	5/10(50%)	0	5/50 (10%)	15/20 (75%)	0	0

a - very major error, b-Major error, c- Minor error

Table 5. Kappa value - Comparison of Disk diffusion with agar dilution method

	Kappa value	Asymp Std error[a]	ApproxT[b]	Approx sig
Measure of Agreement (kappa)	0.706	0.135	5.222	0.000
EUCAST criteria	0.519	0.137	4.187	0.000
MBL- *P. aeruginosa* CLSI criteria	0.412	0.135	4.187	0.000
EUCAST criteria	0.286	0.108	2.887	0.004
Mo of valid cases	50	50	50	50

4. DISCUSSION

One form of fosfomycin is Fosfomycin trometamol which is used in the treatment of uncomplicated urinary tract infections (UTI) as an oral single dose regimen. The other is an intravenous (IV) form of fosfomycin – Fosfomycin di sodium salt, has been used in some European countries and in Japan. Successful outcomes were reported in some studies by using fosfomycin intravenously for infections other than UTI, although fosfomycin has not been approved for conditions other than UTI [16,17,18].

Our study of susceptibility pattern of fosfomycin against drug resistant gram positive cocci like MRSA and gram negative bacilli like ESBL producing *Escherichia coli* and *Klebsiella species*, MBL Producers *Pseudomonas aeruginosa* correlates well with many studies.

All our isolates tested were from the specimens other than urine, mainly the pus, blood, body fluids, and sputum. MRSA is known for its tenacious problem in both hospital and community acquired infections [19]. Fosfomycin was found to be effective in the treatment of experimental MRSA osteomyelitis in rats [20]. Our study showed that all the isolates of MRSA tested were 100% susceptibility to fosfomycin both by disc diffusion and agar dilution methods as per CLSI and EUCAST criteria. This is in consistent with the study conducted by Falgas et al, where 129 of 130 (99.2%) of MRSA isolates were susceptible to fosfomycin [21]. Even study by Lu CL et al observed that MSSA with 100% susceptibility like our observation and MRSA 89% of susceptibility both by CLSI and EUCAST criteria [22]. But it differs from the study by Oksuz et al reported high fosfomycin resistance (58%) in isolates of a ST 239 - MRSA - III clone [23].

The effect of fosfomycin against ESBL producing Enterobactericeae particularly *E. coli* and *Klebsiella species* were studied. In our study *E. coli* isolates were susceptible to fosfomycin 200µg/disc (100%) and also by agar dilution methods as per CLSI and EUCAST criteria. Studies conducted by Adil karadag et al. [18] Pullukcu H et al. [24] Endimiani et al. [25]. Tharavichitkul et al. [26] showed 97.5%, 96.5%, 99.4% and 97.3% respectively. All these studies support our findings and also fosfomycin has high susceptibility against carbapenem resistant *E. coli* (95.1%). Hence fosfomycin can be considered as an alternate drug for ESBL and Carbapenemase producing *E. coli* [27,28].

In our study susceptibility of *klebsiella species* – ESBL producer to fosfomycin is 88% by DD method and 80% & 70% by AD method following CLSI and EUCAST criteria respectively. This is in par with the study observed by Tharavichitkul et al. [26] but the study carried out by Endimiani et al. [25] was showing only 63.2% of susceptibility to carbapenemase (KPC) producing Klebsiella pneumoniae, which was not tested in our study. Recent study by Sodhi K etal showed that even the last choice of antibiotic, colistin resistance has been increasingly reported in *Klebsiella species* for which susceptibility to fosfomycin had not been included in our study [29].

Moreover, study by Chitra et al. [27] in which *Klebsiella species* were showing 64.2% susceptibility by applying CLSI breakpoints and only 36% by EUCAST break point criteria. Also they stated that *klebsiella species* isolated from blood and sterile body fluids showed increased resistance compared to urine isolates, while *E. coli* were unvaryingly susceptible in all isolates. This comparison could not observe in our study as we have done in isolates other than urine. Perdigao et al. [30] in his studies reported in the same way that similar isolates tested by E test showed 85% and 48% susceptibility by CLSI & EUCAST respectively.

The susceptibility pattern of fosfomycin against *Pseudomonas aeruginosa* were 70% and 60% by AD method according to CLSI and EUCAST criteria respectively, whereas 90% by DD method by applying criteria given for *E. coli* of urinary isolates in CLSI. Most of the studies showed less susceptibility of fosfomycin to *Pseudomonas aeruginosa*.

The comparison of disc diffusion method with agar dilution was carried out. The categorical agreement between two methods was 100% for S.aureus (both MSSA & MRSA) and ESBL producing *E. coli* but errors were observed in between these two methods for *Klebsiella species* and *Pseudomonas aeruginosa*. In *Klebsiella species*, the very major error was 40% and 39.2% by CLSI & EUCAST guidelines respectively. The kappa value was 0.706 and 0.519 in accordance with CLSI and

EUCAST guidelines which showed good to moderate agreement. Our findings are different from the study conducted by M de cueto et al. [10] compared the DD method with AD method in which VME 0.7%, Major error 10.9% and Minor error 3.6% was observed and study by Perdigao – Neto et al. [30] perceived very major Error of 4% only, which shows less error rate when compared to our study. To consider a susceptibility test adequate, CLSI recommends that if it obtains < 10% Minor error, < 3% major error and 1.5% VME. [11] Also in our study disk diffusion method reporting greater susceptibility to ESBL - *Klebsiella species* than agar dilution method whereas study by M de cueto et al. [10] reported greater resistance. As our study showed VME of 40% for *Klebsiella species* which concludes that disc diffusion is not satisfactory to study the susceptibility pattern. No breakpoints were given for *klebsiella species* in CLSI and EUCAST guidelines. The study was done by using the criteria given for *E. coli* from urinary isolates in CLSI and Enterobacteriaceae in EUCAST. In our study ESBL *E. coli* and MRSA had significantly lower fosfomycin MICs than *klebsiella species* which is in accord to previous studies [28,31].

For *Pseudomonas aeruginosa* the VME was 50% and 75% as per CLSI and EUCAST criteria respectively and kappa coefficient was 0.412 and 0.286 as per CLSI & EUCAST guidelines. Our study is in accordance with the study by Perdigao – Neto et al. [30] where VME was 80% and 100% following CLSI and EUCAST respectively. Hence disc diffusion is not adequate to test the susceptibility pattern.

5. CONCLUSION

The existing evidences and our study concluded that fosfomycin has a high level of antimicrobial activity against isolates with high level of resistance to antimicrobial drugs such as MRSA, ESBL producing *E. coli*, *Klebsiella species* and MBL producing *Pseudomonas aeruginosa*. Our study has limitations as we tested only for fifty isolates each and clinical outcome following treatment is also not known. The feasible method to know the susceptibility is Disk diffusion method which shows good agreement for *S. aureus* and *E. coli* whereas only moderate agreement for *Klebsiella species* and very poor for *Pseudomonas aeruginosa*. The error rates were also high for these two isolates. To consider a susceptibility test adequate CLSI recommends that if it obtains < 10% Minor error, < 3% major error and 1.5% VME. Therefore, need further studies to establish the breakpoint criteria and to know the clinical outcomes. Hence fosfomycin can be considered as an alternate drug for treatment of infections with multi drug resistant bacteria, not only for the UTI but for systemic infections also. This is achievable with the establishment of breakpoint values and zone diameter for all the common isolates.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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Chapter 11
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Nutrigenomics: An Approach to Understand the Role of Nutrients and Gene Interactions in Periodontal Disease

Aparna Suraj Nellipunath[1*], Suchetha Aghanashini[1], Apoorva Sokke Mallikarjunappa[1], Darshan Basavarajappa Mundinamane[1], Sapna Nadiger[1] and Divya Bhat[1]

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ABSTRACT

Periodontal disease is an inflammatory process that causes periodic destruction of the periodontal attachment apparatus [1]. The advancement of knowledge on the pathogenesis of periodontal destruction and the role of nutrients in it has heightened interest in determining the relationship between periodontal disease and nutrition [2]. It is also critical to understand that genotype and dietary interactions influence periodontal disease risk [3]. Nutrients regulate periodontal health and have a key role in inflammatory and immunological responses [4].
Nutrigenomics focuses on the finding and understanding of molecular-level interactions between nutrients and genomes using genomic methods in nutritional studies [3]. It gives information on the impact of nutritionon metabolic pathways and homeostatic control and allows toknow the disturbances arising in this regulation, at its earliest [5]. This chapter provides a detailed description of role of nutrition in periodontal disease and recommends the daily nutritional intake necessary for prevention of periodontal disease [3].

Keywords: Nutrigenomics; nutrition; periodontal disease; nutrient-gene interactions.

1. INTRODUCTION

Periodontal disease is a progressive inflammatory process, involving periodic destruction of periodontal attachment apparatus and loss of structures of the apparatus, essentially gingiva, periodontal ligament, cementum and alveolar bone, ultimately resulting in loss of tooth in most susceptible patients.[1]Periodontitis, currently shows more than 85% prevalence rate among the general population in India and is considered as the second most-commonest disease around world [5,6,7,3].

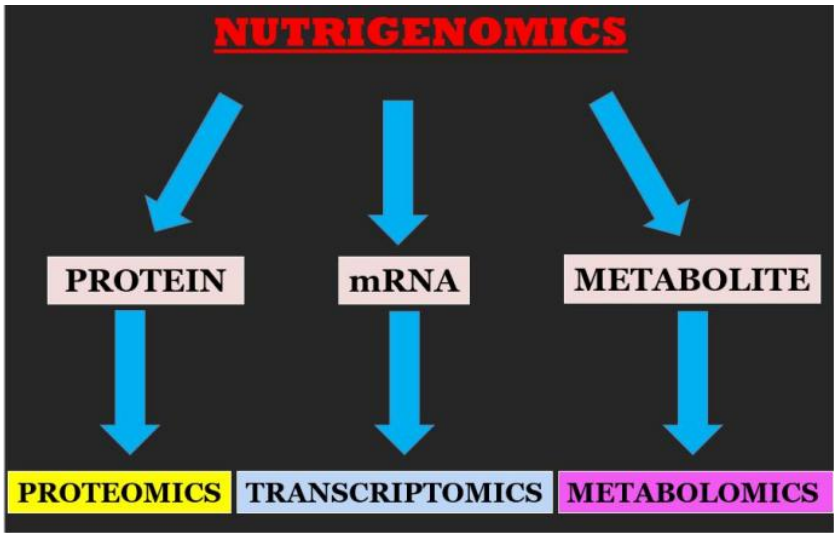
Various biological, environmental and behavioural risk factors, increases inflammation, thereby depleting antioxidant vitamins. These depleted molecules later counteract with reactive oxygen species leading to periodontal changes [8,4]. Crucial interplay between bacteria, environmental, genetic and nutritional factors increase the susceptibility to periodontal disease [3,9].

An emerging branch in the field of science and technology, **"Nutrigenomics"** plays a potential role in bringing about the changes in future guidelines and recommendations of diet [10]. It concentrates on interactions between human genome and nutrients(Fig.1), using modern tools [Flowchart 1].

The first **nutrigenomics company** was launched in **1997** and the term "nutrigenomics" was described by Pelegrin [3,11].

[1]Department of Periodontics, DA Pandu Memorial RV Dental College, Bengaluru, Karnataka, India.
**Corresponding author: E-mail: akku23nellipunath@gmail.com;*

Fig. 1. Illustration of nutrient-gene interaction



Flowchart 1. Tools of nutrigenomics

2. SCOPE OF PERIODONTICS

NUTRIGENOMICS: Is a newly emerging scientific field that is believed to have a serious role in the development of periodontal diseases [12].

EPIGENETICS: Refers to changes in chromatin structure resulting in a somatically heritable state of gene expression, without altering the DNA sequence [13].

PROTEOMICS, TRANSCRIPTOMICS AND METABOLOMICS: Study of proteome, and addressing its biological characteristics constitutes **'Proteomics'**. Transcriptome is the complete set of RNA that can be produced from a genome. Study of transcriptome, at the mRNA level, constitutes **'Transcriptomics'**. The scientific study of chemical processes, involving metabolites is called **'Metabolomics'** [11].

BIOMARKERS: Various proteomic, microbial and genetic biomarkers have been identified for periodontal diseases [12].

2. OBJECTIVES AND APPLICATIONS OF NUTRIGENOMICS

- ✓ To identify genes present in disease and health capable of modifying diet, that are beneficial/harmful to the body [14].
- ✓ To identify transcription factors that function as nutrient sensors.
- ✓ To develop specific biomarkers, using nutritional systems biology [11].
- ✓ To achieve a better understanding on the safer upper/lower limits for essential macronutrients and micronutrients [15].

3. EFFECTS OF NUTRITION ON EPIGENETIC MECHANISMS

DNA Methylation: Any nutrient that can affect S-adenosyl methionine, and S-adenosyl homocysteine metabolite levels in tissue can alter the DNA methylation. DNA hypermethylation status is reduced by genistein present in soyabean, tea polyphenols, thereby inhibiting cancer.

Histone Modifications: Niacin is involved in histone adenosine di-phosphate ribosylation.

Histone Acetylation: Vitamin B3 and B5 are involved in histone acetylation. Enzymes of histone acetylation are histone deacetylase(HDAC) and histone acetyl transferases(HAT). Resveratrol, a bioactive component in grape skins inhibit HDAC. HAT is inhibited by curcumin.

Histone Methylation: Is affected by Vitamin B-9, B-12, choline, methionine and betaine.

Histone Biotinylation: Deficiency of Vitamin B7(biotin) has a significant impact on chromatin structure, gene silencing and DNA repair [13].

4. DISCUSSION

4.1 Nutrient-Gene Interactions

Nutrients can be classified into Macro-nutrients (carbohydrates, fats, proteins and water) and Micro-nutrients (vitamins, minerals and trace elements) [Table 1] [16]. Along with genetics and diet, other environmental factors (smoking, physical activity) need to be considered to optimize health [17].

Table 1. Most-common transcription factors mediating the nutrient-gene interactions

NUTRIENT	TRANSCRIPTION FACTOR
Carbohydrates	ChREBP(carbohydrate responsive element binding protein), SREBP(sterol-responsive-element binding protein)
Proteins	EBPs(enhancer binding protein)
Fats	P PARs(peroxisome proliferator-activated receptor), LXR(liver X receptor), HNF(hepatocyte nuclear factor)
Vitamins-	
Vitamin A	RAR(retinoic acid receptor), RXR(retinoid X receptor)
Vitamin D	VDR(vitamin D receptor)
Vitamin E	PXR(pregnane X receptor)
Minerals-	
Calcium Iron	Calcineurin/NF-ATs(nuclear factor of activated T cells)
Zinc	IRP1 & 2(iron regulatory protein)
	MTF1(metal responsive transcription factors)
Others –	
Flavonoids	ER(oestrogen receptor), NFκB(nuclear factor Kb),AP1(activating protein1)
Xenobiotics	PXR

4.2 Nutrigenomics: Effects on Periodontal Health

Role of Carbohydrates: Artificial sweetener, Xylitol, has an antibacterial effect against periodontal pathogens, Porphyromonas gingivalis and Aggregatibacter actinomycetemcomitans. Reduced intake of sugar, along with scaling and root planing(SRP), and use of xylitol-containing gums effectively improves periodontal health.

Role of Vitamins:

- Improvement in periodontal health upon Vitamin A supplementation.
- Vitamin-B supplementations accelerates the post-surgical healing

- Vitamin C supplementation improves periodontal therapy outcomes.
- Local application of Vitamin D accelerates post-surgical healing/osseointegration.
- Patients undergoing non-surgical periodontal therapy, have shown to increase the total antioxidant capacity in supplementation with vitaminC.

Role of Minerals:

- Local application of calcium enhances osseointegration.
- Magnesium and calcium supplementation improves non-surgical periodontal therapy outcomes.
- Iron and zinc have anti-oxidant effect on periodontium.
- Zinc reduces the severity of diabetes-induced periodontitis [18].

Table 2. Common dietary sources and recommended dietary allowances (RDA*) of nutrients [19-23]

NUTRIENT	DIETARY SOURCES	RDA*/DAY
VITAMIN A	Oily fish, liver, eggs, dark green and yellow fruits and non-citrus vegetables	Men and women:100 µg
NIACIN	Dairy products, eggs, meat, yeast extracts, pulses	Men:16 mg Women:14 mg Pregnant women:18 mg
PANTOTHENIC ACID	Chicken, beef, oats, liver, egg yolk, Broccoli.,	Men and women:5 mg Pregnant women:6 mg
BIOTIN	Liver, fruits, meats	Men and women:30 µg Pregnant women:30 µg
FOLATE	Dark leafy vegetables, enriched cereals grains, bread products.,	Men and women:400 µg
VITAMIN B12	Dairy products, meat, fish, eggs, fortified breakfast cereals	Men and Women:1.8 µg Pregnant women:2.6 µg
VITAMIN C	Citrus fruits, bell peppers, parsley, berries, green vegetables, potatoes	Men:90 mg Women:75 mg Pregnant women:80–85 mg
VITAMIN D	Sunlight, Oily fish, eggs, fortified margarine	Men and women:5 µg
VITAMIN E	Sunflower seeds, vegetable oil, eggs	Men and women:10 mg
IRON	Meat, fish, dark green vegetables, pulses, fortified breakfast cereals	Men:8mg Pregnant women:18 mg
ZINC	Meat, poultry, dairy products, fish, pulses	Men and women:8-11 mg Pregnant women:12 mg
SELENIUM	Animal products	Men and women:55 µg
CALCIUM	Milk, cheese, yogurt, Chinese cabbage, broccoli	Men and women:1,300 mg
FLUORIDE	Fluoridated water, marine fish, teas.,	Men:4 mg Women:3 mg
MAGNESIUM	Green leafy vegetables, unpolished grains, nuts, starches	Men:240 mg Women:240 mg Pregnant women:400 mg

4.3 Effects of Nutrigenetic Interactions in Periodontal Disease

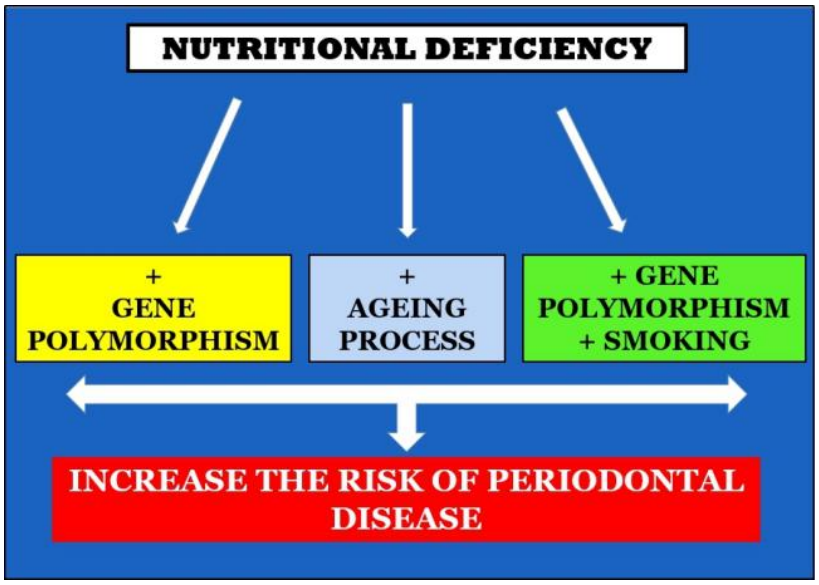
4.3.1 Macronutrients

Proteins: Connective tissue degeneration of gingiva and periodontal ligament, osteoporosis of alveolar bone, impaired cemental-deposition and delayed wound healing are the associated manifestations of protein deficiency [24].

Lipids: A positive modulating effect of lipids have been suggested on gingival inflammation in animals [25]. Kesavalu et al. hypothesized that omega (ω)-3/n-3 fatty acids (FA) dietary supplementation, modulate inflammatory reactions, leading to periodontal disease in infected rats, which were fed with n-3 FA diets for 22 weeks and infected with *P. gingivalis*. Elevated serum eicosapentaenoic and docosahexaenoic acid levels were documented in rats on ω-3 FA diet. PCR was carried out, which showed a less alveolar bone resorption in P. gingivalis induced model of periodontitis [26].

El-Sharkawy et al. evaluated the effects of dietary supplementation on 80 healthy subjects with advanced chronic periodontitis. While control group received SRP and placebo, test group were treated with SRP followed by dietary supplementation of ω3PUFAs and 81 mg aspirin/day. A significant pocket depth reduction and attachment gain were showed after 3 and 6 months in test group compared to control [27].

Micronutrients: Dietary deficiency/lifestyle factors, resulting in decreased serum/plasma micro-nutrient levels, may play a key etiologic factor in development of periodontal disease **[Flowchart 2]** [1].



Flowchart 2. Key etiological factors in development of periodontal disease

4.4 Role of Vitamins and Minerals

Vitamin-A: Deficiency leads to mucosal keratinization and leukoplakia.

Vitamin B-Complex: Vitamin-B12 deficiency causes reversible dysplastic changes to oral mucosa and recurrent ulcers. Vitamin B3 deficiency leads to mucosal atrophy and folate deficiency causes candidosis [23].

Vitamin D and Calcium: Vitamin D receptor (VDR) gene polymorphisms are associated with periodontitis [28]. Miley et al. conducted a study on the effects of Vitamin D and calcium supplementation on 51 chronic periodontitis subjects. 23 subjects received vitamin-D (≥400 international units/day) and calcium (≥1000 mg/day) supplementation, and 28 subjects did not receive such supplementation. Periodontal disease parameters such as, probing depth, attachment loss, bleeding upon probing, were recorded. Better periodontal health was observed in subjects who received periodontal maintenance therapy with adjunctive vitamin D and calcium supplementation [25,29].

Krall et al. obtained the information on tooth loss and oral health status, from 145 subjects aged 65 years/older, who completed a 3-year, randomized, placebo-controlled trial of effect of vitamin D and calcium supplementation on bone loss from hip. Subjects in supplemented group received calcium citrate malate, 500 mg/day, and cholecalciferol, 700 IU/day. Subjects in placebo group received same number of pills, each containing micro-crystalline [30]. Results showed a significant reduction in the osteoporosis and tooth loss, in subjects who received calcium supplements [31].

Vitamin-E: Deficiency in humans is unlikely, as the vitamin is widely distributed in diet [32].

Vitamin-C: Nishidha et al., conducted a study utilizing the survey conducted by NHANES III, on 12,419 adults, with dental measurements and assessment of dietary vitamin-C. Individuals with a clinical attachment level of ≥1.5 mm, were arbitrarily defined as periodontal disease group. A statistically significant dose-response relationship was observed between Vitamin C and periodontal disease, with a low intake resulting in increased risk of periodontal disease [33]. Increased risk of periodontal destruction, is found to be associated with IL-1β and IL-RN genotypes polymorphisms [31].

Blignaut & Grobler compared the periodontal condition of workers in citrus fruit-producing farms with those working in grain-producing farms (controls) [25]. Results showed a less frequency of deeper pockets in subjects who consumed citrus fruit [34].

Relationship between vitamin C deficiency and necrotizing ulcerative gingivitis have also been frequently described [25].

Vogel and Wechsler, studied the effects of mega-doses of vitamin-C on neutrophil chemotaxis and experimental gingivitis, among 24 dental students, divided into two groups [35]. Results demonstrated that the daily dietary intake of Vitamin C was lesser in periodontitis individuals when compared to controls [36].

ZINC: Dang et al. investigated the efficacy of zinc supplementation, modified by SLC30A8 genotype, to improve beta cell function in a rat cell-line model with diabetes. To investigate the potential impact of increased extra-cellular zinc on insulin secretion, control/ZnT8 small interfering RNA knockdown beta cells were incubated 24 hours before incubation with extra-cellular glucose. They hypothesized that zinc supplements may alter periodontal disease progression through changes in expression of ZnT8 transporter gene [2,1].

Others: Makimura et al., conducted a study to examine the inhibitory effects on collagenase activity by catechin derivatives from Japanese green tea *Camellia sinensis*. Among the tea catechins tested, epicatechin gallate and epigallo catechin gallate showed the most potent inhibitory effect on collagenase activity [37]. Decrease in mean pocket depth, clinical attachment loss, and bleeding on probing was associated with every one cup/day increment in green tea intake.

The possible role of flavonoids in relation to periodontal disease was supported by many animal studies, that investigated the effects of a cocoa-enriched diet on gingival oxidative stress in rat-periodontitis model [25]. **Tomofuji** et al., conducted a 4-week study on rats, divided into 3 groups: a control group (regular diet) and 2 periodontitis groups (cocoa-enriched diet). Periodontitis was induced in these models. Consuming cocoa-enriched diet, showed a diminished periodontitis-induced oxidative stress and a suppression in the progression of periodontitis [38].

Isabgol extract has shown effective action against periodontal pathogens and matrix metalloproteins [39].

5. CONCLUSION

Nutrigenomics is currently showing a tremendous success, since the development of personalized nutrition interventions is likely to induce large, appropriate and consistent modifications in eating and lifestyle behaviours, supporting new preventive and therapeutic strategies [40].

The 2011 European Workshop on Periodontology recommends that the dental team, as a part of periodontal prevention/treatment regime, should consider to include fish oils, fruits, vegetables, fibers, and reduce refined sugars in diet [41]. Adequate daily intake of vitamin D and calcium are also recommended, for the prevention/treatment of periodontitis [42].

Thus, a better understanding of inter-relationships between the human genome function and dietary components are necessary to achieve a stable life cycle, optimal human health and disease prevention [43].

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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Biography of author(s)

Dr. Aparna Suraj Nellipunath
Department of Periodontics, DA Pandu Memorial RV Dental College, Bengaluru, Karnataka, India.

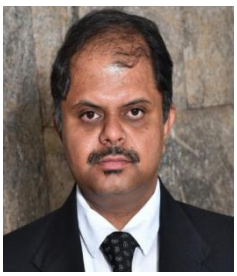
She Completed her graduation from Government Dental College, Bangalore, India in 2017. She is pursuing her Masters in Periodontology at DAPM RV Dental College, Bangalore, India. She has been awarded prizes for her paper and poster presentations in various national conferences. She also has National and International publications to her credit. She has authored the book “Cementum in health and disease”.

Dr. Suchetha Aghanashini
Department of Periodontics, DA Pandu Memorial RV Dental College, Bengaluru, Karnataka, India.

She is a Professor and Head of the the department of Periodontology, at D.A.P.M.R V Dental College, Bangalore, India since 2011. She worked as a lecturer at Rajarajeshwari Dental College, Bangalore during 2001-02 and joined as a Senior Lecturer at D.A.P.M.R V Dental College, Bangalore in 2002. She completed her Masters in Periodontology from A. B. Shetty Memorial Institute of dental sciences, in 2001. She is the editor-in-chief of Rajiv Gandhi University of health sciences dental journal for the period 2019-2021. She has authored more than ten books and has more than 100 national and international publications to her credit.

Dr. Apoorva Sokke Mallikarjunappa
Department of Periodontics, DA Pandu Memorial RV Dental College, Bengaluru, Karnataka, India.

She has been teaching at D.A.P.M.R V Dental College, Bangalore since 2008 and currently is the reader of the department of Periodontology. She Completed her Masters in Periodontology from D.A.P.M.R V Dental College, Bangalore, in 2008. She has authored several books and has several national and international publications to her credit.



Dr. Darshan Basavarajappa Mundinamane
Department of Periodontics, DA Pandu Memorial RV Dental College, Bengaluru, Karnataka, India.

He has been teaching at D.A.P.M.R V Dental College, Bangalore since 2007 and currently is the reader of the department of Periodontology. He completed his Masters in Periodontology from A. B. Shetty Memorial Institute of dental sciences, in 2007. He has authored several books and has several national and international publications to his credit. Currently, he is the executive committee member of South Asian society of oral and maxillofacial implantology (SASOMI).

Dr. Sapna Nadiger
Department of Periodontics, DA Pandu Memorial RV Dental College, Bengaluru, Karnataka, India.

She has been teaching at D.A.P.M.R V Dental College, Bangalore since 2007 and currently is the reader of the department of Periodontology. She Completed her Masters in Periodontology from College of Dental Sciences, Davangere in 2006. She has authored several books and has several national and international publications to her credit.

Dr. Divya Bhat
Department of Periodontics, DA Pandu Memorial RV Dental College, Bengaluru, Karnataka, India.

She has been teaching at D.A.P.M.R V Dental College, Bangalore since 2009 and currently is the senior lecturer of the department of Periodontology. She Completed her Masters in Periodontology from K.L.E. V.K. Institute of Dental Sciences, Belgaum, in 2009. She has authored several books and has several national and international publications to her credit. She has authored the book, “Case history recording in periodontics” in 2014, with a second edition released in 2017.

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Chapter 12
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Exploring the Use of Manual Liquid Based Cytology, Cell Block with Immunomarkers p16/ki67, VIA and HPV DNA Testing as a Strategy for Cervical Cancer Screening in LMIC: A Prospective Study

Nandini Nandish Manoli[1,2*], Devananda Devegowda[2], Ashoka Varshini[1], Pushkal Sinduvadi Ramesh[2], Sherin Susheel Mathew[3] and Nandish Siddappa Manoli[4]

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ABSTRACT

Among low- and middle-income countries (LMIC), cervical cancer is the fourth most common malignancy in women. Cervical cancer is detected using a variety of approaches, including the conventional Pap smear (CPS), Liquid Based Cytology, and supplementary techniques such as Cell Block with immunocytochemistry. Another approach being promoted as a key screening tool is the VIA. HPV DNA testing and other molecular diagnostics have been at the forefront of screening efforts. All of the above methods were used in this study by using cost-effective in-house procedures to see if they could be useful in clinical settings. We found them valuable in light of the need for greater work and people training in order to improve cervical cancer diagnosis. There is a need for a uniform policy of screening of women at the primary health care center level with increasing the awareness of the different methods among the public.

Keywords: Manual liquid based cytology; cervix; immunomarkers.

1. INTRODUCTION

Despite the fact that cervical cancer is the greatest cause of mortality in women in the modern world, the incidence differs greatly between developed and developing countries. India has a quarter of the world's cervical cancer cases, with 20.2 per 100,000 new cases diagnosed and 11.1 per 100,000 deaths each year [1]. Almost 70% of the global burden of cervical cancer falls in areas with lower levels of development, and more than one-fifth of all new cases are diagnosed in India. Cervical cancer is also the second most common cause of cancer deaths when both genders are combined [2-5].

Cervical cancer screening using a variety of methods has been proven to reduce the incidence of the disease in developed countries. However, in countries like India, a lack of infrastructure, national policies, poverty, a lack of financial resources, inadequately trained health care providers, improper implementation of screening programmes, and a lack of education among women, particularly in rural areas, have hampered cervical cancer control. This renders a huge load leading to a major global impact on health care of women [6,7,8].

[1]Department of Pathology, JSS Medical College, JSS Academy of Higher Education & Research, 570015, India.
[2]Centre of Excellence in Molecular Biology & Regenerative Medicine, Department of Biochemistry, JSS Medical College, JSS Academy of Higher Education & Research,570015, India.
[3]Department of Pathology, Dr. Somervell Memorial Church of South India Medical College, 695504, India.
[4]Department of Obstetrics and Gynecology, JSS Medical College, JSS Academy of Higher Education & Research, 570015, India.
**Corresponding author: E-mail: nandinimanoli65@gmail.com;*

Screening tests for cervical cancer include:

- Conventional exfoliative cervico-vaginal cytology i.e. thecervical (Pap) smear.
- Manual liquid-based cytology
- Fluid sampling technics with automated thin layer preparation(liquid-based cytology)
- Cell block technique with immunomarker study
- HPV DNA testing
- Polar probe
- Laser induced fluorescence
- Visual inspection of cervix after applying Lugol's iodine (VILI)or acetic acid (VIA)
- Speculoscopy
- Cervicography

1.1 Exfoliative Cytology (Conventional Pap Smear)

Conventional Pap smear has been the standard screening method for cervical cancer screening from the past several years. It includes sampling of material from the junction between the ecto- and endo-cervix using an Ayre's spatula. The material obtained is spread on to the clean glass slide which is stained by routine pap stain and studied by the Cytopathologist. It has limitations due to many errors either due to sampling (5-10%), interpretation and obscuring factors like blood which hamper the accurate diagnosis. Only 20% of the sample taken gets spread on to the slide which hampers the sensitivity [9,10].

1.2 Manual Liquid-based Cytology

Liquid based cytology is a technique wherein cells are arranged in a single or monolayer on a clean glass slide. It is a method which helps to remove obscuring factors seen in conventional method. There are two automated methods followed in developed countries: the SurePath and ThinPrep. These methods improve the sensitivity and specificity and also help in using the remaining material for ancillary techniques like cell block with IHC and HPV DNA testing. But these automated methods have their limitations as they are expensive to be used in LMIC.

To overcome these limitations, we have utilized an in-house cost-effective method of Manual Liquid Based Cytology (MLBC). We used simple machines like centrifuge and the sample collected in liquid fixative which was processed in a polymer solution prepared in the laboratory [11].

1.3 HPV Testing

Epidemiological studies and mechanistic evidence has led to the conclusion that 70% of cervical cancer cases are attributed to HPV-16 & HPV-18, the high-risk subtypes of HPV. Cytology based cervical screening has led to the reduction in the incidence and mortality rates of cervical cancer and evidence suggests that inclusion of HPV testing could further refine the screening programs. Also, HPV testing has enormous potential to be used as a cost-effective primary screening module, to identify women with greater risk of disease progression and as a test of cure of disease [7,12,13].

1.4 Cell Block Technique

Cell block technique is a method wherein the residual material in a sample can be processed to form a tissue block. The process employed can be varied from alcohol, formalin, agarose or thromboplastin to form a cell pellet which is processed like a tissue. The advantages are, multiple sections can be taken and can be used for immunocytochemistry (ICC) to improve the diagnosis of cervical lesions. Cell block can be used as histopathology tissue for controls in cytopathology laboratories. Only limitation is the time required for processing a cell block and the extra cost for the preparation [14].

1.5 Visual Inspection Tests

Visual inspection tests with 3-5% acetic acid (VIA) and/or Lugol's iodine (VILI) appear to be a satisfactory alternative screening approach to cytology. These tests have been used since the 1990s, mainly in poor resource settings. They are simple, cost-effective with relative ease of use and may be performed by different healthcare workers (physicians, nurse, midwives and technicians). Moreover, this approach does not require high technology or infrastructure and has been shown to reduce mortality in developing countries [12,15,16].

2. MATERIALS AND METHODS

This was a prospective study carried out from January 2017 to June 2018 in a tertiary care hospital of South India. The study was conducted after obtaining Institutional Ethical clearance from the committee. A total of 100 subjects within the age group of 20-70 years attending the OBG department with gynecological complaints were recruited for the study. Subjects with a history of abnormal Pap tests were included in the study and subjects who did not give consent to participate were excluded from the study. Samples were collected from the recruited subjects only after obtaining signed informed consent.

2.1 Sample Collection and Processing

Liquid based cervical cytology samples were collected using an endo-cervical cell collection device (Cervex-Brush®-Rovers medical devices). All the 100 samples were subjected to conventional Pap smear testing and manual liquid-based cytology analysis which was subjected to ancillary tests ieHPV testing and cell block processing. Due to the inadequacy of the sample for DNA extraction, only 68 cases were subjected to HPV DNA detection by Polymerase chain reaction. A total of 25 cases of cell block with HPV correlation were subjected to immune-cytochemical analysis with p16 and ki67 markers.

2.2 DNA Extraction & Polymerase Chain Reaction for HPV Detection

Samples are collected using cyto brush was transferred to a sterile capped container and immediately transported to the hospital's molecular laboratory. Samples were centrifuged at 10,000 rpm for 5min to pellet down the cells and the cells were subjected to DNA extraction. DNA from the samples was extracted using HiPuraTM Multi-sample DNA purification kit (HiMedia, India) according to the manufacturer's instructions. PCR was performed using consensus MY09/MY11 primers that targets a 450bp region in L1 gene of the HPV. PCR was performed on Eppendorf's Mastercycler gradient as described in [17]. The positive DNA samples from the PCR was then subjected to type specific PCR with specific primers. Previously described primers described by [18] were utilized for the PCR experiments.

2.3 p16 and ki67 Immunocytochemistry with Cell Blocks

Immunocytochemistry was performed on the formalin-fixed and paraffin-embedded cell block sections by DAB chromogen method. Mouse monoclonal anti-p16 antibody was used. Scoring was done as following: Negative (no staining or <3 positively stained cells), 1+(3-10 positively stained cells), 2+(>10 positively stained cells). Along with the cell number, staining intensity was also taken into consideration. For ki67 only nuclear staining with less than or more than 10 cells were taken as weak or strong positivity.

2.4 Visual Inspection with Acetic Acid (VIA)

We conducted a study with a primary health centre for correlation of VIA with CPS on 100 cases at healthcare camps. The study took into consideration whether the VIA test was adequate or inadequate with visualization of the squamocolumnar junction or not. Pap smears with Ayre's spatula

were taken by trained health workers at these camps. 5% acetoacetic acid was applied to the cervix which was visualized by a Gynecologist or the health worker

3. RESULTS

A total of 68 samples were subjected to HPV detection and genotyping by PCR in our study. Out of 68 cases, 12 were positive for HPV (8.16%) and out of these 12 HPV positive cases 7 were positive for high risk HPV type 16. None of the samples were HPV 18 positive. Since we did not have positive controls for other high risk HPV subtypes, we could not test the status of these samples for other HPV subtypes.

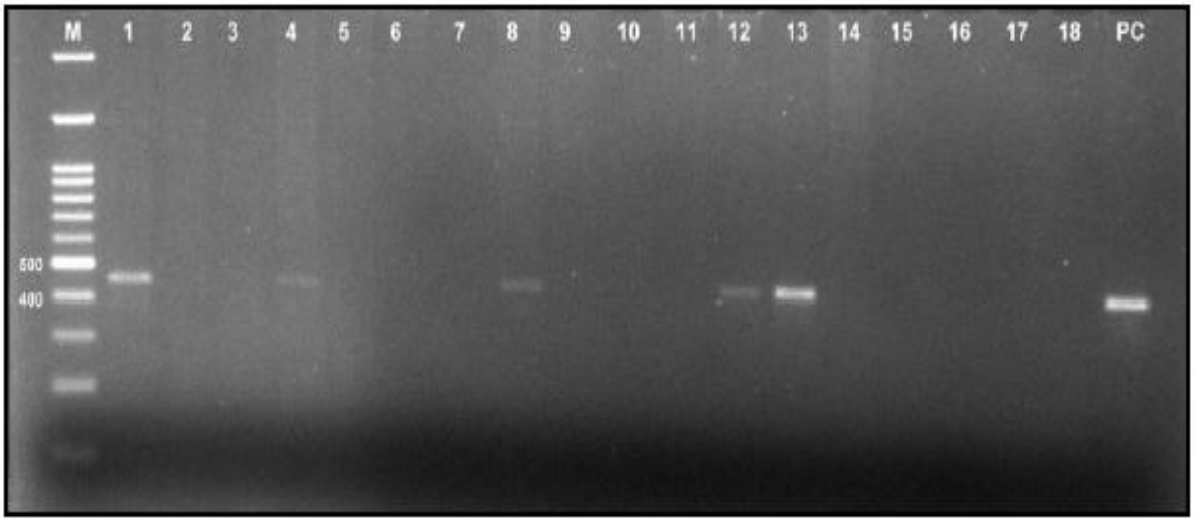

Fig. 1. Representative gel image showing amplified HPV gene product of 450bp (M= Marker, PC= Positive control)

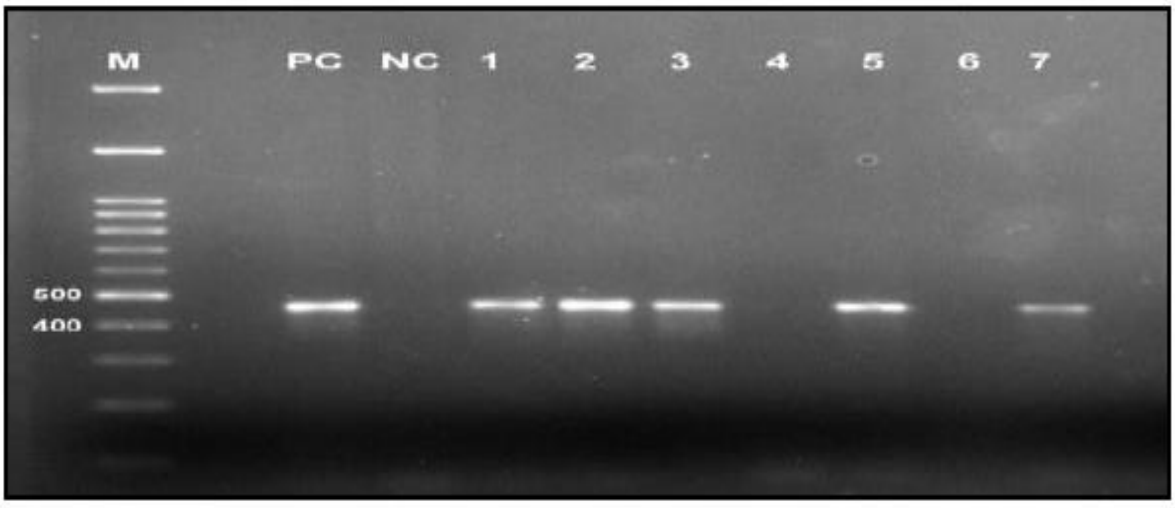

Fig. 2. Representative gel image showing amplified HPV 16 gene product of 468bp (M= Marker, PC= Positive control, NC= Negative control)

Twenty-five cases of cell block with HPV correlation showed 6 cases with HPV positivity. All 25 cases were subjected to p16 ink 4a IHC, of which 18 cases of chronic cervicitis were negative, two cases of koilocytic atypia were negative, one case of LSIL was weak positive, two cases of HSIL were strong positive and two cases of SCC were also strongly positive.

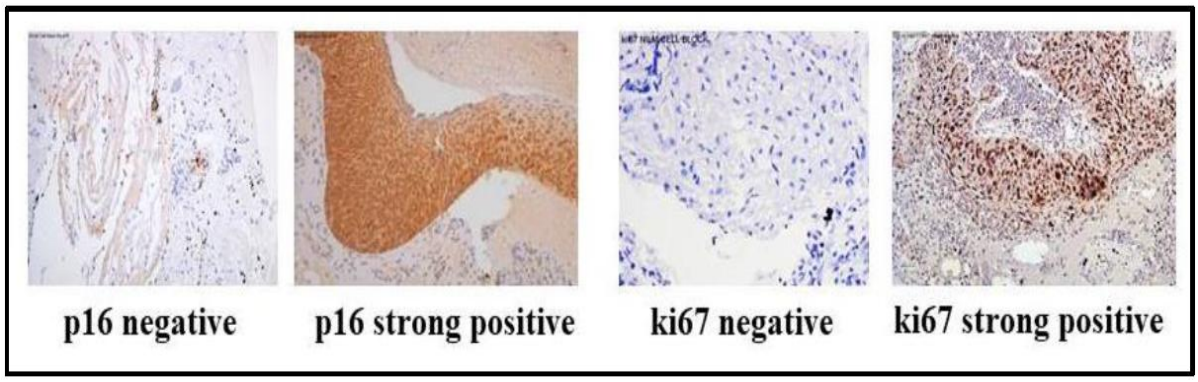

Fig. 3. Representative images of IHC stained for p16 and ki67 immunomarkers

In cell blocks of NILM (negative) and squamous cell carcinoma (positive).

Table 1. Correlation of histopathological characteristics with HPV DNA positivity

	HPV Positive(n=1)		HPV Negative	Total
	Negative HR-HPV (16/18)	Positive HR-HPV (16)		
Chronic Cervicitis	1	0	6	07
LGSIL	3	3	4	10
HGSIL	0	1	6	07
SCC	0	3	2	05
Total	4	7	18	29

Table 2. Correlation of cell block staining with HPV DNA positivity

		HPV	
P16 ON CELL BLOCK		**Positive**	**Negative**
	Positive	5	0
	Negative	1	19

3.1 Statistical Analysis

Correlation of HPV DNA testing with histopathology:

Sensitivity: 45%

Specificity: 85%

PPV: 91%

NPV: 24%

Correlation of Cell block and HPV DNA testing:

Sensitivity: 83.3%

Specificity: 100%

PPV : 100%

NPV: 95%

Table 3. Correlation of histopathology with cell block and conventional Pap smear

CELL BLOCK	CHRONIC CERVICTIS(10)	LSIL (14)	HSIL(12)	SCC(7)
NILM	10(100%)	0	0	0
KOILOCYTIC ATYPIA	0	2(14%))	2(17%)	0
LSIL	0	12(86%)	1(8%)	0
HSIL	0	0	9(75%)	1(14%)
SCC	0	0	0	6(86%)
CPC				
NILM	10(100%)			3(42%)
KOILOCYTIC ATYPIA		2(13%)		
LSIL		8(53%)	10(84%)	1(16%)
HSIL		3(34%)	1(8%)	
SCC			1(8%)	3(42%)

Table 4. Correlation of VIA with conventional Pap smear

Diagnosis	CPS	VIA
NILM	47	35
LSIL	04	01
HSIL	07	01
SCC	12	02
Endo-cervical carcinoma in situ	05	-
ASCUS	06	01
High grade carcinoma	03	01
Unsatisfactory	16	04
Total	100	47

Of the 63 cases, 20 cases are without any correlation wherein 10 cases were VIA negative. Thus, the percentage of missed cases on VIA was 16%.

4. DISCUSSION

Cervical cancer screening and detection has improved from the days of conventional Pap smear screening to molecular tests in developed countries where government national health care policies have initiated screening programs leading to the reduction in the incidence. In developing country like ours, the screening programs have not got its wings due to various reasons. Attempts to make a low-cost method of early detection lead us to start an MLBC technique which reduces obscuring factors and spreads the cells in a monolayer for a clearer viewing of the cells. Similar technique has been used by many investigators as it has additional advantage of ancillary studies like testing for HPV DNA, preparation of cell block for immune-marker studies [11,19,20].

HPV as a primary screening test has been advocated and being followed in European countries it has also found its feasibility in LMIC countries because of availability of many commercially available kits. They have become a milestone in the more effective screening of cervical cancer and prolonging the screening interval for patients.

We have standardized our own in-house HPV DNA testing methodology with a turnaround time of one day. The World Health Organization (WHO) recommends targeting HPVscreening to women who are 30 years of age and older because of their higher risk of CC, and that priority should be given to screening women aged 30-49 years (WHO screening recommendation update 2014) [7].

4.1 Triage of HPV-Positive Women

HPV-based screening has a low positive predictive value for CC because it does not directly test forcancer, but for HPV infection instead. At the present time, three test methods can potentially be

used as triage test: visual methods (VIA/VILLI); cytology; and molecular testing. To date, there is no clear evidence to determine which strategy should be prioritized. Therefore, the choice of test essentially depends on the available resource [16].

4.2 Triage with Cytology

Cytology is the most widely recommended test to triage HPV-positive women, where quality-assured cytology is available. HPV-positive women with a cytology diagnosis of ASCUS or worse are referred for colposcopy, and the rest are advised to have repeat HPV testing after 1 year. Cytology performs better in a triaging scenario, since the prevalence of disease is high in the sample and cytologists have a limited number of specimens to evaluate. The current recommendations by the American Society for Colposcopy and Cervical Pathology (ASCCP) are direct referral to colposcopy for HPV 16/18 positive women and repeat testing after 1 year for women positive for other HPV types [21].

4.3 Triaging with Biomarkers

LBC with immunocytochemistry and cell block sections with immunohistochemistry result in enhanced specimen quality, and accurate diagnosis, and diminished false negative cases. LBC has potential as a screening tool for cancer and precancerous lesions in several tissues other than gynecologic organs.

Cell block tissues made from remnants and residual LBC samples, aspirates, and fluid samples may also have applications for practice in the field of cytopathology. We have used a cost effective method by using MLBC in our set up to be used for HPV and cell block with p16 and ki67 as immune markers [22]. These markers are known to highlight the HSIL and squamous cell carcinoma cases of cervix. p16 can also diagnose LSIL cases even though it also gives positivity for endometrial cell tubal metaplasia and squamous metaplasia which will not be given positive by ki67 [19]. Thus, the use of p16inka4a and ki67 on cell blocks will enhance high grade/malignant lesions of the cervix from the non-neoplastic conditions and thus improve diagnostic accuracy as we found in our study.

4.4 Triaging with VIA

VIA which is a good approach for screening and treating in resource poor settings where cytology and HPV testing cannot be done is useful when the skill and knowledge about the technique is good. We found that it has its limitations as found by many workers [21].

5. CONCLUSION

Cervical cancer screening in low to middle income countries still needs to be refined in terms of affordability and accessibility. Current strategies for cervical cancer screening are not being implemented to its full potential mainly due to the lack of training, high cost and need of well set-up screening centers. There are various methods for screening cervical cancer in LMIC, which are being done in a small scale either in the form of research studies or by NGOs with whom we joined hands and did a study on VIA. In our study we explored the usage of multi-algorithm screening strategy for the screening of cervical cancer in a tertiary care hospital.

There is a need for a uniform policy of screening of women at the primary health care center level with increasing the awareness of the different methods among the public. Also, there is a need for well-trained health workers and Cytopathologists to diagnose and maintain follow up about cervical cancer with a cancer registry.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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Chapter 13
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Study on Meningitis Outbreaks due to *Neisseria meningitidis* in 2010 and 2012 in Burkina Faso

M. Savadogo[1*], D. Yelbeogo[2], S. Zabsonre[3], F. Tarbagdo[2], D. Koussoube[2] and A. Ouedraogo[4]

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ABSTRACT

During the epidemic seasons in 2010 and 2012, epidemic outbreaks were recorded in several health districts in Burkina Faso. Climatic conditions, poverty and poor population immunity make it possible for the emergence of recurrent meningitis outbreaks by Neisseria meningitidis.
Objective: Describing the epidemiology characteristics and response during epidemics of 2010 and 2012.
Patients and Methods: It consisted in a descriptive cross-sectional study which was conducted during epidemics of 2010 and 2012. Data were collected in health districts. Meningitis cases are defined as suspect, probable or confirmed cases according to WHO definitions. The data were entered and analyzed using Epi Info 3 5 1.
Outcomes: Twelve districts had crossed their epidemic threshold in 2010 and thirteen in 2012. The isolated bacteria were dominated by *Neisseria meningitidis* X in 2010 and *Neisseria meningitidis* W in 2012. The average age of patients was 12 years in 2010 and 9 years in 2012. The sex ratio was 1.3 in 2010 and 1.3 in 2012 in favor of the male. The modal age group was that of 5 and 14 years respectively in 2010 and 2012. Three health districts had a vaccine response with uncombined polysaccharide vaccine ACYW.
The appropriate management of cases and children immunity strengthening remain major strategies against meningitis epidemics. It is important to acquire combined tetravalent ACYW vaccine for adapted vaccine response in case of epidemics.

Keywords: Meningitis; epidemics; Neisseria meningitides; Burkina Faso.

1. INTRODUCTION

Burkina Faso is located in the middle of West Africa, within the band expanding from the Red Sea to the Atlantic, described in 1963 by Lapeyssonnie as the African meningitis belt. In this area are regularly recorded epidemic outbreaks occurring periodically. In Burkina Faso these epidemics are a real public health issue [1]. Climatic conditions, poverty and poor population immunity make it possible for the emergence of recurrent meningitis outbreaks by *Neisseria meningitidis*. *Neisseria meningitidis* serogroup W has been associated for several decades with sporadic cases and variable amplor epidemics. Between January and May 1992, Burkina Faso experienced the first large-scale epidemic caused by *Neisseria meningitidis* W, with more than 13,000 cases, including 1,500 deaths [2]. *Neisseria meningitidis* of serogroup A was formerly responsible for most outbreaks [3-5], but vaccination of the population in 2010, with the new combined antimeningococcal A vaccine (MenAfriVac) significantly reduced this form of meningitis in favor of other serogroups of *Neisseria meningitidis*. Different vaccination strategies, such as changing the infant vaccination schedule or extending vaccine coverage to older children and adults, are needed, in addition to stronger

[1]*Department of Infectious Diseases, Yalgado Ouédraogo University Hospital, Burkina Faso.*
[2]*Direction de la Protection de la Santé de la Population, Ministère de la Santé du Burkina Faso, Burkina Faso.*
[3]*Neurosurgery Department, Yalgado Ouédraogo University Hospital, Burkina Faso.*
[4]*Psychiatric Service, Yalgado Ouédraogo University Hospital, Burkina Faso.*
**Corresponding author: E-mail: savadoma@gmail.com;*

surveillance [6]. Topaz et al. [7] shows that the importance of carriage studies to track the outbreak-associated strains circulating within the population in order to inform future vaccination strategies and molecular surveillance programmes. We describe the characteristics of the outbreaks which took place in 2010 and 2012 in Burkina Faso.

2. PATIENTS AND METHODS

Data were collected at health districts. Meningitis cases are defined as suspect, probable or confirmed cases [9] corresponding to the inclusion criteria. Weekly attack rates are obtained by the ratio of the number of cases on the district population, and are expressed in number of cases per 100,000 inhabitants. They allow the definition of epidemic thresholds at district level. Thus, a district is on alert when it shows 5-9 cases per 100 000 inhabitants in a week and in epidemics when it showed at least 10 cases per 100 000 inhabitants in a week. Data were entered and analyzed using Epi Info 3 5 1.

3. OUTCOMES

Epidemiological aspects: 12 health districts crossed their epidemic threshold in 2010 and 13 in 2012. The average age was 12 years and 9 years respectively in 2010 and 2012. The sex ratio was 1.3 in 2010; and 1.3 in 2012 in favor of men. The modal age group was that of 5 to 14 years. Fig. 1 shows the cases, deaths and lethality due to meningitis in 2010 and 2012.

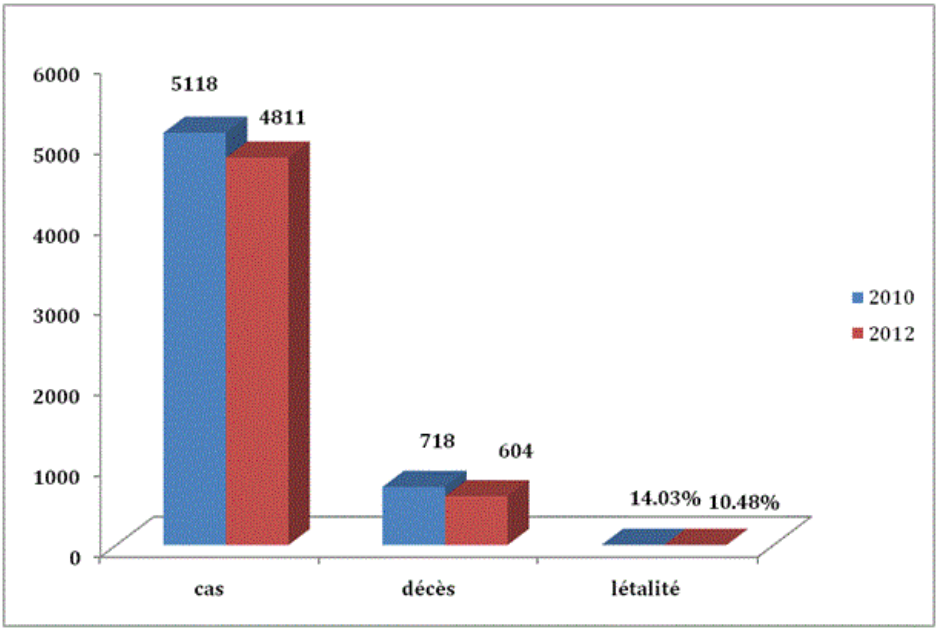

Fig. 1. Distribution of cases and deaths in 2010 and in 2012. *Neisseria meningitidis* X was the dominant organism in 2010. This organism was responsible for epidemiologic outbreaks in 12 health districts out of 63. In 2012, it was *Neisseria meningitidis* W that was responsible for epidemiologic outbreaks in 13 out of the 63 health districts

4. RESPONSE TO EPIDEMICS

Three health districts benefited from a reactive vaccination campaign with the uncombined polysaccaridique vaccine (A, C, Y, W) in 2012. In the absence of vaccine against serogroup X, health officials had suppliedthe health districts withspecific drugs (ceftriaxone and ampicillin, according to national guidelines) allowing free treatment of meningitis cases. Deficiencies in the management were noted such as late diagnosis, a non-compliance with treatment protocols by health workers, deficiency in managing treatment drugs and late consultations of patients.

5. DISCUSSION

Neisseria meningitidis X was for the first time responsible for meningitis epidemics in Burkina Faso in 2010, with 14.03% lethality. In the absence of vaccine against serogroup X, the main strategy used by health authorities was the free treatment of cases and awareness raising for early use of health structures. In 2012, it is *Neisseria meningitidis* W that was responsible for several outbreaks in 13 districts, with lethality of 10.4%. This lethality has not varied much over the years since it was 11.5% in 1992 and 2002 [2]. All epidemics due to *Neisseria meningitides* W had the same difficulty, that was the one related to lack of vaccine containing the W valence for rapid and adapted immunization response as recommended by WHO. As Bertherat [8], we find that the occurrence of outbreaks by *N. meningitides* W, is a challenge in terms of epidemic response due to the poor production capacity of the tetravalent vaccine [8]. In addition, the unconjugated polysaccaridique vaccine (including the quadrivalent A, C, Y, W) does not offer an extended individual immunity. The short duration of immunity makes it possible to limit the spread of the epidemic for one or two transmission seasons, but no more [9]. Moreover, unlike the combined vaccine that controls nasopharyngeal carry [10], the polysaccharide vaccine induces low group immunity, reducing its impact on the spread of the epidemic. The high cost of tetravalent vaccine (A, C, Y, W) and supply problems make it necessary to strengthen the monitoring and improving the management of meningitis cases in high-risk countries such as Burkina Faso [8]. Vigilance will be particularly in districts that have not undergone recent outbreak by *N. meningitidis* W because the population is less immunized there and therefore is more exposed [10,11].

6. CONCLUSION

The emergence of *N. meningitidis* W and *N. meningitidis* X changed the epidemiological situation of meningitis in Burkina Faso. There remains a risk of occurrence of these outbreaks due to the absence of a vaccine against *N. meningitidis* X and allow immunization coverage against *N. meningitides* W. It is important to build the capacity of laboratories and health care facilities for appropriate case treatment.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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Study of Nadi's in Tantric Literature and Their Relation to Neurons

K. V. Mini[1*] and H. H. Awasthi[2]

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ABSTRACT

In the Rig-veda, the word "nadi" signifies "stream." It is important to understand the term "Stream," which underpins the entire Nadi concept. In other terms, every channel through which energy flows is referred to as Nadi. Tantra's artistic side has its own set of rules. It isn't a tradition without rules, nor does it approve of anything dramatic, sensational, novel, or unconventional. Tantra offers a structured and orderly view of art based on an understanding of the universe's occult and spiritual rules.
[1] Yogis made the fewest anatomical correlations on this subject because it was well known at the time that these are the main path or tract through which consciousness travels, or because it was not necessary for them. Tantric and Yogic Gurus both mention the conducting pathways, but they do so in distinct ways. Because they are made up of subtle matter, they cannot be seen with the naked eye, and we are unable to conduct any tests at this level to demonstrate that they are the nerves that we met during the dissection of the human body. So the quest for "Nerves" in the physical body can be clearly identified, but those Nadis or Nerves in the astral body have yet to be identified.

Keywords: Nadis; Tantra; Sushumna; Chakra; Yoga Nadis; Neurons etc.

1. INTRODUCTION

Scientific research has been carried out to verify the existence of the *Nadis*. Dr Hiroshi Motoyama pioneered this research and found stable voltages of electromagnetic currents flowing within close proximity to the nervous system, which he cited as evidence for the existence of *Nadis*. The network of *Nadis* is so subtle and vast that even the yogic texts differ in calculation of their exact number. References in the *Goraksha Samhita* and *Hatha Yoga Pradipika* place the number at 72,000; the *Prapanchasara Tantra* gives the number of 300,000; while the *Shiva Samhita* states that 350,000 *Nadis* emerge from the navel center.

In Yoga, Nadis are channels of Kundalini energy. Many Yogic texts such as Siva Samhita, Gheranda Samhita, Goraksa Samhita, Hatha Yoga Pradipika, Goraksa Paddhati, Sat Chakra Nirupana, Rudrayamal Tantra, Sandilya Upanishad, Dhyanabindu Upanishad, Saradatilaka Tantra etc. have detailed explanation about Nadis. The important Nadis are explained in Siva Samhita Dvitiya Patala. 14 important Nadis are given in Siva Samhita.

The term *Nadi* is the most controversial term in the field of *Ayurveda & Yoga.* It is not an easy subject & cannot be understood by studying Ayurveda texts only. To get into the depth it is necessary to consult texts on *Tantra, Yoga* texts, Acupuncture in addition to Nervous system, Psychology and Ayurveda. *Nadi* means stream in *Rig-veda* (Mac Donnel and Keith) [2]. It is very important to understand this term stream on which the whole concept of *Nadi* stands. In other words we can said that, any channel through which anything substance or energy flows is called *Nadi* and the flow through it is known as *Srotas* [3]

[1]P G Department of Rachana Sharir, Vaidya Yagya Dutt Sharma Ayurved Mahavidyalaya Khurja, Bulandshahr. U P., India.
[2]Department of Rachana Sharir, Faculty of Ayurveda, IMS BHU, Varanasi, U. P., India.
**Corresponding author: E-mail: minikv69@gmail.com;*

*Harihara*n*anda Aranya* (1938) mentiond that *Nadi* means "*Nala*".The (channel) through which anything (energy, substance) flow. This is the reason why nerves, veins, arteries are called *Nadi*. Yogis did least anatomical correlations on this subject as this was known to all at that time as these are the principle path or tract through which consciousness travels or it was not much necessity to them. The conducting pathways are quoted by Tantric as well as Yogic Gurus, but they give this in a different way. According to Professor Guar, the term *Nadi* has been used, commonly as a vessel, cord, meatus, canal or tube & *Vata Nadi* was considered as Nerve.

2. SYNONYMS OF NADIS

According to *Kanada* Synonyms of *Nadi* are given below:

Hinsra, hansi, snayu, dhamani, dhar, tantuki, jivitajnya, jivanjnyana, vasa & sira [4].

2.1 Meanings of Synonyms [5]

Hinsra & *Hansi*: These words are derived from the verb '*Han'* which means to destroy. It also means movement or speed. If the vessels are diseased or do not pulsate normally, life is in danger.

Snayu: This word is derived from the word '*Shna'* which means to bath or to clean. Thus *Snayu* means that which bathes & cleanses various tissues.

Dharani and *Dhara*: Both of these words are derived from verb '*Dhuy'* which means to support or sustain. This *dhamani* or *dhara* means one which supports & sustains life.

Tantuki: This word is derived from '*Tanu'* which means to spread. Thus *Tantuki* indicates the wires spreads all over the body.

Jivitajnya: The word joint means life & "*Jnyana"* means knowledge . Thus it is through the *Nadis* the physician gets the knowledge of life.

Vasa: *Vasa* is the word derived from the verb '*Vas'* meaning to stay or to cover. It covers or encloses the nutrient fluids & helps in their distribution to various organs.

Sira: The word *Sira* is derived the verb" Sra", which indicates movements.

The main function of *Nadi* is to receive and convey the *Prana* in the body from one place to another. In other terms we can say *Nadis* are transport system which travels through their own routes/paths which are present in the body, transmit the *Prana* [6].

2.2 *Nadis in Tantra Grantas*

In *Kundalini Yoga* by Swami Sivananda gives the idea of some important *Nadis* as *Gandhari, Hastijihwa, Kuhu, Saraswati, Pusha, Sankhini, etc*. These have their origin in *Kanda.* All these *Nadis* placed on the sides of *Sushmna*, *Ida* & *Pingala*, & proceed to different parts of the body to perform some special functions. These are all subtle *Nadis*. All the *Nadis* from the *Kanda* which is located in the space between the origin of the reproductive organ and the anus. Innumerable minor *Nadis* spring from these as the leaf of an *Aswatha* tree & is covered with minute fibers. So also this body is permeated with thousands of *Nadi*.

Chakras or *Padmas* are present in the *Linga Sharira* or Astral body –which is made of 17 *Tatwas*-*5Jnanendriyas*, 5 *karmendrias*, 5 *Pranas*, *Manas* & *Buddhi*. These have their corresponding sites in the centers in the spinal cord & the nerve plexus are in gross body. These centers or *Chakras* cannot be seen by the naked eye, but the nerve plexuses at the corresponding sites can be viewed with our naked eye. *Sukshma Prana* moves in the *Nadis* of the *linga sharira*. *Sthoola Prana* moves in the nervous system of the gross physical body. These two courses are intimately connected. We can see

so many plexuses in the *Sthoola* or physical body or physical gross plexuses as interlacing of several nerves, arteries, veins etc. Well known plexuses are, Cervical, Brachial, Lumbar, Hepatic, Cardiac, Epigastric, Pharyngeal, Sacral, Cocygeal etc. These are considered as centers of *Sukshma Prana* in the *Sushmna Nadi* & these are subtle centers of vital energy [7].

Nadis are those cord like structures through which, the traditional Indian Medicine & spiritual science, the energies of the subtle body flows. According to the *Tantras* there are 72,000 more such cords or channels or their networks are present through which the stimuli flow like an electric current from one point to another. *Nadis* are thought to carry a life force energy, known as *Prana* in Sanskrit. The word *Nadi* comes from the Sanskrit root "*Nad*" meaning Flow, Channel, Stream. The rhythmical breathing and special breathing techniques are supposed to influence the flow of these *Nadis* or energetic currents.

Showing the total number of *Nadis* mentioned in different texts.

S. No.	Name of the texts	Total number of *Nadis* mentioned
1	*Prasnopanishad*	720,000,000
2	*Gautamiya Tantram*	35,000,000
3	*Rudrayamala Tantram*	35,000,000
4	*Siva Samhita*	3,50,000
5	*Prapancasara Tantram*	3,00,000
6	*Yajnavalkya Smriti*	72,000
7	*Bhutasuddhi Tantram*	72,000

(Awasthi H.H., Ph.D.(Ay). Thesis, Study on neuro-physio-anatomy in Ayurveda w.s.r. VAta Dosha, page no. 85)

Those main *Nadis* are *Sushmna*, *Ida* & *Pingala*, known as "*Trinadis*".The *Nadis* are stated the conduits of *Prana*. Through them, the solar& lunar currents flow. They are therefore vital forces of life. *Sushmna Nadi* is situated within the spinal column, in the spinal canal. Within the *Sushmna Nadi* there is the *Nadi* by name *Vajra*. *Chitra Nadi*, a minute channel which is also called *Brahma Nadi* is with in this *Vajra Nadi*. *Kundalini* awakens & passes through this *Chitra Nadi*. These are all *Sukshma* centers and we cannot have any laboratory tests & test- tube experiments to identify these .Without these subtle centers, the gross physical body cannot exist & function [8].

2.3 *Yoga Nadis*

According to *Yogis*, *Nadis* are the astral tubes made up of astral matter that carry psychic currents. The Sanskrit term "*Nadi*" comes from the root *"Nad*", which means motion or sound. It is through these *Nadis* that the vital force or *Pranic* current moves or flows. Since they are made up of subtle matter they cannot be seen by naked eyes & we cannot make any test at this level to show scientifically these are those nerves which we met when we do the dissection of Human body. So the quest of "**Nerves"** in the physical body can be clearly identified, but those *Nadis* or Nerves in the astral body is yet to be identified.

The gross **Nerves & Plexuses & Tracts** have close relationship with the subtle body. So we can understand that the physical centers have close relationship with astral centers, the vibrations that are produced in the physical centers by Yogic methods have desired effect in the astral centers.

The interlacing of several arteries, veins are called plexuses. For example pampiniform plexuses of veins, plexuses of arteries are also seen in the body. Likewise there are plexuses or centers of vital forces in the *Susuhmna Nadi* . They are known as *Padmas*, (lotuses) or *Chakras*. Detailed information on all these *Chakras* gives us an idea of those *Nadis* present in them as the 50 Sanskrit Syllable and they are the *Yoga Nadis*.

2.4 *Khanda*

All the *Nadis* spring from the *Kanda*. It is in the junction where the *Sushmna Nadi* is connected with the *Muladhara Chakra*. This is 12 inches above the anus. It is like the shape of an egg & is covered

with membranes. This is just above the *Muladhara Chakra*. The four petals of the *Muladhara Chakra* are on the sides of this *Kanda* and the junction is called *Granthi-Sthana*, where the influence of *Maya* is very strong. In some *Upanishads* you will find that *Kanda* is of 9 digits above the genitals.

Kanda is the center of the astral body from where the subtle channels arise and carry the *Sukshma* Prana (vital energy) to the different parts of the body. Corresponding to this part is the Cauda equina in the gross physical body. The astral center of cauda equine is the *Kanda*. The spinal cord extending from brain to the end of the vertebral column tapers into a bunch of nerves. This bunch of nerves is Cauda equina in the gross body [9].

2.5 Some Important Nadis

Ten principal *Nadis* are enumerated in *Trisikhi- Brahmanyopanishada*, *Yogachudamaniyupanishad* and in several treatises on sphygmology. Only nine *Nadis* were enumerated in the first chapter of *Vaidya Sastra*. Only eight *Nadis* were enumerated in two treatises, *Kalajanana Nadi-Pariksha* and *Nadi-Pariksha*. *Nadi Sastra Samgraha* enumerated twenty *Nadis*, *Gadasanjeevani Nadivijnana* enumerated sixteen *Nadis*, *Brahmavaivarta purana* (*Brahma Kanda*) enumerated sixteen *Nadis*, *Siva Samhita* enumerated fourteen *Nadis*, *Nadi Sastra Samgraha*, *Nadichakra Vidhi*, *Nadi- Nidana* also enumerated fourteen *Nadis*, *Yogasikhopanishad* enumerated twelve *Nadis*, *Vaidya Sastra* enumerated eleven *Nadis* in total. (Awasthi H.H, Ph.D. (Ay) Thesis, Study on neuro-physio-anatomy in Ayurveda w.s.r.to *Vata Dosa*, page no. 85-86) [10].

2.6 Available Description on Some of the *Nadis*

Alambusha - which connects the mouth and anus.

Candra- *Starting from Left nostril moving to the crown of the headand descending to the base of the spine.*

Citra- One of the *Nadi* emanating from the heart through which the creative energy (*Sakti*) of *Kundalini* passes to reach the *Sahasrara*(crown). {Of the 101 *Nadis* only the *Citra Nadi* splits into two parts at the root of *Sushmna*}. One part of the *Citra* moves within it, extending upwards to the *Randra* (aperture) of *Brahma* at the crown of the head above *Sahasrara Chakra*. This is the gateway to the *Parabrahma* (supreme spirit). The other part of *Citra* moves down to the genital organs for discharge of semen. It is said that at the time of death, *Yogis* and *Saints* consciously leaves through the *Brahmarandhra*. Since the aperture is in the *Karana Sarira* (spiritual or causal body), it cannot be seen or measured, when the *Prana* rises upwards ,via the *Citra*, through the *Chakras* it takes with it *Ojas*(radiance), a creative energy latent in semen. The *Citra* is transformed into the *Brahma Nadi* or *Para Nadi*.

Gandhari- One of the *Nadis* said to be behind the *Ida Nadi*, terminating near the left eye, regulating the function of sight.

Hastijihva – Located in front of the *Ida Nadi* , terminating near the right eye, regulating the function of sight, seeing.

Ida – Starting from left nostril, moving to the crown the head and descending to the base of the spine. In its course it conveys lunar energy and is therefore called *Chandra Nadi*. Its function is cooling, *Tamas*(inertia).

Kausiki- One of the *Nadi* terminating in the big toes.

Kuhu- One of the *Nadi* located in front of *Sushmna*, its function is to evacuate feces.

Kurma – Subsidiary *Nadi* whose function is to stabilize the body and mind.

Payasvini- One of the *Nadi* terminating in the right big toe, said to be located between the *Pusha* which is behind the *Pingala Nadi* and the *Sarasvati* (behind *Sushmna*.)

Pingala- (tawny or reddish) starting at the right nostril moving to the crown and down the spine to the base. As the solar energy flows through it, it is also called *Surya Nadi* . Its function is burning,(*Rajas*), action.

Pusa- *Nadi* situated behind *Pingala*, terminating at the right ear. Function is hearing.

Rakta- *Nadi* creates hunger and thirst and collects mucus at the sinuses.

Sankhini- Terminates at the genital organs, situated between *Gandhari* and *Sarasvati*. It carries the essence of food.

Sarasvati- *Nadi* which is behind the *Sushmna Nadi*, terminating at the tongue, controlling speech and keeping the abdominal organs free from diseases.

Soma- The *Nadi* which is very much related to *Ida Nadi*.

Sura- *Nadi* which lies between the eye brows.

Surya- The *Nadi* of the sun as functions as *Pingala Nadi*.

Sushmna- Starting from the base of the spine to the crown of the head, up the center of the spine. Its function is *Agni*,fire (*Satva*), illumination.

Varuni- The *Nadi* which flows throughout the body. Its function is to evacuate urine. Its position is between *Yasasvini* and *kuhu*.

Vijnana- The *Nadi* is the vessel of consciousness.

Visvodari- *Nadi* having the function of absorption of food. Its position is between *Hastijihva* and *Kuhu*.

Yasasvini- *Nadi* situated between the left ear and the left big toe. (before *Pingala* and between *Gandhari* and *Sarasvati*).

In addition to the various primary and minor *Nadis*, the *Sakta Tantra* and *Kundali*ni/*LayaYoga* traditions emphasis was placed on central *Nadi* which represented concentric (hence increasingly subtle)channels along or in front of the spine and along which are sprung the 7 *Chakras*. The four central *Nadis* are *Sushmna Nadi*, *Vajra Nadi*, *Citra Nadi* and *Brahma Nadi* [11].

3. DISCUSSION

Nadi – Literally means a river, a channel or passageway; the pulse; there are innumerable *Nadis* in the human body, from the very subtle to the very gross, carrying substances into, out of, or throughout the body. (Lad, M.A.Sc, Vasant, 2002)

Rivers: Rivers carry not only liquid water, but also some solids, specifically, suspended solids (sediments) and dissolved solids (mostly salts). Here we focus on the dissolved solids. **The natural function of rivers is to carry these solids to the ocean.** To put it in simple terms, the first and foremost role of rivers is to export the solids produced by the watershed into the ocean.

3.1 Neurons

When we look on to the functions of Neurons they are carrying the sodium, potassium, calcium from the CSF to the different parts to make you energetic. The neuron utilizes a process known as axonal

transport for moving vesicles and other organelles to regions remote from the neuronal cell body. Proteins called molecular motors make use of the energy released by hydrolysis of ATP to drive axonal transport. Thus the neuron has evolved unique mechanisms to establish and maintain the form required for its specialized signaling functions. Neurons, like other cells, exhibit a voltage difference across their plasma membranes. Rapid changes in this trans- membrane voltage, called action potentials, can be propagated from one part of the cell to another and are used by neurons (but not by most other cells) to encode information. There are membrane ion channels, a ubiquitous class of highly specialized membrane proteins that have evolved to provide exquisite control over the movement of ions across the plasma membrane [12].

4. CONCLUSION

The structure we met with dissection along with the Vessels (Arteries,Veins)are termed as Nerves according to Modern Science. What is the name given to those structures in Ayurveda? The answer to this question is being answered with different names. *Tantrika, Nadi, Dhamani, Sira, Vasa, Tantuki, Jivitajnya* etc. To clear the concept of *Nadi*, we want to search The *Puranas*, *Upanishads*, *Yoga Granthas*, *Tantra Granthas* etc. To understand these *Granthas*, first of all we want to understand Saktism and Saivism, in which Lord Siva clears the doubts of Parvati Devi about those diseases prevailed at that time. As this was a secret medicine, the knowledge did not flourish among common people. The knowledge spreads among Yogis and by doing the Yoga in proper way they attain a higher energy level which gives them all types of Satisfaction.

So the Yogis found energy flowing inner path as ***Nadis***. They explained the *Yama, Niyama, Asana, Pranayama, Pratyahara, Pranidhana, Dhyana and Samathi,* The *Ashtangas* of *Yoga* by *Patanjali* for the fruitful growth of body and mind in *Yoga Granthas*. So the Tantric literature deals with the *Yoga* and the way of living happily with a great energy level which they feel through the structure called ***Yoga Nadis***. The important *Nadis* are *Sushmna*, *Ida* and *Pingala*. The total number of *Nadis* given in *Siva Samhita* was 3,50,000 [13].

The main function of Nadi is to receive and convey the Prana in the body from one place to another. Nad's are subtle cylindrical cords that branch out from centers in the physical and astral bodies and transmit psychic currents or impulses to different areas.

A short History of Aryan Medical science describes the wind diseases of the Hindus are mostly treated by the western writers as diseases of the Respiratory system. The bile diseases are generally corresponding with diseases of the Circulatory system. The disorders of phlegm as the diseases of Alimentary system. The Demonical diseases of the Hindus are but other words for Hysteria, E Macdonell A.A and Keith A.B, Vedic index, Hindi Translation by Rati Rama Kumara, the Caukhambha Vidyabhavana, Varanasi,1962.epilepsy, Dancing Mania and other disorders as the disorders of ***Nervous system*** [14].

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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Biography of author(s)

K. V. Mini
P G Department of Rachana Sharir, Vaidya Yagya Dutt Sharma Ayurved Mahavidyalaya Khurja, Bulandshahr. U P., India.

She is a traditional Marma Practitioner from Nangelil Ayurveda Marma Chikitsalaya, Thodupuzha , Kerala State, India, under Vd. Dileepkumar. She is working as Associate professor in PG Department of Rachana sharir, Vaidya Yagya Dutt sharma Ayurved Mahavidyalaya Khurja, Bulandshahr, U P, India. She has a Ph.D in Rachana Sharir From Faculty Of Ayurveda, Banaras Hindu University, Varanasi U P under the Guidance of Professor Hari Hridya Awasthi. She is Teaching U G and PG students. Throughout her carrier she has been involved in teaching students to perform dissection to make the structures of our body Clear and to show them how each and every organ is related and the importance of this type of relationship in day today life. She gives students the idea behind the normal way of presentation of our body parts and to live the life normally. She has presented papers in various National and International Seminars, and published the work in reputed journals. She is also the member of Nangelil Ayurveda Medical college, Kothamangalam , Ernakulam, Kerala, India. She is interested to do more work on the effect of Yoga Asanas and Pranayama to Maintain the Health of the Body to control Prana Through the Nadis (Swasthasya Swastyarakshanam).

H. H. Awasthi
Department of Rachana Sharir, Faculty of Ayurveda, IMS BHU, Varanasi, U. P., India.

He is an eminent speaker, a great motivator, who guided more than 13 MD and 7 Ph. D scholars. He is working in the Department of Rachana Sharir, Faculty of Ayurveda, Institute of medical Sciences, Banaras Hindu University, Varanasi, Uttar Pradesh, India. He has teaching experience of (UG) 25 years and (PG) 17years. He has BAMS, MD, and Ph.D degrees from reputed organization. He Published and Presented more than 100 National and 25 International Papers in reputed journals and in seminars. He also authored and edited 6 Books.

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https://docplayer.net/59633566-Nadis-in-tantric-literature.html.

Chapter 15
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Study about Artificial Intelligence: An Approach towards Robots and Philosophy in Surgery

Jaime Hinzpeter[1*]

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ABSTRACT

In terms of AI (Artificial Intelligence), so much progress has been made in two years ... The first thing that comes to mind is mathematician Alan Turing who, when philosophizing about a problem that pretended not to attend a part, concluded with: Writing papers that could be the cornerstone of current computer technology [1]. All efforts to equip computer systems with certain capabilities are summarized under the term AI (Artificial Intelligence) [2]. The subject is intriguing and can even terrify you, because of course conflict arises as events and potential dangers loom around the corner. It is not that AI (artificial intelligence) opens a door that is forbidden a priori (if a metaphysical similarity is possible), but rather that it reveals a very fundamental challenge, namely its correct development. The task of infusing an intelligent machine with complicated concepts like justice, kindness, and love is already complicated, but work is already being done on it. What would Turing think of an intelligent machine?

High-performance machines are present in medicine today and will continue to be so. Jesús Moreno, doctor of the Spanish Society for Laparoscopic and Robotic Surgery says: "In 10 years, robots will be used more often in the operating room." 4 The updated history of robotic surgery [3] begins with the PUMA 560 (° r), which has developed into 2 prototypes, PROBOT (° r) and ROBODOC (° r) for prostatectomy or trauma surgery. The AESOP, an endoscopic system for abdominal surgery, is primarily a robotic arm that carries a laparoscopic camera. Towards the end of the century, the ZEUS (° R) robot appeared and expanded its range of application to include the integration of urology. The Zeus robot is a large 3-armed robot; a left and a right arm simulate the surgeon's arms plus a third arm controlled by an AESOP voice control. This is how the DA VINCI (° r) robotic surgery system was created, consisting of 3 elements: a visualization trolley, the surgeon's console (arms with 7 sliding areas and a computer system with 3D images) and the mobile car (with more arms). This robot was developed by the SRI (Standford Research Institute), the first copy of which came on the market in 1997 [4] and was visibly approved by the FDA. Perhaps its downside is that it is still a large robot with many connections.

In the 21st century, we've seen gigantic advances in robotic surgery, both in terms of models and sizes.

Also impressive. (I would say downright scary) Or the author Isaac Asimov? Who was still far from the digital age when he published his book I Robot in 1950? Today, the two of them could be witnesses of AI (artificial intelligence) in any of its magnitudes and magnificence, which act as digital assistants not only for mobile phones, but also for social networks such as Facebook, Microsoft or Google [5].

But be careful, Nick Bostrom emphasizes in an interview this year:

If we cannot control it, we could give room to the reality of a super-intelligent system that could prioritize the achievement of its own values at the expense of our own [6]. Two years ago, before this interview, the Swedish philosopher had already said about superintelligence

Keywords: Artificial intelligence; robotic surgery; prototypes.

[1]*Department of Traumatology, Clinical Hospital, University of Chile, Chile.*
**Corresponding author: E-mail: jhinzpeter@hcuch.cl;*

1. INTRODUCTION

Without a doubt, there is a lot of literature on the subject. That much progress has been made in terms of Artificial Intelligence in just a couple of years... as well. If we add numbers, we would say that more progress has been made than in the 50 previous years. Let us propose a name. The first one that comes to mind is mathematician Alan Turing who while philosophizing about a problem that seemed to go nowhere, ended up composed writings that would be the cornerstone of modern computer technology [1]. The Turing test, among others, is worthy of another article in itself. Currently, we find definitions of AI in indexed journals, publications printed on paper, social media and others obtained through search engines. Google Scholar, for example, led me to a definition that I liked and that dates back to 1987. All the efforts made in order to equip the computer systems with certain capacities are grouped under the term artificial intelligence [2]. Then, this brief article lists them: vision, recognition ... speech; human characteristics! The subject is fascinating and may even terrify you because, of course, conflicts arise parallel with developments, and potential risks are just around the corner. It is not that artificial intelligence opens a forbidden door a priori (if I may make a metaphysical analogy) but it poses a very important challenge, which is its proper development. Moreover, in the words of the Swedish philosopher and professor of the U of Oxford Nick Bostrom, there are no opportunities for rehearsals and he adds: "I do not think we have a second chance [7]." This refers to the fact that there is only one opportunity to develop technology aligned with human values and civilization. The task of how to introduce an intelligent machine, complex concepts such as justice, kindness, and love, is already difficult, however, work is already being carried out on that. Many groups of scientists simultaneously collaborate with each other. According to the Swedish philosopher, this refers to the fact that work is being done on scalable control methods. What would Turing think of an intelligent machine? I believe his taciturn visage[1] would have a great smile etched upon it. Let us leave these reflections for a moment and move on to the chronological existence of AI in the medical field.

Today, intelligent machines are very much present in medicine and will continue to be so. Dr. Jesús Moreno, doctor of the Spanish society of laparoscopic surgery and robotics says, “In ten years’ time, robots in the operating room will be even more commonplace.”[4] The modern history of robotic surgery [3] begins with the PUMA 560 (°r) which evolved into two prototypes, PROBOT (°r) and ROBODOC (°r) used for prostatectomy and trauma surgery respectively. In 1994, we arrived at the digestive system field. The AESOP, an endoscopic system for abdominal surgeries, is basically a robotic arm that holds a laparoscopic camera. Near the end of the century, the ZEUS robot (°R) was born, which extended the target to include urology. The Zeus robot is a large robot with three arms; a left arm and a right arm simulate the arms of the surgeon plus a third arm that an AESOP controls by voice. Its main difficulty was its size that made it difficult to use in an operating room. This is how the DA VINCI robotic surgery system (°r) came about, consisting of three components: a visualization cart, the surgeon's console (arms with 7 ranges of movement and a computer system with 3D images) and the mobile cart (with more arms). This robot was developed by the SRI (Standford Research Institute) whose prototype was launched in 1997 [4] and approved, evidently, by the FDA. Its handicap, perhaps, is that it is still a large robot with many interconnections.

During the 21st century, we have witnessed great developments in robotic surgery, both in models and sizes. In 2018, the Nature Journal of Biotechnology published that nanoscale robots will be used to fight cancer [8]. Promising results have been obtained in model animals, such as BAMA pigs, which show greater similarity with humans than rodents [9]. Amazing indeed.

Going back to the restlessness in the initial paragraphs, I ask myself whether machines will end up deciding what needs to be done, and who will do it ... In the near future, will more engineers than surgeonsemerge from the medical profession?

Amazing too. (I would say downright terrifying)

The prologue of the Bioinformatics book by thebiologist Rafael Lahoz-Beltran 2004, states:

The effects of technology on society will extend from the most common tasks such as home chores to commercial, social and political discussions [10].

What would Alan Turing say today? Or the writer Isaac Asimov?, who was far from the digital age when he published his book I Robot in 1950. His literary fiction challenged Turing's tests, but today's reality surpasses his stories. Both would be a witness today to artificial intelligence in all its dimensions and splendor, colonizing the system as digital assistants not only for cell phones, but for social networks such as Facebook, Microsoft, or Google as well [5]. Or also for evaluation and employmentplatforms (AURA system created by telephone).

Maybe there are only a few years left for them to become partners in daily life, algorithms, nanorobots, and virtual pets. Perhaps, as well, for the eradication of diseases such as cancer. But beware, Nick Bostrom in an interview this year emphasizes:

"You are creating something that would be very intelligent (the AI) and could also be very powerful. If we are not able to control it, we could give rise to the existence of a super intelligent system that could prioritize achieving its own values to the detriment of ours [6]. A couple of years ago, before this interview, the Swedish philosopher had already spoken of super intelligence [11,12,13]... and says that something has not been answered yet: "superintelligence is an intellectual who is much smarter than the best human brains..." As a society-I wonder- How smart are we? You be the judge.

2. CONCLUSION

So much progress has been made in terms of AI (artificial intelligence) in just two years...

Jesús Moreno, a doctor at the Spanish Society of Laparoscopic Surgery and Robotics, says: “In 10 years, robots in the operating room will be even more common”. 4 The up-to-date history of robotic surgery [3] begins with the PUMA 560 (°r) which evolved into 2 prototypes, PROBOT (°r) and ROBODOC (°r) used for prostatectomy and trauma surgery respectively. The AESOP, an endoscopic system for abdominal surgery, is primarily a robotic arm that supports a laparoscopic camera. Towards the end of the century, the ZEUS robot (°R) appeared, which expanded the purpose of integrating urology. The Zeus robot is a large 3-arm robot; a left arm and a right arm simulate the surgeon's arms, plus a third arm controlled by an AESOP voice. Thus emerged the DA VINCI robotic surgery system (° r), composed of 3 elements: a viewing cart, the surgeon's console (arms with 7 displacement ranges and a computerized system with 3D images) and the mobile cart (with more arms). Its downside, perhaps, is that it is still a large robot with a lot of interconnections.

In the 21st century, we have witnessed giant advances in robotic surgery, both in models and sizes. Impressive too.

The two could now witness AI (artificial intelligence) in all its magnitude and splendor, colonizing the system as digital assistants not only for cell phones, but also for social networks like Facebook, Microsoft or Google [5].

If we are not able to control it, we can provide space for the life of a super-intelligent system that prioritizes the achievement of its own values over ours [6].

COMPETING INTERESTS

Author has declared that no competing interests exist.

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Biography of author(s)

Jaime Hinzpeter
Department of Traumatology, Clinical Hospital, University of Chile, Chile.

Research and Academic Experience:

- Postgraduate Studies: University training in the specialty of Orthopedics and Traumatology, Training Center University of Chile, Clinical Hospital.
- Studies in Arthroscopic Knee Surgery.
- Sub-Specialty Studies: Training in Shoulder and Knee Surgery, Sports Medicine, Orthopaedic Institute of the Americas.
- Tutor: Phil Davidson M.D.
- Sub-specialty studies, Training in Arthroscopic Hip Surgery, Hospital for Special Surgery.
- Tutor: Bryan Kelly M.D. New York, N.Y, USA.

Research Area:

- Arthroscopic Knee Surgery.
- Shoulder and Knee Surgery, Sports Medicine.
- Arthroscopic Hip.

Number of Published Papers:

- Author of more than 50 research papers as author, among others.
- Medical Area: Traumatology and Orthopaedics
- Specific Med. Area: Sports Traumatology. Arthroscopic knee, hip and shoulder surgery.

Special Award: Award for the best manuscript of the “Sports Health Magazine” A Multidisciplinary Approach, entitled “Effect of Active Versus Passive Recovery on Performance During Intrameet Swimming Competition” that was selected to receive the 2015 Sports Health T. David Sisk Award for Best International Paper.

Any Other Remarkable Points:

1. Member Editorial Board: Journal of Orthopedic Research and Therapy. November 2019. Reviewer Articles for publication
2. Member of the Chilean Society of Physiological Sciences, August 2019.
3. Member Editorial Board: International Journal of Biochemistry & Physiology (IJBP). May 2019, Reviewer Articles for publication.
4. Editorial Team Member: EC Clinical and Experimental Anatomy, March 2019, Reviewer Articles for publication
5. Member of the Editorial Committee of the Journal “International Journal of Biochemistry and Physiology” since March 2019. Reviewer Articles for publication.
6. Member of the Editorial Committee of the Journal "EC Clinical and Experimental Anatomy" since March 2019. Reviewer Articles for publication.
7. Member of the Editorial Committee of the Journal "International Physiology Journal" since August 2018. Reviewer Articles for publication.
8. Member of the Editorial Committee of the “KSSTA” Journal, since April 2017. Reviewer Articles for publication.
9. Member of the Editorial Committee “Global Journal of Health Science, Canadian Journal, since June 2016. Reviewer Research Articles.
10. SOCHMEDEP Member. Chilean Society of Sports Medicine 2013.
11. Member of the International Society for Arthroscopic Hip Surgery ISHA, since 2012.
12. Member of the Colombian Society of Orthopaedic Surgery and Traumatology, since 2012.
13. Member of the Latin American Society of Arthroscopic Surgery SLARD, since 2011.
14. Active member of the Chilean Society of Traumatology and Orthopedics, since 2011. SCHOT.
15. Member of ISAKOS, since 2010.
16. Member of the Editorial Committee of the Journal "Archives of the Chilean Society of Sports Medicine" Years 2007 - 2008 -2009- 2010- 2011.

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Mobile Genetic Elements in the Human *MGMT* Gene and their Regulatory Potential

O. V. Pidpala[1] and L. L. Lukash[1*]

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ABSTRACT

Mobile genetic elements (MGEs) make up a large part of the DNA of eukaryotes, in particular, almost 45 % of the human genome. Numerous data show the diverse role of these elements in the genome - from plasticity factors to mutability or stability. They discuss their role in the evolution of genomes and the evolution of gene regulation. The purpose of this work was to investigate the distribution of MGEs in human *MGMT* gene and their regulatory potential. It is shown that in the human *MGMT* gene MGEs are present both in the intron sequences and in the promoter region. In the intron sequences, MGEs form composite cluster structures that are the source of various regulatory sequences and have the potential to form alternative promoters. In the promoter region, three sequences of MGEs were identified: two LTR-repeats and a fragment of the DNA-transposon. The MGEs fragments in the promoter region of human *MGMT* also enriched with potential cis-regulatory sequences that may be involved in the regulation of this gene.

Keywords: Human MGMT gene; mobile genetic elements (MGEs); composite cluster structures; regulatory elements; promoter; alternative promoters.

1. INTRODUCTION

The eukaryotic genome is a complex and dynamic structure. About 50 % of the human genome, and possibly more [1,2], is covered by mobile genetic elements (MGEs), which for a long time were considered unnecessary ballast. Studies of recent years convincingly testify to the important role of these elements in the evolution of genomes [3-8] and in the evolution of gene regulation [9-15]. They are not only mutagenic factors [16-18], but they can also be the source of a variety of regulatory sequences, such as sites of alternative splicing [19,20], cis-regulatory modules, which are clusters of binding sites of transcription factors [21-23], or play the role of alternative promoters [24]. Also they are vital source of different genome innovation [25-28] and subject to epigenetic silencing by histone modifications and DNA methylation [29-31]. MGEs tends to integrate into non-coding genome sites (introns, flanked genes and intergenic sites) [32]. On average, in the human introns, MGEs are approximately 89.5%; in exons, they account for slightly more than 10%, in particular about 4% for protein-encoding genes [33]. Up to 20% of the genes contain MGEs in non-translated regions of mRNA, where they can affect the regulation of gene expressions, in particular, the MGEs in 5'UTR affects the initiation of translation [34]. It is known that about 25% of human genes contain MGEs in promoter regions, and today there is convincing evidence of their involvement, in particular promoters of retro-elements, in the regulation of gene transcriptional activity [21,33]. There are cases where MGEs plays the role of alternative promoters, which leads either to increase the level of expression of the corresponding gene, or to change the tissue specificity of its expression [35].

Human tumor suppressor *MGMT* gene encodes a reparative enzyme called O6-methylguanine-DNA methyltransferase, which removes alkylated groups from the O6 position of guanine in DNA and protects cells from their toxic and mutagenic effects [36,37]. Expression of this gene and the activity of the enzyme themselves have wide limits both in-between and intrinsically-individual variations,

[1]*Institute of Molecular Biology and Genetics, National Academy of Sciences of Ukraine, Ukraine.*
**Corresponding author: E-mail: lukash.imbg@gmail.com;*

indicating that its regulation is complicated [38]. Given the complexity of this problem, it might be worthwhile to focus on MGEs, the role of which in gene regulation has recently been discussed quite widely. Numerous databases today have enormous material on the availability of mobile elements, but the data array is not characterized and not generalized. Perhaps the MGEs of this gene is also a source of a variety of regulatory sequences, which led us to focus our attention on the study of this issue.

2. MATERIALS AND METHODS

The nucleotide sequence of the *MGMT* gene is taken on the Ensembl site. Data on the promoter region of the gene were obtained from GeneBank (X61657), about the potential promotor regions of the gene being studied - from the AceView database. The results of the search and identification of MGEs are done with the CENSOR program. The homology between the sequences studied was determined by the BLAST 2.2.32 program. Functional sites are defined by TFSEARCH: Searching Transcription Factor Binding Sites (ver 1.3). The search for potential regulatory sequences was performed by SITECON, SiteGA, BLASTN 2.2.26 using the TRRD database resources (Transcription Regulatory Regions Database) and Cister: Cis-element Cluster Finder, WWW Signal Scan and Tfsitescan. These methods and databases were used in the relevant sections of the article.

3. RESULTS AND DISCUSSION

3.1 Distribution of MGEs in the Gene of Human MGMT Reparative Enzyme

Human *MGMT* gene is localized on telomeric region of chromosome 10 at position 10q26 and consists of one non-coding and four coding exons and four introns.

Mobile genetic elements in human *MGMT* gene are present in all intron sequences and absent in exons (Fig. 1). Most of their fragments were detected in the intron 3 (44.74%), and the smallest in the intro 4 (8.26%). In most of the introns, non-LTR retrotransposons predominate among MGEs classes (in particular LINE-elements). The share of endogenous retroviruses as well as DNA-transposons is insignificant in all introns (Table 1).

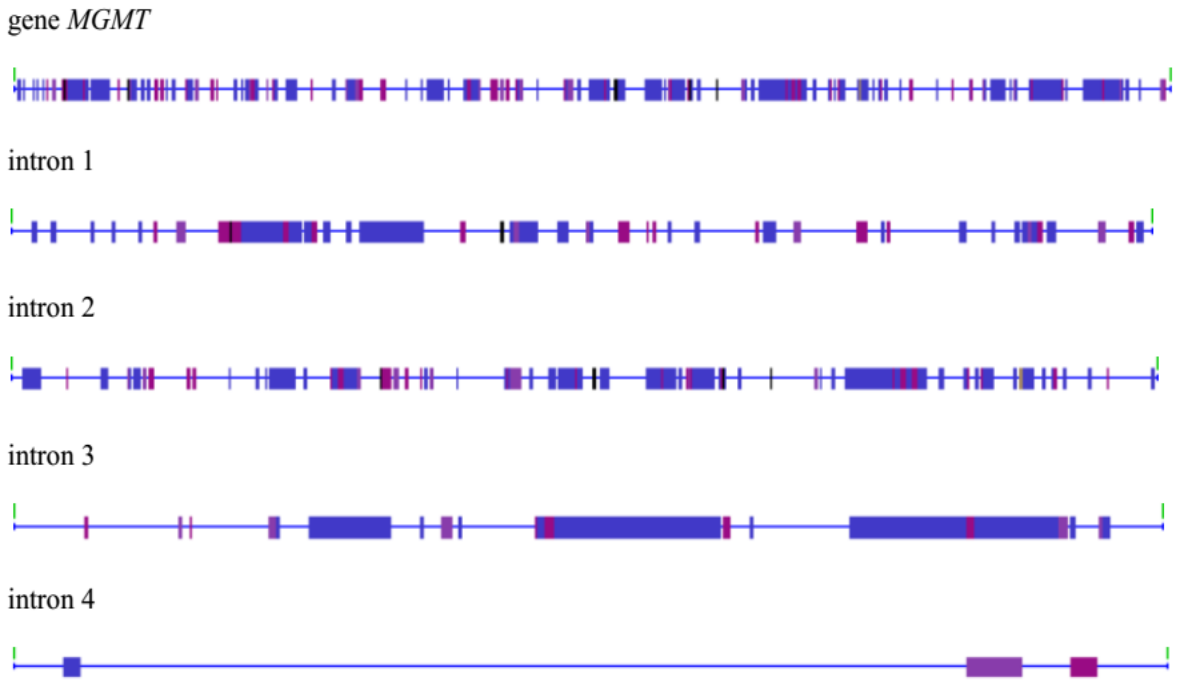

Fig. 1. MGEs in the introns of the human *MGMT* gene

Table 1. The proportion of MGEs in the intron sequences of the human *MGMT* gene

Intron number (bp)	% MGEs, (bp)				
	DNA-transposons	Endogenous retroviruses	LTR retrotrans-posons	Non-LTR retrotransposons	In total
intron 1 (68944)	6.05 (4169)	2.36 (1625)	-	19.26 (13281)	27.67 (19075)
intron 2 (171517)	4.14 (7102)	2.19 (3762)	0.24 (409)	20.75 (35590)	27.32 (46863)
intron 3 (51158)	2.26 (1158)	2.63 (1345)	-	39.85 (20388)	44.74 (22891)
intron 4 (7446)	2.25 (167)	4.71 (351)	-	1.3 (97)	8.26 (615)
gene (300824)	4.21 (12676)	2.35 (7083)	0.24 (409)	23.03 (69275)	29.73 (89443)

Clusters of MGEs were found in the intron sequences of the studied gene. Thus, in intrusion 2 there are three compositional clusters, which, in addition to LINE-elements, include representatives of other classes (Table 2). Intron 3 also revealed three clusters of LINE-elements, one of which is composite (Table 3).

Table 2. MGEs clusters in the intron 2 of the human *MGMT* gene

Coordinates within the intron	MGEs	Length, bp	Class / family	Chain	Number cluster
103739-104018	AluSg	280	NonLTR/SINE/SINE1	-	1
104019-104712	L1ME_ORF2	694	NonLTR/L1	+	1
104713-105077	L1ME_ORF2	365	NonLTR/L1	+	1
125707-125739	MuDRF-2_MLP	33	DNA/MuDR	+	2
125742-125938	L1MC4_5end	211	NonLTR/L1	+	2
125941-126057	MER81	115	DNA/hAT	-	2
126058-126151	L1MC4_5end	101	NonLTR/L1	+	2
150912-151320	MER61E	414	LTR	-	3
151322-152319	L1HS	1005	NonLTR/L1	-	3
152320-152398	L1-2_Cja	79	NonLTR/L1	+	3

It is known that MGEs tends to cluster in intragenic sites of genes and introns [39]. Among the MGEs, clustering tends to Alu-repeats and suggests that these clusters are involved in chromosomal rearrangements [40]. In the case of the *MGMT* gene of the individual, all identified clusters include fragments of LINE1-elements alone or in combination with representatives of other classes of MGEs (Tables 2, 3).

Table 3. MGEs clusters in the intron 3 of the human *MGMT* gene

Coordinates within the intron	MGEs	Length, bp	Class /family	Chain	Number cluster
13431-14056	L1MEC_5	626	NonLTR/L1	+	1
14060-14677	L1ME_ORF2	618	NonLTR/L1	+	1
14681-14963	AluSz6	283	NonLTR/SINE/SINE1	+	1
24109-24489	L1PA6	378	NonLTR/L1	-	2
24490-30537	L1HS	6049	NonLTR/L1	-	2
30538-30654	L1PA6	120	NonLTR/L1	-	2
30655-31384	L1-2_Cja	733	NonLTR/L1	+	2
42743-42794	L1MC1_EC	52	NonLTR/L1	-	3
42795-45806	L1MB3_EC	3223	NonLTR/L1	-	3
45807-45933	L1MEf_5end	140	NonLTR/L1	-	3
45941-46419	L1MD1_5	476	NonLTR/L1	-	3

Recently, the literature actively discusses the role of MGEs in regulation of genetic activity. For retrotransposons, in particular for LINE-elements located in intron sequences, it has been shown that

they can interfere with the transcription of the gene, causing exonization, introns content, crypt polyadenylation [41], or act as a "downtrodden policeman" by regulating the passage of RNA polymerase II [42]. A "gene breakdown" hypothesis is also proposed, according to which the LINE1-element, which is located in the intron in the opposite direction of transcription, can "break" the transcript into two parts [43].

Consequently, in the human *MGMT* gene the MGEs are present in the intron sequences. In two introns, they form composite cluster structures that can be the source of a variety of regulatory sequences and potentially affect its expression.

3.2 Composite Cluster Structures of MGEs in the Introns of the Investigated Gene as a Source of Potential Regulatory Sequences

Of the six clusters we identified, within the introns of the human *MGMT* gene, for the further analysis, those that, in addition to the fragments of LINE-elements, also contain Alu-repeats. Such compositional clusters revealed two of them, one in the intron 2 (Table 2, cluster number 1) and another in the 3rd one (Table 3, cluster number 1). The length of the first cluster is 1339 bp. In addition to the full-length AluSg-repeater, which is in the opposite direction to the transcription, there are fragments of two LINE-elements. Within the AluSg-repeat, homology with binding sites for four transcription factors, namely YY1, p300, C / EBP and for transcription activator of HSF2 heat shock genes, has been detected.

Interestingly, the analysis of the consensus sequence of Alu-repeats showed that it contains conservative sites that the YYI transcription factor [44] can theoretically bind to. It is known that there are a large number of consensus sites in the human genome to bind the transcription factor YY1, and some of them (24 %) are localized in Alu-repeats. It is noted that about 50 % of Alu- repeats contain a potential site for binding YY1 [44]. This allowed to propose the assumption about the role of the Alu-repeats in the activation and in the suppression of transcription. The assumption is indirectly confirmed by the data on the ability of the YY1 protein to bind to regulatory regions of the gene and thereby increase or suppress transcription [45]. The YY1 property to attract other proteins to the site of its binding to DNA also can affect the activity of the genome. For example, YY1, specifically binding to the hRPD3 protein, which detects histone deacetylase activity, may initiate complexation in certain regions of the genome of the complex that translates chromatin into an inactive state [46]. Such interaction can lead to changes in the level of transcription. In addition, YY1 protein may interact with ADPRT protein, which has ADP-ribosyl transferase activity [47]. It is shown that YY1 protein, interacting with the protein ADPRT, stimulates its auto-ribosylation [48]. On the basis of these data, the mechanism by which the negative regulation of transcription with the participation of Alu-repeats can be carried out is proposed. The YY1 protein binds to the Alu-repeat, "attracts" the ADPRT protein and initiates its auto ribozylation. In a certain region of the gene there is a complex that sterically opposes the formation of the complex of transcription initiation. According to the authors in a similar way, the regulation of genes that are under the control of hormone-associated elements can be carried out. This will reveal data on the interaction of the ADPRT protein with the TR/ RXR complex, which complicates the binding of nuclear receptors to the complex of initiation [49].

In the consensual sequence of Alu-repeats, a block of AGGTCA with which the transcription factors that belong to the family of nuclear hormonal receptors can be identified. The bulk (more than 70 %) of the potential hormone-associated elements for the thyroid hormone, retinoic acid, and estrogens is located exactly in the Alu-repeats. CV-1 cells show that binding to nuclear receptors, the Alu-Associated DR-4 element can regulate transcription activity depending on the presence of the thyroid gland hormones [44]. Proteins of a large family of nuclear hormonal receptors can bind not only to the AGGTCA block but also to the various variants that arise due to its duplication. It is suggested that the Alu-repeats are the "containers" which contain sets of potential sequences for the binding of various transcription factors [50,51].

Within the fragments of the LINE-elements that are part of the composite cluster structure within the intron 2 *MGMT* gene, homology with binding sites for 22 transcription factors was detected. Among the number of identified potential sites, I would like to highlight the sites of heat shock protein binding

(HSF2), C/EBP, SRY, STAT, Oct, GATA and AP-1. In addition, TATA box and glucocorticoid receptor (GR) and RORalpha1 (orphan hormone nuclear receptor) binding sites have been identified. This is particularly interesting as previously the presence of sites for glucocorticoid receptor binding within Alu-repeats [44] has been shown. It has also been found that hormone-acceptor elements for the thyroid hormone, estrogen and retinoic acid are mainly localized in Alu-repeats [44].

The presence of TATA box can be a prerequisite for the existence of an alternative promoter in the human *MGMT* gene within the intron 2. It is worth noting that in addition to the internal promoter, L1 contains an antisence promoter (ASP) [52]. The analysis of the database of the expressed gene sequences of the man revealed 49 chimeric transcripts, which begin in LPAA and are part of the mRNA of known genes [53]. In 45 cases, the direction of transcription from the ASP and the promoter of the gene coincided, and the four ASP activity led to the formation of the corresponding complementary RNA. It is assumed that the L1-compatible ASP gene may serve as an alternative promoter and may either lead to the appearance of a chimeric mRNA that is translated to form the same protein (in the case of L1, "topically" relative to the point of transcription), or to the formation of 5'-truncated mRNAs , the translation of which leads to the appearance of various N-terminal forms of protein (in the case of the location of L1 in the intron of the gene). In addition, the ASP activity deserves special attention to be aligned with the L1 gene located in the intron, since in this case, chimeric RNAs that contain sequences complementary to the exons of the gene that potentially can regulate the activity of the corresponding gene through the mechanism of RNA interference are formed.

It is worth mentioning also the "gene-breaking" hypothesis, according to which L1, located in the introns of the gene in the opposite direction of tracing the orientation gene, can "break" the transcript into two parts: 1 - the RNA, which covers "above lying" exon and ends in the antisense site polyadenylation L1 and 2 - a transcript consisting of an ASP L1 and encompassing "non-existent" exons [54].

The second composite cluster we investigated is located within the intron 3 (Table 3, cluster number 1). It has a length of 1527 bp and consists of two fragments of LINE-elements and a full-length AluSz6-repeat. The transcription direction of all the constituent parts of the cluster composite structure in this case coincides with the direction of transcription of the gene. Interestingly, as in the case of the AluSg-repeat from cluster 1 in intron 2, the AluSz6-repeat also contains an element of response to the HSF2 heat shock proteins. Within the fragments of LINE-elements among other potential sites, as in the previous cluster, the homology with binding sites for C/EBP, SRY, STAT, Oct, GATA and AP-1, TATA box and sites for binding to RORalpha1 (orphan hormone nuclear receptor).

As can be seen from the results presented in Table 4, for some transcription factors, sequences that are homologous to their binding sites are present in retroelements belonging to different families. There are those that are present only in the sequence of fragments of LINE- elements, including the TATA box and the potential hormone-associated element for the retinoid orphan receptor RORalpha1 (orphan hormone nuclear receptor).

Thus, having analyzed the two compositional cluster structures in the intron 2 and intron 3 the human *MGMT* gene, which include Alu-repeats and fragments of LINE-elements, it was found that both Alu-repeats contain sequences that are homologous to the response elements to the heat shock proteins, and cluster structures within the fragments of LINE-elements have TATA box sequences. This gives grounds for considering analyzed composite cluster structures of MGEs within the intron sequences of human *MGMT* gene as potential alternative promoters.

3.3 Motives of Regulatory Sequences in the Promoter Regions of the MGMT Gene within the MGEs

The gene promoted gene promoter (X61657) has a length of 1157 bp and covers exon 1 part intron 1. It is devoid of TATA or CAAT sequences, contains CG-rich regions and is structurally reminiscent of genes of the household. It also contains SP1, AP-1 and AP-2, NF-kapB sites, two elements that bind

the glucocorticoid receptor (GRE) and a 59-bp size element, which is located on the first eccentric-intron boundary, necessary for effective transcription of the reporter designs [55,56].

Table 4. Representation of potential sites of binding in boundaries of composite cluster structures of MGEs in the human *MGMT* gene introns

Trans-cription factor, name	Ingredients of composite clusters					
	intron 2 (cluster 1)			intron 3 (cluster 1)		
	AluSg	L1ME_ORF2	L1ME_ORF2	L1MEC_5	L1ME_ORF2	AluSz6
C/EBP	+	+	+	+	+	
Oct-1		+	+	+	+	+
SRY		+	+	+	+	+
GATA		+	+	+	+	+
HSF2	+	+	+			+
AP-1		+		+		+
Pbx-1		+		+	+	
MZF1		+	+	+		
TATA		+			+	
STAT			+		+	
RORalp		+			+	

Fragments of MGEs in the promoter region of the investigated gene. By analyzing the promoter region of the human *MGMT* gene, sequences of two fragments of LTR repeats of mammalian retroviruses were identified, namely, LTR-repeats of endogenous retroviruses ERV3 MLT1C2 and MLT1C, which are located in the distal part of the promoter sequence (Table 5) and are about 23 % of the total length of the promoter. It should be noted that the described response elements for glycocorticoid hormones (GRE) with coordinates 28-42 and 63-77 [55] are localized within one of the fragments of the LTR-repeat, namely, MLT1C2.

In addition, a sequence of the fragment of the Mutator-like non-autonomous DNA transposon of SETARIA1 was found (Table 5). Interestingly, the minimum promoter (886-955) and the sequence of SP1 of the site (862-867) [55] are within the given sequence SETARIA1. Thus, in the case of the human *MGMT* gene, the promoter region contains fragments of three MGEs, which is almost half of its length.

Table 5. Fragments of MGEs in the promoter region of human *MGMT* gene

Referential promoter, symbolic	Data on MGEs				
	Element	Class	Length	Direction	Coordinates within promotor
hO^6P	MLT1C2	ERV/ERV3	117	c	-949/-839
	MLT1C	ERV/ERV3	147	c	-813/-667
	SETARIA1	DNA	341	d	-265/+76

Notes: c - complementary; d - straight

It is known that up to 25 % of genes contain MGEs in promoter regions [21]. Especially enriched by MGEs genes which are associated with metabolic processes [57]. For human *DNAseII* and *CAML* genes, it has been shown that the presence of Alu-repeats in the promoter may affect the expression of these genes [58]. It is known that the human gene *MSLN* (Mesothelin, a megakaryocyte-potentiating factor) has two promoters, one of which is formed by the sequence of LTR, and the other is a MIR element (tRNA-like SINE) [34]. The only currently known promoter of the *BAAT* gene, specifically expressed in the liver and involved in the development of an hereditary disease associated with bile metabolism, is also the sequence of LTR-repeats [59]. An interesting case of regulation of the transcription of the *NAIP* gene encoding one of the inhibitors of apoptosis is interesting. It has been shown that the promoter portions of this human gene and rodents do not have homology, but in one and the other, LTR are alternative promoters [60]. In humans, the integration of LTR has led to the formation of a tissue-specific promoter that is active mainly in the testicles,

whereas in rodents two LTRs are described that are capable of initiating transcription. One of them is the main, constitutive promoter, active in all rodents, and the other - an alternative, found only in the mouse. It is important to note that LTRs of humans and rodents that are in the promoter region of the *NAIP* gene are not related. This case is an example of the independent involvement of LTR in regulating the transcription of orthologic genes [60].

Potential cis-elements within the fragments of MGEs in the promoter regions of the investigated gene. Among the potential cis-regulatory sequences within the LTR-repeats, an element of response to the HSF2 heat shock proteins, binding sites for several representatives of the GATA family of proteins (important regulators specification and differentiation of various tissues), MZF1 (involved in the control of cell proliferation and carcinogenesis), NF-kappaB (participation in the activation of transcription of numerous cytokine and immunoregulatory genes), AML-1 (regulation of hematopoiesis, angiogenesis and neurogenesis), C/EBP (control of the differentiation of various cell types and a key role in regulating cellular proliferation through interaction with cell cycle proteins), CRE-BP (an important role in the development and functioning of the nervous system), Nkx-2.5 (the regulation of the expression of tissue-specific genes, the development of the heart, the time and spatial development models), CDP (regulates gene expression, morphogenesis and differentiation, the role in the progression of the cell cycle, especially in the S-phase) and the potential hormone-associated element for the retinoid orphan R receptor RORalpha1 (orphan hormone nuclear receptor) (Fig. 2).

As part of the sequence of the DNA fragment of the transposition of SETARIA1 (Fig. 2), apart from the numerous potential binding sites for the Sp1 factor (one of the major transcription factors involved in the regulation of the cell cycle, chromatin structure changes and DNA methylation regulation), the motives for recognition for transcription factors GATA-2 (hematopoietic transcription factor), c-Rel (participation in immune and inflammatory reactions, developmental processes, cell growth and apoptosis) and USF (one of the key regulatory elements of gene expression).

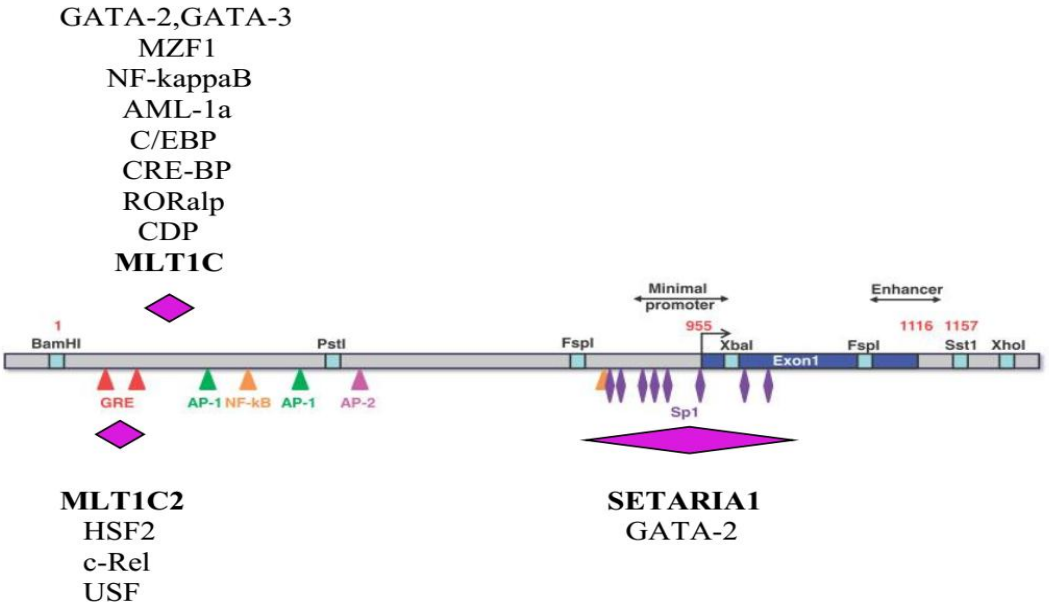

Fig. 2. Potential cis-regulatory elements in the MGEs fragments identified within the sequence of the refracted promoter of the human *MGMT* gene

Recently, not only for LTR elements, called "regulatory information packages" [61], but also for other MGEs, the presence of numerous regulatory sequences in their structure has been shown. Integrating in the promoter regions of the genes, MGEs can be the source of cis- regulatory modules, which are clusters of transcription factor binding sites [21,57,62]. In particular, in the consensus Alu-repetition sequence, there were found binding sites for 20 transcription factors, the functional activity of most of which has been proved experimentally [57]. In addition, Alu-repeats identified functional binding sites for retinoic acid receptors [63,64] and hormone-acceptor elements [65,66]. As already noted, the bulk

of the potential hormone-associated elements for the thyroid hormone, retinoic acid and estrogens is located exactly in the Alu-repeats [44].

The direct involvement of Alu-repeats in expression regulation is shown for genes that are associated with differentiation and development, namely for *PTH*, *FcεRI-γ*, *CD8α*, *CHRNA3*, *BRCA-1* and *PLOD-1* genes [67]. In Alu-repeats, sites of transcription factor binding have been identified which are involved in hematopoiesis, T-cell differentiation, and the development of various organs (eyes, teeth, heart, lungs, brain) [57], which is another evidence of Alu-repeats participation in ontogenesis.

Thus, in the referenced promoter of the *MGMT* gene of the human, three sequences have been identified that are homologous to the MGEs fragments, namely two LTR-repeats and a fragment of the DNA-transposon. The fact that one of the LTR-repeats contains the previously described response elements for glycocorticoid hormones (GRE) and that the known minimal promoter and sequence of the SP1 site are located within the fragment of the DNA-transposon, confirms the important role of MGEs in gene regulation. Also, the identified MGEs fragments in the promoter region of human *MGMT* enriched with potential cis-regulatory sequences that may be involved in the regulation of this gene.

3.4 Fragments of the MGEs as Components of Potential Alternative Promoters

In addition to the referenced promoter, the AceView database contains information on eight potential promotor sites (length of 2.000 bp) for the gene in question. For four, there is information that these sequences may contain a promoter (aAug10, cAug10, eAug10, iAug10). According to BLAST, the analysis of potential promotor regions of the human *MGMT* gene revealed that of the eight potential promoter regions, three sequences (cAug10, hAug10 and iAug10) had reflex sequence homology and no five homology sequences. In particular, the dAug10 sequence is localized within the intron 1. Four other sequences (aAug10, eAug10, fAug10, and gAug10) are located within the intron 2.

Fragments of the MGEs in alternative promoter regions of the investigated gene. It has been shown that four of the eight potential promotor regions studied contain fragments of MGEs (Table 6, Fig. 3). It is interesting that these are the exact sequences that may include the promoter, as indicated in the AceView database. Therefore, these sequences were analyzed in detail. Two sequences of possible alternative promoters (cAug10 and iAug10) overlapping with the referenced sequence (hO6P) contain additional fragments of MGEs - MIRc (NonLTR-retrotransposon) and MER117 (DNA-transposon), whereas the sequences of two fragments of LTR-repeats of mammalian retroviruses, namely the LTR-repeat of the MaLR retrovirus-like element (MLT1C) and the LTR-repeat of the endogenous retrovirus ERV3 (MLT1C2) identified by the CENSOR program as one fragment of the endogenous retrovirus due to the larger size of the nucleotide sequence. This feature of the program should be taken into account in further research. In the sequences aAug10 and fAug10, located within the intron 2, fragments of MGEs of different families were identified (Fig. 3). Particular attention deserves two sequences: Tigger15a (DNA-transposon) and AluSx1 (NonLTR-Retrotransposon), which are specific to MGEs for mammals and primates.

3.5 Potential Regulatory Sequences within Identified MGEs Fragments

All of the MGEs fragments we investigated in the alternative human *MGMT* gene promoters are enriched in a variety of regulatory sequences (Table 7). In particular, the AluSx1-repeat contains a number of new, besides the described [57,63-66], transcription factors binding sites, as well as tissue-specific promoter, enhancer, and seylener sequences (Table 7). It is known that Alu-repeats can affect cellular processes by providing new transcription termination sites or splice sites or acting as alternative promoters [16,19,21,62]. Such situations may interfere with the normal functioning of the gene, but may sometimes lead to the formation of proteins with new functions [68]. DNA transposons are also enriched by regulatory sequences [69]. The fragment of the Tigger15a element we examined contains binding sites for a number of transcription factors (ERE, HRE, MEF-2 and C/EBP), as well as promoter, enhancer, and seylerange sequences and the locus control region (Locus Control Region-like region).

Table 6. Sequence of MGEs in potential alternative promoter regions of the human *MGMT* gene

Symbolic designation for the promoter	Data on MGEs				
	Element	**Class**	**Length**	**Direction**	**Coordinates within promoter**
aAug10	Tx1-11_Crp	NonLTR/Tx1	60	c	320-379
	Tigger15a	DNA/Mariner	105	c	448-552
	Gypsy-54_PIT-I	LTR/Gypsy	54	d	1278-1331
	DNA4-8_CGi	DNA	104	d	1425-1528
cAug10	MIRc	NonLTR/SINE/SINE2	53	c	115-167
	MER117	DNA/hAT	120	d	202-321
	MLT1C2	ERV/ERV3	397	c	771-1167
	SETARIA1	DNA	341	d	1576-1916
fAug10	ERV-1_Crp-I	ERV	38	c	19-56
	AluSx1	NonLTR/SINE/SINE1	285	c	374-658
	Gypsy-57_GR-I	LTR/Gypsy	44	c	1777-1820
iAug10	MIRc	NonLTR/SINE/SINE2	53	c	208-260
	MER117	DNA/hAT	120	d	295-414
	MLT1C2	ERV/ERV3	397	c	864-1260
	SETARIA1	DNA	314	d	1685-1998

Notes: c - complementary; d - straight.=

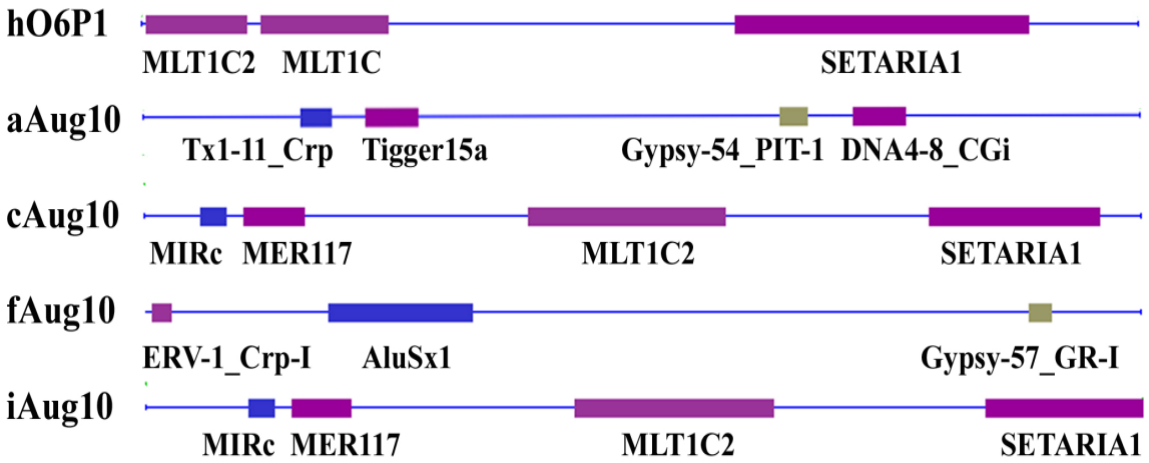

Fig. 3. Fragments of MGEs in a referenced human *MGMT* promoter (hO6P1) and in its potential promoter sequences (aAug10, cAug10, fAug10, iAug10)

The human genome is not only specific to mammals, primates or humans, or their fragments or copies, but also fragments of MGEs of other organisms: animals, plants and even bacteria [70-73]. Such sequences are also enriched in a variety of regulatory elements and can affect the transcriptional activity of the human *MGMT* reparative gene. It is worth noting that the presence of TATA box within the MGE of the potential promoter region of fAug10 (indicated in bold in Table 7) may be a prerequisite for the existence of an alternative promoter.

Thus, analyzing the nucleotide sequences of the potential alternative promotor regions of the human *MGMT* gene from the AceView database revealed that the two sequences (cAug10 and iAug10) are overlapped with the reflux promoter and the other two sequences (aAug10 and fAug10) are located within the intron 2. All of them contain in its composition fragments of MGEs that are enriched in a variety of regulatory sequences and can affect the regulation of the transcriptional activity of the human *MGMT* gene.

Table 7. Regulatory potential sequences of MGEs, inherent in the genome of the man, within the potential promoters of the human *MGMT* gene

Conditional designation of the promoter	Mobile genetic elements		Regulatory sequences	
	Name	**Species affiliation**	**Linking sites for transcription factors**	**Regulatory elements**
aAug10	Tigger15a	Mammalia	ERE; HRE; MEF-2; C/EBP	promoter; enhancer; silencer; Locus Control Region-like region
fAug10	AluSx1	Primates	**TATA box**; YY1; GATA; SF1;Sp1;CAAT box; OCT1; SREBP; PAX2; NF-kappaB; PBX2; SOX9; SRY; WT1; AP-2; C/EBP; P53;CRE; CREB; MEF-2; nCaRE;TRalpha; RARalpha/RXR; IFN-stimulated response element	promoter; myeloid-specific promoter; erythroid-specific promoter; enhancer; lymphocyte-specific enhancer; H_2O_2-inducible enhancer; non-renal silencer cytokine responsive element; Ets-responsive region PMA and cAMP responsive region

There is no doubt that MGEs can affect the expression of eukaryotic genes. First and foremost, these processes involve their promoters and their associated regulatory sequences. A global analysis of retrotransposon expression in the human genome revealed ~ 275.000 TSSs (transcriptional start sites), which is ~ 31 % of all known TSSs human genomes, although their level of activity is significantly lower than that of conventional genes. Transcription of sequences of MGEs also affects the transcript of encoding proteins [74]. It is shown that 576 promoters of human retrotransposons or their fragments are used as alternatives to the transcription of known genes. Also described are cases of enhancers and MGEs infectious agents in the transcriptional networks of human genomes, animals and plants [75]. Thus, the regulatory potential of MGEs are enormous, since they are not only a source of regulatory innovations, but can also participate in the epigenetic regulation of the genome [76]. In addition, an imbalance in the management of MGEs, which leads to genomic instability, can lead to the development of malignant neoplasms [77]. All these facts indicate the important role of MGEs in ontogenesis and phylogeny of eukaryotes, but their global significance still needs confirmation [78].

4. CONCLUSION

In the human *MGMT* gene, MGEs are present both in the intron sequences and in the promoter region. In the intron sequences, MGEs form composite cluster structures that are the source of various regulatory sequences and have the potential to form alternative promoters. In the promoter region, three sequences of MGEs were identified: two LTR-repeats and a fragment of the DNA-transposon. The fact that one of the LTR-repeats contains the described response elements of the glycocorticoid hormones (GRE) and that the minimal promoter and sequence of the SP1 site are located within the fragment of DNA-transposon confirms the important role of MGEs in gene regulation. In addition, the MGEs fragments in the promoter region of human *MGMT* enriched with potential cis regulatory sequences that may also be involved in the regulation of this gene.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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Skincare Products – A Risky Path

Atefa Noorain[1*]

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ABSTRACT

Skin is the largest organ of the human integumentary system, that interacts with the environment and role in protecting the body from pathogens and toxic chemicals. It comes into touch with the environment directly or indirectly in daily life from many sources such as skincare products available in the markets. In this work, we look at a list of a few chemicals that have been shown to treat skin disorders but also cause serious acute diseases. Most skin care products contain sodium lauryl sulphate, diethanolamine, nanoparticles (zinc oxide, titanium oxide), monoethanolamine, triethanolamine, propylene glycol, and butylene glycol, which have both beneficial and harmful effects. The main objective of this study is to give clarity about the skincare products that involve certain chemicals which have been proven to be harmful to the human body.

Keywords: Skincare products; Sodium lauryl sulphate; diethanolamine; nanoparticle, monoethanolamine; triethanolamine, propylene glycol; butylenes glycol.

1. INTRODUCTION

Human skin is the largest organ of the body is often taken for granted. Approximately 15-20% of the total body weight is comprised of skin. According to the researchers, 6 million cells are present in each square centimetre.

Skin is known to be the most sensitive part of the human body. According to the researchers, everything that's applied to the skin passes through a layer stratum corneum. People generally use a wide variety of cosmetic products like moisturizers, face creams, lotions, lipsticks without knowing the sensitivity of their skin and how these products will harm them. All these cosmetic products have certain allergens present in them which become the main cause of allergy. To name a few of these harmful chemicals that act as allergens: Acrylamine, Ethylene Oxide, Phthalates and Formaldehyde. They are the things that cause an allergic reaction in the human body.

It is no doubt to say that beauty means good health. But some of the harmful synthetic cosmetics containing various chemicals affect the body health and the beauty of many persons who are hypersensitive to those things.

The study aims to differentiate between the useful & toxic chemicals present in various skincare products & how the human skin reacts to those chemicals when comes in contact.

2. SODIUM LAURYL SULPHATE (SLS) [1]

One of those compounds that are formed when the sulphuric acid reacts with certain other chemicals is sodium lauryl sulphate (SLS). SLS is known to be produced from plant sources like palm kernel oil or coconut oils and petroleum. It is one of the essential ingredients in cleansing products that are used to create lather which in turn gives an effective impression of cleaning power to the general public.

[1]Thomson Reuters, India.
**Corresponding author: E-mail: atefanuri@gmail.com;*

2.1 Products containing SLS

Face wash, body wash, shampoo, bubble bath, bar soap, moisturizers, dish soap, toothpaste & mouth wash.

2.2 SLS Chemistry

SLS exert its effect on proteins by forming a chemical bridge between the fat-soluble & water-soluble parts of the protein molecule. This disrupts the hydrophobic forces needed to maintain protein structure & the molecule collapses, rendering it useless. This effect is usually reversible.

- The result of this is 2-fold, firstly existing proteins are damaged leading to an increase in the amount of healing required by the body. Secondly, new proteins can be damaged & cells disrupted while they are under construction. It is exactly this type of activity that can lead to the early stages of skin cancer.
- The process can be severe, that skin layers may separate & inflame due to its protein denaturing property.
- Carcinogenic nitrates can form in the manufacturing of SLS or by inter-reaction with other nitrogen-bearing ingredients within a formulation utilizing this ingredient.
- A single shampooing can produce more cancer-causing nitrates in the body than eating a pound of bacon, which is very high in nitrates.

2.3 Harmful Effects of SLS

- It is a highly toxic synthetic substance
- It can lead to health issues ranging from eczema to canker sores.
- It causes inflammation of the skin & can weaken the immune system.
- Known to causes intestinal toxicity, urinary tract infection, bladder & kidney infection, genital disorder, eye irritation, skin rashes, hair loss & allergic reactions.
- It is used to violate the skin's natural barrier function in a lab test.
- SLS is known to cause stronger reactions when certain allergens and irritants come in contact with the human body.
- One study showed 41.8% of 160 people showed an irritant reaction after exposure to 0.5% SLS.

3. DIETHANOLAMINE (DEA) [2]

Generally when ethylene oxide reacts with ammonia three products are formed in a crude mixture namely: ethanolamine, triethanolamine and diethanolamine. Diethanolamine is mainly used in cosmetic products to give a creamy texture and nice consistency to skincare products.

3.1 Products Containing DEA

Moisturizers, sunscreen, hair conditioners, shampoos, dishwashing detergents and soaps.

3.2 DEA Chemistry

Diethanolamine is not used in its original form in skin care products as it is combined with certain other chemicals resulting in the formation of a completely new ingredient (for ex: Cocamide diethanolamine) that is not identical to DEA chemically. Usually, these chemical reactions result in the formation of a new substance that is chemically stable does not easily be separated.

3.3 Harmful Effects of DEA

- It has been linked with kidney, liver & other organ damage according to research done.

One study found that topical application of DEA in rodents resulted in anaemia, kidney degeneration & nerve damage

- to the brain & spinal cord.
- It is readily absorbed through the skin & has been linked with stomach, oesophagus, liver & bladder cancer.
- Around 200 million pounds of DEA is produced annually in the U.S most of which goes to personal care products.
- It can react with other ingredients in cosmetic formulas to form an extremely potent carcinogen called NDEA (nitrosodiethanol amine).

3.4 Cosmetic Ingredients Contaminated with DEA

- Cocamide DEA
- DEA lauryl sulphate
- Lauramide DEA
- Linoleamide DEA
- Oleamide DEA

4. MONOETHANOLAMINE (MEA) [3]

When aqueous ammonia reacts with ethylene oxide it results in the formation of monoethanolamine (MEA) along with other products like triethanolamine and diethanolamine. The production ratio of these products can be controlled by the stoichiometry of the reactants.

4.1 Products Containing MEA

Soaps, lotions, shampoos, hair conditioners, shaving creams, eye shadows, blush and household cleaning products.

4.2 MEA Chemistry

Apart from sharing few reaction pathways with other ethanolamines, it is also one of the most commonly studied alkanolamines. MEA is known to be bi-functional as it contains both primary alcohol and primary amines.

4.3 Harmful effects of MEA

- It is a corrosive liquid, harmful if swallowed.
- It can cause skin burns & can lead to corneal burns with possible permanent damage & CNS depression.
- Ingestion may result in irritation of the mouth, respiratory tract & digestive tract & may cause drowsiness & dizziness.
- It is a potential skin sensitizer.
- Inhalation of MEA may cause asthma-like symptoms.

5. TRIETHANOLAMINE (TEA)

Triethanolamine is one of those compounds which is a tertiary amine and a triol both. TEA sample generally appears yellow but in reality, it is a colourless liquid and the yellow colour appears because of the impurities in the sample.

5.1 Products Containing TEA

Eyeliner, mascara, eye shadow, blushers, make-up bases & foundations, as well as fragrances, hair care products, wave sets, shaving products, sunscreen & skin cleansing products.

5.2 TEA Chemistry

Triethanolamine is one of those compounds that have the potential to convert itself into a carcinogen N-nitrosodiethanolamine. Triethanolamine can also acts as an antioxidant against the auto-oxidation process of vegetable and animal fat.

5.3 Harmful Effects of TEA

- TEA is an amine, an organic compound derived from ammonia which is used in low concentrations as an alkalizing agent to increase the pH of cosmetic formulations.
- It is a possible human carcinogen.
- It is used to adjust the pH balance, but toxic & causes eye problems, & dryness of skin & hair.
- TEA in sunscreen occasionally causes contact allergies like dermatitis.
- TEA causes an increased incidence of tumour growth in the liver via a choline-depletion mode of action & that this effect is likely caused by inhibition of choline uptake by cells in female B6C3F1 mice, but not in Fischer 344 rats.

6. PROPYLENE GLYCOL (PG) [4]

Propylene glycol is one of the ingredients which are colourless and is added to skincare products to elevate moisture retention.

6.1 Products Containing PG

Shampoo, deodorant, detergent, styling mousse, mascara, soap, skin cream, baby powder, conditioner toner, aftershave, baby wipes.

- It is also found in tyre sealant rubber cleaner,de-icer, stain remover, fabric softener, degreaser, paints, adhesive.

6.2 PG Chemistry

- It is a chemical made by the reaction of propylene oxide with water.
- It is chemically neutral
- It is a cosmetic form of mineral oil found in automatic brake & hydraulic fluid.

6.3 Harmful Effects of PG

- It is a petroleum by-product & is a synthetic ingredient used as a humectant that causes retention of moisture content of skin or cosmetic products by preventing the escape of moisture & water.
- It is used as an industrial anti-freeze to de-ice aeroplanes.
- It easily penetrates skin can weaken protein & cellular structure.
- PG is a solvent that dissolves through a stainless steel tank in 48 hours.
- PG is so toxic that it requires workers to wear protective gloves, clothing & goggles.
- It acts as an immune toxicant that causes many allergic reactions & can cause liver abnormality & kidney damage.
- It is found to cause skeletal muscular damage in rats & rabbits.
- It is a known irritant & sensitizer causing dryness, erythema & even blistering.

- It directly alters the cell membrane to cause thickening of skin, skin dehydration & chronic damage to the skin.
- Patch test helps to diagnose the toxicity of PG.

7. BUTYLENE GLYCOL (BTG) [5]

- It is used as solvents & viscosity decreasing agents in cosmetics & personal care products.
- It is been proven to be gentle & non-irritating to the skin, even in higher concentrations.

7.1 Products Containing BTG

Shampoo, conditioner, moisturizer, foundation, cleanser, eye cream & sunscreen.

7.2 BTG Chemistry

It is an organic molecule with 2 alcohol groups, used in cosmetics as a humectant to bind moisture & hold water to the skin.

8. TITANIUM DIOXIDE (TIO2)

- It is one of the commonly used ingredients in sun protection creams.
- It has the characteristics that make it act as a physical sunscreen that reflects UV light before it causes any damage to the skin.
- It reduces the risk of skin cancer.
- It cannot penetrate through the normal skin.
- It can lead to detrimental cellular effects only if the skin is lacerated or exposed to micronized TiO2.
- It also acts as a mild skin irritant.
- It is listed as a safe pigment in skincare products.

9. CONCLUSION

Thus in the summary of the paper, we get a clear picture of how different chemicals can act as Friend or Foe to the human body.The 2 chemicals Titanium dioxide & Butylene glycol are proved to be helpful to the human skin without any severe side effects.
Hence in future, the detailed composition of the skin products needs to be checked before commencement of its use. Future research can be done taking particular skin products and testing their quality and beneficial effects on the human body.

COMPETING INTERESTS

Author has declared that no competing interests exist.

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Biography of author(s)

Dr. Atefa Noorain
Thomson Reuters, India.

She is a former Head of the Department of Life Science and has also served as an Assistant Professor for Microbiology. She had been felicitated as the Youngest Speaker at an international conference and has also won the Best Scientist of the Day Award for the research project on Nanoparticles. She is an active editorial board member of various Research Journals. She has been in the research field for over five years and has presented research papers at different international conferences. She has submitted a dissertation in collaboration with renowned hospitals like Nizams Institue of Medical Sciences. She has spent around four years of her career in the marketing field as an Editor and a copywriter and was also involved in the end-to-end content management for internal and external communication. She is a creative writer with a keen proficiency in editing and proofreading content. Being a conference reporter and a corporate trainer has always been her forte.

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Registered offices
India: Guest House Road, Street no - 1/6, Hooghly, West Bengal, PIN-712410, India, Corp. Firm Registration Number: L77527, Tel: +91 7439016438 | +91 9748770553, Email: director@bookpi.org, (Headquarters)
UK: 27 Old Gloucester Street London WC1N 3AX, UK
Fax: +44 20-3031-1429 Email: director@bookpi.org, (Branch office)