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CUPRINS

REVIEW

THE ANALYSIS OF MUSCLE FORCE DEVELOPMENT WITH TRAINED AND ELITE ATHLETES

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ABSTRACT

The goal of this review was to investigate whether maximal rate of force development (MRFD) represents an excellent indicator for training planning. The review underlines the influence of the speed of activation, synchronization and optimization of motor units on MRFD, as well as that MRFD represents an outstanding discrimination factor for achieving optimal sports performance. The analysis focused on research involving force development in athletes who won gold medals in Olympic, world, European and national competitions as well as non-medal athletes. According to the analyses, MRFD from the beginning of the movement for elite athletes was produced in time interval ranges from 0.063±0.007s to 0.115±0.016s, using 56% to 89% of maximal force. This data suggests an emphasis on training to attain MRFD within time intervals of 0.130s. Analyses showed that MRFD developed within this duration represents an excellent discriminator (P<0.01) between elite athletes competing in different disciplines and between elite athletes and well-trained individuals. To conclude, MRFD is related to (P<0.01) the conditions and regimes of muscle work, level of force developed by individual groups of motor units, speed of activation, synchronization and optimization of motor units at intramuscular and inter-muscular levels.

Key words: motor units' activation speed, motor-unit optimization, maximal force in motor unit groups, model and discrimination value

INTRODUCTION

The time necessary to develop the maximal rate of force is shorter than the time necessary for developing maximal force (2, 18, 47, 55, 56, 58, 59, 60). Studies have revealed that a relatively small number of sport disciplines use an athlete's maximal capacity for developing force (18, 47, 55, 57, 58, 59, 60). The majority of sports apply force during intervals ranging from 0.05s to 0.17s (2, 18, 23, 47, 54, 55, 58, 59, 60). Hence, the success of elite athletes depends more on the maximal rate of force development (MRFD) and factors that influence its level (18, 26, 46, 47, 51, 54, 57), than on the maximal force. Consequently, it implies that the MRFD could be an excellent discrimination measure. Thus MRFD should be able to discriminate between elite and recreationally trained athletes. Furthermore, it should be possible to distinguish between elite athletes competing in different disciplines based on the MRFD in time intervals up to 0.13s.

The verification of these hypotheses was performed using data gathered from the results of elite athletes, winners of national, European, world championships and Olympic games in basketball, volleyball, soccer, handball, athletics, boxing, weightlifting, karate, and data gathered from testing performed on non-medal winning professional athletes, using the difference model. Data was analyzed regarding maximal force and MRFD in different muscle groups. The literature was also analyzed for the influence of the following factors on the MRFD: time and type of work of motor units, types of muscle contraction, muscle length, time (speed) necessary for contracting/extending a muscle, time necessary for transferring from eccentric to concentric contraction, external conditions and regime of muscle work.

MATERIALS AND METHODS

Subjects

The studies analyzed in this review include force measurements of professionals athletes that trained daily but did not achieve a medal standing (N=300) and medal-winning, elite athletes (N=208) competing in different sports of Olympic, world and European championships (Table I).

Table I. Characteristics of the subjects

	Age (years)	Body Height (m)	Body Weight (kg)	N
Olympic judo champion	23	1.728	73.65	1
World judo champion	25	1.91	97.8	1
Mediterranean judo champion	24	1.77	80.3	1
World karate champion	25	1.878	90.2	1
Basketball players	23.4±3.37	1.957±0.09	88.1±8.46	38
Handball players	22.76±5.2	1.821±0.05	81.82±5.47	30
Volleyball players	25.0±2.75	1.871±0.03	84.62±1.35	32
Soccer players	22.94±2.61	1.725±0.02	68.23±0.14	36
Athletics: middle and long distance runners	22.94±2.61	1.753±0.02	62.8±6.91	30
Athletics: sprint runners	21.76±3.48	1.763±0.017	71.5±1.36	12
Athletics: athletes competing in long jump, high jump and triple jump	22.37±3.26	1.83±0.03	75.5±0.72	24
Athletes in good shape	21±0.65	1.836±0.05	79.92±8.79	300

Note: All results are mean ± SD

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Methodologies for Diagnosing Force-related Measurements

Measurements in studies analyzed in this review were collected using a variety of hardware-software systems (5, 12, 13, 30, 53, 54, 55). Measurements included tensiometeric probes with measurement range of 0-15000 decanewtons (daN) and measurement accuracy of 0.0002daN (1 daN = 10^1N), free weights with equipment enabling time measurements (s), vertical and horizontal changes in bar position at each 1mm applied to the entire lifting range, tensiometric platform, electro-optical system for measuring movement parameters during testing (1, 2, 4-18, 20, 24, 27-38, 41-43, 49, 50, 52, 53, 56).

The Belt method with a sampling rate of 1000 Hz is used for measuring isometric force for all muscle groups. The force (**F_t**) is measured for 1%, 2%... 99% of the maximum force expressed in daN as well as the time of development. The maximum force (**F_{max}**), also expressed in daN, is measured as well as the time to peak force. The system allows muscle length to be controlled and varied during the measurement process (the angle of muscle activity), the time and mode of muscle activity as well as the number of muscles – joints included in the measuring process. Each test is repeated three times and the best result is taken into account (4, 5, 8-13, 15, 16, 18, 31, 32, 34, 36, 38, 42, 43, 49, 52).

Dynamic measurements of force can be applied during weight training (6, 35, 36, 37) and using tensiometric platform (7, 10, 12, 18). With the help of tensiometric platforms, muscle force development during different types of drop rebound jumps, drop jumps or vertical jumps can be measured. Piezoelectric sensors embedded in the platform register at a high speed (500 Hz) the compression forces of jumps allowing detailed dynamic analysis of all jump phases and a high validity for each movement. Apart from standard parameters, a tensiometric platform allows tracking of the dynamics of the force development, the speed of executed jumps, the depth of a drop jump (or depth jump), the effects of stretching, the ability of rapid replacement of eccentric and concentric contractions as well as the transfer of elastic energy (7) and a number of other parameters.

By using weight lifting, the system allows muscle length (angle), time, the mass of free weights, duration and speed of activity, velocity of shortening (concentric contraction) and muscle elongation (eccentric contraction), the time of transition from eccentric to concentric contraction (reversible contraction) to be controlled and varied during the measurement process. When lifting any weight, the timing of vertical and horizontal change in the position of the barbells can be measured: vertical change was measured at 5.8 mm and horizontal was measured at 1 mm, throughout the whole range of muscle activity. Each test was repeated three times and the best result was taken into account (6, 8, 17, 28, 30, 35, 37, 49, 50, 53). Those attempts during which the horizontal change in the weight position was not in the permitted zone (4-5 cm) were rejected. On average, 100 points are observed per measurement. The data on length and time were used to calculate the vertical velocity and acceleration for each measuring section as the first and the second derivative of path by time. The results of velocity and acceleration were fitted before the calculation of force. After that the force was calculated from the fitted data in each measuring section throughout the entire process of weight lifting according to the formula (6, 35, 36, 37):

$F = m (a + 9.81) / 10$, where **F** represents the force expressed in daN, **m** – the mass of the weight expressed in kg and **a** – acceleration (m/s^2) observed within a specific sample. In both modes, the ratio between the force and time was measured at each 1% of the maximum force as well as the maximum force and time during which it was developed. From the data obtained on force and time, the rate of force development is calculated as their derivatives by time, for all muscle groups involved in the testing.

Both in the isometric and dynamic assessment mode, throughout the entire force development process, at each percentage, the rate of force development were calculated using the formula:

RFD=F/t, where **RFD** represents the rate of force development, expressed in daN/s, **F** - the appropriate level of force expressed in daN, and **t** – the force observation time expressed in seconds (s).

The obtained data on time and force, for both assessment modes, were used to calculate the change in the rate of force development, using the formula:

$CRFD = (F_2-F_1) / (t_2-t_1)$, where **CRFD** is a change in the rate of force development expressed in decanewton per second (daN/s) for each percent of the measured force (F) from 1% to 100% and the time (t) during which it is happening, **F_1** and **F_2** - the appropriate level of maximal force expressed in decanewtons (daN), and **t_1** and **t_2** - force observation time expressed in seconds (s).

The data on force and time, obtained during the application of the Belt method and weightlifting were used to calculate the speed of activation of motor units for each F_t using the formula (5, 13, 31, 54, 56):

$C = - (1/t) * \ln ((1 - F_t/Fmax))$, where **$F_t$** is a level equaling to 1%, 2%... 99% of maximum force expressed in daN; **F_{max}** - maximum force generated by the engaged muscles, expressed in daN, **C** – a constant which characterizes the speed of activation of motor units expressed in index units (IJ), **t** - time during which an adequate level of maximum force is achieved, expressed in seconds (s).

When using the tensiometric platform for drop jumps, drop rebound jumps; the speed of activation of motor units is calculated using the following formula (18, 54, 56):

$C = Ln (1 – F_{exp} / A / e^{B X \beta exp}) \Delta t_{exp}$, where **C** is a constant which characterizes the speed of activation of motor units expressed in index units (IJ); **F_{exp}** - the level of force registered in the dynamic muscle activity over a period during which the muscle strain appears in isometric conditions, expressed in dN, **βexp** - the angle of the knee joint at which the angular velocity equals to zero, expressed in degrees; **Δt_{exp}** - the time interval starting from the contact with the tensiometric platform to the point where the angular velocity of the knee joint equals to zero, expressed in seconds.

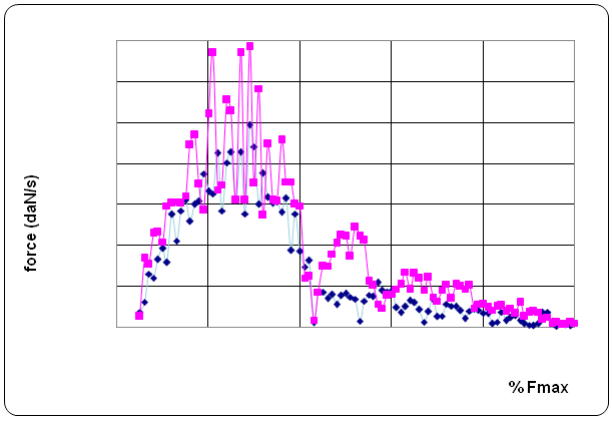

Fig.1. Speed of change in rate of force development for leg extensors for two judo champions

Data Analysis

The data were processed by using descriptive statistics, discriminatory analysis, cluster analysis, factor analysis and fitting by using least-squares method. The data on force, time and the rate of inclusion of motor units were used to determine, by cluster analysis, the class of motor units (36, 54, 56) according to the level of the force development in any given moment of observation or measuring section. From the ratio between force and time, the rate of inclusion and force levels of individual motor units throughout the force development process, the level of synchronization of motor units (36) was calculated. By fitting the data on the force level of individual motor units and time period needed to achieve it throughout the entire force development process for one or more muscles, the optimization of their activity (36, 38) on the intramuscular or the intermuscular level was calculated.

The Analysis of Results Relating to the Rate of Force Development in Muscles

Maximal Rate of Force Development (MRFD) in Muscles

The results (1, 2, 4, 6, 7, 10, 12, 18, 24, 28, 29, 33, 36, 37, 43, 45, 51, 56, 59) of most studies point to the fact that the RFD differs according to sports discipline, athlete, muscular group, regimes, types and speeds of work.

With non-medal winning athletes (BH=1.836±0.05m; BW=79.92±8.79kg; Age=21±0.65 years), isometric contraction MRFD is shown in Table II.

Table II. Descriptive Characteristics MRFD isometric contraction with non-medal winning athletes

	Mean	SD
Back extensors (angle of 135º) in dead lift (daN/s)	268.32	26.79
Right hand flexors (daN/s)	630.83	44.15
Both knee extensors (angle of 125º) (daN/s)	595.07	35.70
Hip flexors (sitting position, at the angle of 90º) (daN/s)	332.24	29.90
Hip extensors (sitting position, at the angle of 90º) (daN/s)	257.58	25.76
Hip rotators to the left (sitting position, at the angle of 90º) (daN/s)	155.13	13.96
Hip rotators to the the right (si. position, at the angle of 90º) (daN/s)	295.64	25.41

In comparison, an elite male judo competitor, winner of Olympic gold medal, (BH = 1.728m; BW = 73.65kg; Age = 23 years) achieves much greater RFD with an isometric regime (Table III). Other elite athletes also achieve much better results when the RFD is concerned (P<0.01) than non-medal winning athletes, which is obvious if we take a look at the results of leg extensor testing at the angle of 112^0. Middle and long distance runners achieve an MRFD of 1382±152.02daN/s in leg extensors. Basketball, volleyball, handball and soccer players achieve an MRFD up to 1727.5±103.65daN/s in leg extensors, while athletes competing in long jump, high jump and triple jump, achieve an MRFD at 2320±150.8 daN/s. With isometric contractions, for both non-medal winning athletes and elite medal athletes, the highest RFD is recorded in hand flexors, while the lowest results are typical of left (weaker side) hip flexors.

Table III. Descriptive Characteristics and function of development of MRFD isometric contraction with elite male judo competitor, winner of Olympic gold medal

	Mean	RFD =
Back extensors (angle of 135º) in dead lift (daN/s)	4720.40	$14990\,t^{0.35}$
Right hand flexors (daN/s)	4633.33	$7098\,t^{0.62}$
Both knee extensors (angle of 125º) (daN/s)	1740.01	$7441\,t^{0.33}$
Hip flexors (sitting position, at the angle of 90º) (daN/s)	4050.03	$10049\,t^{0.74}$
Hip extensors (sitting position, at the angle of 90º) (daN/s)	1720.04	$4856\,t^{0.45}$
Hip rotators to the left (sitting position, at the angle of 90º) (daN/s)	1323.07	$5635\,t^{0.65}$
Hip rotators to the the right (si. position, at the angle of 90º) (daN/s)	3440.00	$7583\,t^{0.55}$

With dynamic contractions, the RFD displays even better results; nevertheless these results are different for each individual and sport. Table IV illustrates the MRFD in leg extensors with an eccentric contraction regime (drop jump – amortization from 110cm). The relation between the angle (the length of muscles) where the amortization ends (X) and the MRFD in leg extensors is nonlinear (MRFD = $4.674X^2 – 965.3X + 53909$ for $R^2 = 1$). Table V illustrates the MRFD in leg extensors with stretch-shortening cycle (SSC) or reversible type jumps (drop jump-depth jump from the elevation of 80cm).

Table IV. Descriptive Characteristics MRFD eccentric contraction in leg extensors with elite athletes

	M	SD
Drop jump-amortization from the elevation of 110 cm		
Athletics: middle and long distance runners		
MRFD (daN/s)	4570.3	776.9
Amortization time (s)	0.115	0.016
Angle (º)	113.6º	11.68º
Level of force (daN)	525.59	78.83
Athletics: sprint runners		
MRFD (daN/s)	6235.54	997.69
Amortization time (s)	0.101	0.012
Angle (º)	124.78º	15.63º
Level of force (daN)	629.79	100.77
Athletes competing in long jump, high jump and triple jump		
MRFD (daN/s)	7985.85	1105.42
Amortization time (s)	0.082	0.020
Angle (º)	132.2º	10.85º
Level of force (daN)	654.84	91.68

If our test sample includes elite athletes competing in different sports (47, 54, 55) and we change the elevation for performing drop jump-depth jump we will get the following function, which defines well the relation between the MRFD in leg extensors and the elevation from which the subjects jump (X):

$MRFD = 0.015x^3 - 3.700x^2 + 278.4x - 1E\text{-}10$ for $R^2 = 1$

In a SSC or reversible activity such as lifting weights (70% of 1RM), a junior medal-winner competing at a European championship (BH=1.675 m; BW=58.2 kg; Age =21 years) achieved the MRFD in clean phase (shoulder muscles) at 1984daN/s; 2778daN/s in half squat (leg extensors); 1628daN/s in bench press (arm extensors) and 964daN/s in dead lift (back extensors). MRFD in leg extensors during SSC contractions in standing high jump starts at 1294±181.16daN/s in non-medal athletes and 1582±189.84daN/s in soccer players, to 2000±180.0daN/s in volleyball players and 2432±243.2daN/s in athletes competing in high jump, long jump and triple jump. Considering blows in martial sports, (30, 56, 57) when executing mae geri, world karate champion (BH=1.878 m; BW=90.2 kg; Age = 25 years) achieved MRFD in hip flexors at 2132.8daN/s, in 0.050s with the force level at 106.64daN; in knee extensors at 1035.0daN/s, in 0.050s with the force level at 51.75daN; in foot extensors at 500.0daN/s, in 0.05s with the force level at 25.0daN. In an experiment involving a junior medal winner at an European championship (BH=1.675 m; BW=58.2 kg; Age = 21 years), where examiners changed the weight and the speed of lifting weights from deep squat (Table 6), it was concluded that the MRFD is achieved when lifting 80% of the maximal weight in one lift (1RM) with maximal speed of lifting (6, 34, 35, 37).

The analysis of the results enables us to conclude that the MRFD is much greater in elite champion athletes than in non-medal winning athletes. With dynamic contractions, the results may be as much as 12 times greater. With isometric contractions, differences range between 3-17 times, while dynamic vs. isometric regimes display results that vary 26 fold ($P<0.000$). Differences are significant ($P<0.01$) between athletes competing in different sports, which imply that the MRFD is an excellent discrimination factor in both regimes. Differences in the MRFD for the same muscle groups, measured during different movements, range up to 31.22 times. The highest RFD is achieved with SSC activities with high movement dynamics. In an eccentric-concentric cycle during the phase of the concentric contraction, more force is generated over a shorter interval of time (in weight lifting with acceleration exceeding 25 ms^{-2} one develops 1450.0daN of force in 0.100s with the MRFD at 14500daN/s or during drop jump-depth jump where one develops up to 983.6±113.11daN of force in 0.063±0.007s with the RFD at 15612.69±1795.46daN/s) (1, 18, 23, 28, 35, 41) than eccentric or concentric only muscle contraction for several reasons. First, at the highest point of the cycle, period between the eccentric and concentric contraction, the force is being developed in isometric conditions. Secondly, since the force development starts in the eccentric phase (in drop jump-depth jump it develops up to 661.89±39.71daN in 0.075±0.004s with the RFD at 8825.08±529.50daN/s) the time available for developing force in a SSC or reversible contraction is extended (18, 28, 34). Third, the level of force is influenced by muscle-tendon elasticity (the accumulation of elastic energy during the eccentric contraction in muscles and tendons) and fourth, reflex muscle contraction (58, 59, 60). All these effects are present when measuring drop jump-depth jump, weight lifting or other sports movements (1, 18, 23, 28, 35, 41). The muscle length and the level of force, after making contact with the ground in drop jump-depth jump and after stopping the first phase of movement in weight lifting or other sports movements, changes abruptly. Muscles are forced to extend and at the same time, their tension rises. These changes are at the same time, controlled and partially balanced by the joint effect of two motor reflexes: myotatic reflex, maintaining an optimal length of muscles, and the Golgi tendon organ reflex, preventing extremely high and harmful muscle tension. Myotatic reflex has a positive effect (the increase in discharge), while Golgi reflex predominantly has a negative or inhibitory effect influencing the applied force. The most important thing for learning reversible contraction during substantial muscle strain is diminishing the activity of the Golgi tendon organ. Nevertheless in drop jumps with amortization or block, in drop jumps-depth jumps from high elevations or when lifting heavy weights from half squat, bench press, pullover, or during torso rotations with additional weight, due to the possibility of harming the integrity of one's body (1, 3, 18, 28), the CNS reacts by applying facilitation and disinhibition effects (1, 3, 18, 26, 28, 53) on different levels. In these cases, the reticular system acts as an amplifier increasing the intensity of efferent signals (58, 59, 60). Furthermore an inhibition of Renshaw's inhibitory interneurons as well as other inhibitory motoneurons can also magnify the facilitation of the system. Pre-synaptic inhibition diminishes Golgi tendon organs inhibition thus minimizing peripheral afferent inhibition permitting higher levels of MRFD (59). Neural disinhibition, from the abovementioned conditions (1, 3, 18, 22, 26, 28, 41, 44, 60), influences the increase in myotatic reflex (the rate of contraction is significantly (several times) faster and more powerful than a completely voluntary muscular contraction). Furthermore, neural disinhibition causes an increase in the speed of activation of motor units and the level of their synchronization, as well as the reprogramming (by increasing) of the limit of force for all motor units. Therefore, the biggest influence on the force increase and at the same time, the decrease in the RFD in reversible contractions, comes from the neural component of muscle contraction – most of all, from the disinhibition process applied at all levels (1, 3, 18, 22, 25, 26, 28, 41, 47, 49, 51, 54, 55, 57, 59). The biggest challenge for athletes determined to learn and master reversible (SSC) activities is to learn and master disinhibition (1, 3, 18, 22, 26, 28, 41, 46, 47, 48, 49, 51, 54, 55, 57, 59).

Table V. Descriptive Characteristics MRFD in SSC in leg extensors with elite athletes

	M	S
Reversible type jumps - drop jump depth jump from the elevation of 60 cm		
Athletics: middle and long distance runners		
MRFD (daN/s)	9234.42	1246.65
Time necessary to switch from eccentric to concentric contraction (s)	0.077	0.009
Level of force (daN)	711.05	95.99
Athletics: sprint runners		
MRFD (daN/s)	11195.65	1231.52
Time necessary to switch from eccentric to concentric contraction (s)	0.069	0.007
Level of force (daN)	772.5	84.95
Athletes competing in long jump, high jump and triple jump		
MRFD (daN/s)	15612.69	1795.46
Time necessary to switch from eccentric to concentric contraction (s)	0.063	0.007
Level of force (daN)	983.6	113.11

Moreover, the analysis of the results enables us to conclude that a high level of MRFD (isometric) enables elite athletes to generate in 0.097s, from 56% of maximal force in right hip rotators to 89% in hip flexors. In contrast, non-medal athletes in the same time interval achieve from 33% of maximal force in hip rotators to the right, to 64% in hip flexors. With dynamic movement (amortization, reversible contractions) due to the high MRFD, elite athletes achieve much greater values of maximal force in a much shorter time interval than the values recorded with isometric contractions (P< 0.000). The relation between MRFD, time and angle at which the force is developed (length of muscles) is nonlinear. The relation between MRFD and the elevation one jumps from is also nonlinear. In the case of elite athletes, the RFD enables them to develop maximal force in the amortization period in the time interval ranging from 0.075s to 0.106s, while the time interval for reversible contractions is 0.063s to 0.077s.

In the end, we can conclude that the MRFD in all types of muscle contraction (isometric, concentric, eccentric, reversible) enables elite athletes to develop, in the time interval ranging from 0.063±0.007s. to 0.115±0.016s, levels of force that are necessary and sufficient to win medals at prestigious competitions. These results also permit us to recommend the best choice for developing MRFD in leg extensors: reversible contractions during drop jump-depth jump from 60cm and drop jump-amortization from 120cm (5, 19, 46, 47, 48, 51, 54, 55, 57). For other muscle groups, we recommend weight lifting with 80% of 1RM and maximal speed (8, 19, 46, 47, 48, 51, 54, 55, 57). When lifting weights (80% of 1RM) different muscle groups develop up to 78.5% of maximal force in a time interval ranging from 0.097 to 0.172s (32, 33, 35, 37, 50).

Change in Rate of Force Development (CRFD)

Sometimes, in sport, we need to develop additional, high forces within a certain time interval, with the aim of achieving an optimal result. Figure 1 clearly shows that the biggest CRFD in isometric conditions (36), in leg extensors, i.e. the biggest increase in force, takes place in the interval between 18% and 32% of maximal level of force. Maximal CRFD in the case of the first competitor is 7000daN/s, while in the case of the second competitor it is at 5000daN/s. At that moment, the level of force was from 94.57daN to 215daN in a time interval ranging from 0.059s to 0.086s at the angle of 125^0. The level of change is different from athlete to athlete. Non-medal athletes achieve much lower values, ranging from 1441daN/s (right hand flexors) to 3947daN/s (leg extensors). The speed of CRFD is different up to 0.200s and after 0.200 s. The changes are much greater up to 0.200s than after 0.200s. An experiment performed on elite judo competitor's leg extensors (BH=1.91m; BW=97.8kg; Age=25 years) confirms this, i.e. it confirms that the speed of change in rate of force development (CRFD) in (t) for every % of force measured (F) from 1% to 100% with isometric contractions at an angle of 115^0, can be described up to 0.200s and after 0.200s (R^2 = 0.997 and R^2 = 0.998) through different formulas (34):

$CRFD_1(t<0.2) = -179437t^2+44792t-428.6$.

$CRFD_2(t>0.2)=254.52t^4-1555.2t^3+3484t^2-3655.4t+2045.4$

Table VI. Rate of change for force development and its' dimensions with various weight lifts and leg extensors reaction

Weight load and lifting speed	50 %			70 %			80 %			90 %		100 %
	High speed	Submaximal speed	Maximal speed	High speed	Submaximal speed	Maximal speed	High speed	Submaximal speed	Maximal speed	Submaximal speed	Maximal speed	Maximal speed
Maximal force F_{max} (daN)	151	176	163	165	191	217	271	298	305	186	181	239
Time t_{max} (s)	0.19	0.14	0.12	0.24	0.20	0.23	1.36	1.41	1.45	0.80	1.16	0.81
Force production development rate/Rate of change of force at 1%VF_1 (daN/s)	1133	1883	1136	699	1328	2178	5682	7579	18372	497	607	897
level of force F_1 (daN)	145	143	117	143	163	154	247	272	204	170	156	201
Time t_1 (s)	0.18	0.11	0.16	0.15	0.16	0.17	1.35	1.40	0.74	1.21	0.86	0.72
Max Production development rate force VF_2 (daN/s)	903	148	1147	973	986	2347	185	245	305	517	769	1096
level of force F_2 (daN)	93.1	105	89.4	132	163	202	135	204	228	166	153	216
Time t_2 (s)	0.10	0.07	0.04	0.14	0.16	0.21	0.73	0.83	0.77	0.32	0.19	0.20

Note: 50%, 70%, 80%, 90%, 100% - percentage of maximal weight lifted in one attempt (% of 1RM).

In an experiment that included a junior medal-winner at a European championship (BH=1.675m; BW=58.2kg; Age=21 years), where scientists changed the weight and speed of weight lifting (Table 6), it was concluded that the biggest CRFD is achieved with 85% of maximal weight in one attempt (1RM) with maximal lifting speed (6, 35, 37). In a kettlebell clean, with maximal lifting speed at 1.68m/s his shoulder muscles achieve CRFD equaling 5832daN/s at 0.150s.

His arm extensors in bench press achieve CRFD equaling 4200daN/s at 0.158s with lifting speed at 1.69 m/s. His leg extensors in deep squat, with a lifting speed at 1.74 m/s, achieve CRFD equaling 7000daN/s at 0.120s. His back extensors in dead lift achieve CRFD equaling 1660daN/s at 0.200s with lifting speed at 1.7 m/s. With reversible or SSC contractions, during drop jump-depth jump, leg extensors achieve much greater values in RFD in much shorter time ($P<0.01$) than with isometric contractions (37, 54, 55). With 85% of maximal weight in one attempt (85% of 1RM) with maximal lifting speed (6, 35, 37) and drop jump-depth jump from 80cm, we detect the most intensive changes in RFD, the most intensive processes for producing proteins actin and myosin (17), but, at the same time, we detect an improvement in intramuscular coordination.

The research results (1, 2, 4, 5, 7, 8, 9, 10, 11, 12, 13, 14, 18, 26, 29, 31, 33, 34, 35, 41, 45, 46, 47, 49, 50, 51, 53, 54, 57) tell us that the RFD directly depends on the level of force in certain groups of motor units, their activation speed, synchronization, optimization of their work at intermuscular and intramuscular levels, as well as their ability to sustain high changes in RFD.

Practical Applications

In certain sports activities, the result exclusively depends on maximal force, which means that the training has to be organized accordingly. In most sports and disciplines, the time interval for developing force is limited to 0.050s - 0.170s. This is why outstanding results rely on the MRFD and factors that influence it (maximal force in individual groups of motor units, motor units' activation speed, synchronization and optimization of motor units' work) more than on the maximal force. The training for developing MRFD should be designed with the aim to achieve maximal values in the time intervals (0.063s - 0.130s.) as recorded for elite athletes and medal winners. The best results in developing MRFD and the factors that influence it, are achieved with maximal speeds, by using free weight jumps, vaults, drop jumps, drop jump-depth jumps and sprints with variations in direction. MRFD is best developed with reversible contractions, in drop jump-depth jump from 60cm, drop jump-amortization from 120 to 200 cm and weight lifting at 80% of 1RM. With 85% of maximal weight in one attempt (85% of 1RM) with maximal lifting speed and drop jump-depth jump from 80cm, we detect the most intensive changes in RFD, the most intensive processes for producing proteins actin and myosin (17), but, at the same time, we detect an improvement in intramuscular coordination. The best training results are achieved if we apply the abovementioned rules for constructing training programs for each athlete.

REFERENCES

1. Aagaard P, Simonsen EB, Andersen JL, Magnusson P, Halkjær-Kristensen J, Dyhre-Poulsen P. Neural inhibition during maximal eccentric and concentric quadriceps contraction: effects of resistance training. *J. Appl. Physiol.* 2000; 89: 2249-57.

2. Aagaard P, Simonsen EB, Andersen JL, Magnusson P, Halkjær-Kristensen J, Dyhre-Poulsen P. Increased rate of force development and neural drive of human skeletal muscle following resistance training. *J. Appl. Physiol.*2002; 93: 1318-1326.

3. Anohin PK. The theory of functional systems: general questions of physiological mechanisms. Moscow: *Sciences,* 1970.

4. Blagojevic M, Milosevic M, Dopsaj M, Arlov D. Mechanic parameters of trunk flexor and extensor muscle force with well-trained young males. *Exe & Soc. J. of Sports Sci*. 1997; 17: 128-129.

5. Blagojevic M, Milosevic M, Dopsaj M. The effect of muscle length on the change in muscle involvement velocity. *Exe & Soc .J. of Sports Sci.* 1997; 21: 213-214.

6. Blagojevic M, Milosevic M, Aleksic V, Papadimitrioiu A, Dopsaj M. The comparative analysis of force generation velocity and its dimensions at maximal voluntary contractions in isometric and dynamic muscle work regime. In: Proceedings of the International Conference on Weightlifting and Strength Training. Editor: Keijo Hakkinen, Lahti, Juvaskyla, Finland, 1998; 273-274.

7. Bobbert MF, Gerritsen KG, Litjens MC, Van Soest AJ. Why is countermovement jump height greater than squat jump height? *Med & Sci in Sports & Exe.* 1996; 28: 1402-1412.

8. Dopsaj M, Milosevic M, Arlov D, Blagojevic M, Stefanovic D. The structure of changes in mechanic contractile characteristics of leg extensor muscles caused by combined strength training during one-year motor learning program in special physical education. In: Proceedings of International Congress on Sports Psychology. Editors: Yannis Theodorakis & Athanasios Papaionnou, Komotini, Greece: Democritos university of thrace department of physical education& sport science, 1996; 313-318.

9. Dopsaj M, Milosevic M, Matkovic I, Arlov D, Blagojevic M. The relation between sprint abilities in freestyle swimming and force characteristics of different muscle group. In: Proceedings of the VIII International Symposium of Biomechanics and Medicine in Swimming. Editors: Keskinen KL, Komi PV, Hollander AP, Jyvaskyla, Finland, 1998; 58-59.

10. Dopsaj M, Milosevic M, Blagojevi M. Metrological values of selected muscle force mechanical characteristics during isometric multi-joint test. 6th International Congress of Northern Greece Sports Medicine Association. Thessaloniki, Greece, 1999a.

11. Dopsaj M, Milosevic M, Matkovic I, Arlov D, Blagojevic M. The relation between sprint ability in freestyle swimming and force characteristics of different muscle groups. Biomechanics and Medicine in Swimming VIII, Editors: Keskinen KL, Komi PV, Hollander AP. Department of Biology of Physical Activity, University of Jyväskylä, Gummerus Printing, Jyväskylä, Finland, 1999b; 203-208.

12. Dopsaj M, Milosevic M, Blagojevic M. An analysis of the reliability and factoral validity of selected muscle force mechanical characteristics during isometric multi-joint test. In: Proceedings of the XVIII International Symposium of Biomechanics in Sports. Editors: Youlian H & David PJ, Hong Kong, 2000; 146-150.

13. Dopsaj M, Milošević M, Blagojević M, Mudric R. A new approach to discriminating athletes according to their specific fitness status when considering isometric force. In: Abstract Book of the 3th International Conference on Strength Training -"Strength Training in Sport and in Rehabilitation". Budapest, Hungary, 2002; 15-16.

14. Dopsaj M, Milosević M, Blagojevic M, Mudric, R. Analysis of the effects that one academic year of Spe has on the characteristics of maximal and explosive force of the trunk extensor in policemen. 3th International Conference on Strength Training - "Strength Training in Sport and in Rehabilitation". Budapest, Hungary, 2002; 17-18.

15. Dopsaj M, Milosević M, Blagojevic M. The effects of a one-year swimming course on the swimming skills of police academy students. Biomechanics and Medicine in Swimming IX, Edited by Jean-Claude C, Department of Biology and sport medicine, University of Saint-Etienne, Publications de l'Universite de Saint-Etienne, Saint-Etienne, France, 2003; 445-450.

16. Dopsaj M, Milosevic M, Rajic B, Abella CP, Blagojevic M, Vucković G. Characteristics of different isometric legs extensors explosive muscle force parameters at female athletes. *Exe & Soc. J. of Sports Sci*. 2003; 34: 303-304.
17. Furandžijev V, Abadžijev I. Osnovi na podgotovkata na elitni i podrastvašći sportisti – Adaptacija na kletčno i molekuljarno nivo. Sofija: Tip-top pres, 2003.
18. Gavrilovic P. Comparative analysis of transitional regime of force development in athletes competing in different sports with willing contractions and dynamic muscle work. PhD Thesis, Beograd: Faculty of Sport and Physical Education, 1992.
19. Gavrilovic P, Svraka M, Milosevic M. Monitoring Physical Fitness using Biomechanical Methods. Paper presented at Athletics 92. *J.of Athl. 92 Belgrade.* 1992; 48-57.
20. Grimby L. Firing properties of human single motor units during locomotion. *J. Physiol*. 1984; 346: 195-200.
21. Hannah R, Minshull C, Buckthorpe MW, Folland JP. Explosive neuromuscular performance of males versus females. *Exp .Physiol.* 2012; 97: 618-629.
22. Ivancevic T, Jovanovic B, Jovanovic S, Đukic M, Đukic N, Lukman A. Paradigm shift for future tennis: the art of tennis physiology, biomechanics and psychology. New York: Springer, 2010.
23. Jelen K. Biomechanical estimate of output force of ligamentum patellae in case of its rupture during jerk. *Acta. Uni. Caro. Gym* 1991; 2: 71-82.
24. McLellan CP, Lovell DI, Gass GC. The role of rate of force development on vertical jump performance. *J. Strength. Cond. Res* 2011; 25: 379-385.
25. Milosevic M, Gavrilovic P. Latent dimensions of strength area in policemen. *13 May.* 1985; 2: 79-88.
26. Milosevic M. Determining the structure motor abilities in policemen. Belgrade: Police College, 1985.
27. Milosevic M, Lazendic O. The relations between the general factor if specific motor activity of police officers and strength tests. *13 May.* 1986; 1: 53-61.
28. Milosevic M, Gavrilovic P, Ivancevic V. Modelling and control of the self-defense system. Belgrade: Scientific Book, 1989.
29. Milosevic M, Dopsaj M, Blagojevic M, Arlov D. Indicator factor structure of force-time characteristics of muscle contraction under isometric conditions. XIV International Symposium on Biomehanic, Portugal, 1996.
30. Milosevic M, Jovanovic S, Arlov D, Blagojevic M, Dopsaj M. The methodology of assessing the adoption of motoric programs in special physical education. In: Proceedings of International Congress on Sport Psychology. Editors: Yannis Theodorakis & Athanasios Papaionnou, Komotini, Greece, 1996; 297-303.
31. Milosevic M, Laparidis C, Dopsaj M, Arlov D, Blagojevic M. The analysis of changes of muscle involvement velocity characteristics of leg extensors by linear and nonlinear methods. *Exe & Soc. J. of Sports Sci*. 1997; 17: 167-168.
32. Milosevic M, Takac-Kostic M, Laparidis C, Dopsaj M, Arlov D, Blagojevic M. The structural change of leg extensor muscle involvement speed indicators influenced by eight-month strength training. *XVII Pan American & XIII Brazilian Congress of Sports Medicine*, Gramado (Porto Alegro), Rio Grande do Sul, Brazil, 1997,
33. Milosevic M, Blagojevic M, Dopsaj M. Analysing the characteristic of transitory of leg extensor force generation in dynamic strain conditions. *Science-Security-Police, J. of Pol. Aca-Belgrade.* 1998; 3: 34-40.
34. Milosevic M, Blagojevic M, Dopsaj M. Determining the functions upon which force generation velocity and its dimensions are changed in leg extensors. In: Proceedings of XVI International Symposium on Biomehanic in Sports. Editors: Riehle HJ, Vieten MM. Konstanz, Germany, 1998b: pp. 204-208.
35. Milosevic M, Cirkovic Z, Mihajlovic M, Blagojevic M, Dopsaj M. The analysis of changes in the parameters of velocity, force and its dimensions at lifting different weights from deep squat at different velocities**.** In: Proceedings of the International Conference on Weightlifting and Strength Training. Editor: Keijo Hakkinen, Lahti, Jyvaskyla, Finland, 1998; 268-270.
36. Milosevic M, Dopsaj M, Blagojevic M. Comparative analysis of force generation velocity and its dimensions in leg extensors in top judoists. *Exe & Soc. J. of Sports Sci*. 1998; 20: 220-221.
37. Milosevic M, Stefanovic D, Dopsaj M, Blagojevic M. The change in leg extensor muscle involvement velocity at weightlifting from deep squat, at different weights and maximal velocity. In: Proceedings of the International Conference on Weightlifting and Strength Training. Editor: Keijo Hakkinen, Lahti, Juvaskyla, Finland, 1998: 271-272.
38. Milosevic M, Takac-Kostic M, Blagojevic M, Cvjetkovic M, Jovanovic B. Force distribution of motor units of leg extensor muscles. In: Proceedings of the 3rd International Scientific Conference on Prevention of Work-Related Musculoskeletal Disorders, I3th International Symposium on Epidemiology in Occupational Health. Helsinki, Finland, 1998; 111-116.
39. Milosevic M, Blagojević M, Tošić B, Pilipović S. Muscular deformations caused by powerful instantaneous force. *Science-Security-Police, J. of Pol. Aca-Belgrade.* 1998; 3: 31-41.
40. Milosevic M, Milic Z, Stefanovic D, Cirkovic Z. Methods and tools for diagnosing and developing speed dimensions for special police movements. *Bezbednost.* 1998; 4: 42- 52.
41. Milosevic M, Dopsaj M, Blagojevic M, Papadimitriou K. The analysis of leg extensor muscle involvement velocity at lifting weight from deep squat in eccentric and concentric contraction phases. *Exe & Soc. J. of Sports Sci*. 1999: 21: 130-131.
42. Milosevic M, Blagojevic M, Dopsaj M. Defining the functions that affect the muscle involvement velocity of leg extensors, back extensors and hand flexors. 6th International Congress of Northern Greece Sports Medicine Association. Thessaloniki, Greece, 1999.
43. Milosevic M, Blagojevic M, Dopsaj M. Analysing the characteristics of transitory of leg extensor force generation in dynamic strain conditions. *Science-Security-Police, J. of Pol. Aca-Belgrade.* 1999; 3: 34-40.
44. Milosevic M, Blagojevic M, Ciric D, Pilipovic S, Tosic B. Muscles in isotropic regime. *Science-Security-Police, J. of Pol. Aca-Belgrade.* 1999; 4: 45-55.
45. Milosevic M, Blagojevic M, Pilipovic S, Tosic B. The muscle contraction and the force production. In: Proceedings of the XVIII International symposium of biomechanics in sport. Editors: Youlian, H & David, P. J. Hong Kong, 2000; 183-186.
46. Milosevic M. Physical trainings for top athletes**.** *SQ sports. J-Belgrade.* 2001; 14: 70-74.
47. Milosevic M. Analysis of the creation of muscular force. *SQ sports. J. Belgrade.* 2002; 16: 68-69.
48. Milosevic M. Methods for developing muscle force in top athletes**.** *SQ sports. J. Belgrade.* 2002; 17: 70-71.
49. Milosevic M, Mudric R, Dopsaj M, Blagojevic M**.** The functions that define maximal values of motor unit involvement velocity in leg extensors while lifting varied weight at maximal velocity in deep-squat. In: Abstract Book of the 3th International Conference on Strength Training -"Strength Training in Sport and in Rehabilitation". Budapest, Hungary, 2002; 100-101.
50. Milosevic M, Dopsaj M, Blagojevic M, Mudric R. Changes in force and motor unit involvement velocity (muiv) induced by plyometric training and the method of incomplete eccentric muscle response**.** In: Abstract Book of the 3th International Conference on Strength Training -"Strength Training in Sport and in Rehabilitation". Budapest, Hungary, 2002; 99-100.
51. Milosevic M. Creation and development of muscle force. *SQ sports. J. Belgrade*. 2002; 16: 24-27.

52. Milosevic M, Dopsaj M, Blagojevic M, Mudric R. The dynamics of developing the maximum force in a basketball centre player by applying modern training technology: One cese study. *Exe & Soc. J. of Sports Sci*. 2003; 34: 372-373.
53. Milosevic M, Mudric R, Dopsaj M, Blagojevic M, Papadimitriou E. The control of force creating in function of the muscle contraction intensity. In: Book of Abstracts of the 4th International Conference on Strength Training. Edited by: Kellis E, Amiridis I and Vrabas I. Serres, Greece, Aristotle University of Thessaloniki, Department of Physical Education and Sport Science at Serres, 2004; 320-321.
54. Milosevic M. New training technology for top athletes. Belgrade: Police College, 2005.
55. Milosevic M. Physical preparation of elite athletes: standardization of management processes. Belgrade: APP, 2010.
56. Milosevic M, Mudric R, Mudric M. The biomechanical analysis of the karate kick (mae geri) in the function of defining educational training aims and methods. *Sport-sci & pra.* 2012; 2: 5-14.
57. Mudrić R, Milošević M, Jovanović S. Attack in karate: education and training. Belgrade: Police College, 2004**.**
58. Thomas R. Physiology of Sports. New York: Taylor & Francis, 2004.
59. Zatsiorsky VM, Kramer WJ. Science and practice of strength training. Belgrade: Data status, 2009.
60. Wilmore HJ, Cosstill D, Kenney WL. Physiology of Sport and Exercise. London: Human Kinetics, 2007.

ANALIZA DEZVOLTARII FORTEI MUSCULARE LA SPORTIVII ANTRENATI SI LA ATLETII DE ELITA

REZUMAT

Scopul acestui review a fost de a investiga daca rata maxima de dezvoltare a fortei (MRFD) reprezinta un indicator bun pentru planificarea antrenamentului. Review-ul subliniaza influenta vitezei de activare, sincronizarea si optimizarea unitatilor motorii la MRFD, precum si faptul ca MRFD reprezinta un factor de discriminare important pentru obtinerea performantei sportive optime. Analiza este axata pe cercetarile referitoare la dezvoltarea fortei la sportivii care au castigat medalii de aur la Olimpiada, la competitii mondiale, europene si competitiile nationale, precum si la sportivii care nu au fost medaliati. Conform acestor analize, MRFD de la inceputul miscarii in cazul atletilor de elita a fost produsa intr-un interval de timp cuprins intre 0,063±0,007s si 0,115±0,016s, folosind intre 56% si 89% din forta maxima. Aceste date indica o crestere a antrenamentului pentru a tinge MRFD intr-un interval de timp de 0.130s. Analizele au aratat ca MRFD dezvoltata in acest interval de timp reprezinta un factor de discriminare semnificativ ($P<0.01$) intre atletii de elita din diferite discipline si intre atletii de elita si indivizii bine antrenati. In concluzie, MRFD este corelata ($P<0.01$) cu conditiile si regimuirle de travaliu muscular, cu nivelul fortei dezvoltate de grupuri individuale de unitati motorii, viteza de activare, sincronizarea si optimizarea unitatilor motorii la nivel intra- si inter-muscular.

Cuvinte cheie: viteza de activare a unitatilor motorii, optimizarea unitatilor motorii, forta maxima in grupurile de unitati motorii, valoare model si de discriminare

PREVALENCE OF POLYGLANDULAR AUTOIMMUNE SYNDROME TYPE III IN A GROUP OF CHILDREN WITH THYROID DISEASES AND DIABETES MELLITUS

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ABSTRACT

Background&Aims: Polyglandular autoimmune syndromes (PAS) (otherwise known as polyglandular failure syndromes) are a constellations of multiple endocrine gland insufficiencies.Polyglandular autoimmune syndrome type III (PAS III) comprises autoimmune thyroiditis, immune-mediated diabetes mellitus, celiac disease, hypogonadism, myasthenia gravis, sarcoidosis, Sjogren syndrome, rheumatoid arthritis, gastric neoplasia, and malabsorption. The purpose of this study is to determine the prevalence of PAS typeIII in a group of children with thyroid diseases and diabetes mellitus (DM) type 1. Methods: The studied group was of 83 children with DM type 1 (71 girls and 12 boys), aged between 7 and 17 years.The methods of investigation were represented by clinical, imaging, biochemical, hormonal and immunological parameters.Results: The prevalence of PAS III in the study group was 59.03 % (85.71% girls and 14.29% boys, $p < 0.001$, $X^2 = 50$). Also, the subtype was III a (autoimune thyroiditis + DM type 1), and prevailed in girls. In 1.2% cases were associated non endocrine disease as vitiligo and decalvant pelada.Conclusions: In the children group we have PAS III a, which prevailed in girls. Because the frequent association was between Autoimmune thyroiditis and DM type 1, if we have a child with an autoimunne disease is better to investigate him for another autoimmune disease, because these can coexist and be asymtomatic.

Keywords: diabetes mellitus, autoimmune thyroid disease, polyglandular autoimmune syndromes type III, children.

INTRODUCTION

Polyglandular autoimmune syndromes (PAS) (otherwise known as polyglandular failure syndromes) are a constellations of multiple endocrine gland insufficiencies.

Essentially, 2 types of PAS exist, type I and the more common type II, also known as Schmidt syndrome. A third type (type III), which occurs in adults, has been described. Type III does not involve the adrenal cortex, but it includes 2 of the following: thyroid deficiency, pernicious anemia, type 1A diabetes mellitus, vitiligo, and alopecia. Other disorders also have been described in association with the PAS syndromes; pulmonary hypertension in association with PAS syndrome type II (PAS-II) is one example (1).

In 1980, Neufeld and colleagues distinguished two major PAS that contained Addison's disease (PAS I and PAS II) and one PAS that was like PAS II but without the involvement of Addison's disease, which was classified as PAS III (2, 6). In retrospect, the latter two PAS are sufficiently related that PAS II a with Addison's disease and PAS II b without Addison's disease would seem more appropriate and have been used herein.

PAS 2 a/2 b appeared biased to female patients with components expressed in successive generations, suggesting a dominant mode of transmission.

The evidence supporting the autoimmune nature of the component diseases of the PAS is compelling: (1) affected organs demonstrate a chronic inflammatory infiltrate composed mainly of lymphocytes, sometimes aggregating into follicle formation; (2) some of the component diseases are associated with immune-response genes encoded by class-II loci of the HLA complex and more recently, the cytotoxic T lymphocyte antigen-4 (CTLA-4) locus(2); and (3) the syndromes are replete with auto antibodies reacting to targeted tissue-specific antigens, which often are targeted organ-specific enzymes, secretor products of the cells or their receptors.

PAS III (or PAS II b) is characterized by the presence of autoimmune thyroid disease in association with one of the other organ-specific autoimmune diseases such as atrophic gastritis/ pernicious anemia, vitiligo, primary hypogonadism (female>male) and/or DM type 1, but in the absence of Addison's disease (3). Frequent associations common to PAS II b as centered around DM type 1, myasthenia gravis (5), and vitiligo (7) have been well documented. Graves' disease and Hashimoto's thyroiditis are both frequent in PAS II, as are vitiligo and pernicious anemia. The authors recommend routine thyroid autoantibody screening of all DM type 1 diabetes patients and full endocrine autoantibody testing in those found to be positive. However, physicians should routinely elicit historical and physical features relevant to the diagnostic triad in all patients with DM type 1 and/or autoimmune thyroiditis. A family history of poly-glandular failure is often present in past generations that can serve as a flag for those patients who need extra monitoring. Close to half of cases are familial, though the patterns of heritability are variable. (4) The presence of non-endocrine autoimmune disease, such as alopecia or vitiligo, is less common than in PAS I. When such manifestations are present, however, they are important

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clinical indicators, especially if they are profound. The mortality risk of untreated adrenocortical failure in the 2% of patients with myasthenia gravis who develop associated endocrinopathies requires that all these patients under 40 years should be assessed closely for endocrinological disorders during their initial investigations.

So, polyglandular autoimmune syndrome type III (PAS - III) (9) is associated with the following diseases: celiac disease, hypogonadism, myasthenia gravis, sarcoidosis, Sjogren's syndrome, rheumatoid arthritis, gastric cancer, malabsorption due to the pancreatic exocrine deficiency, and can be classified in three subcategories:

- PAS III a - autoimmune thyroiditis with DM type 1
- PAS III b - autoimmune thyroiditis with pernicious anemia
- PAS III c - autoimmune thyroiditis with vitiligo and / or alopecia and / or other autoimmune diseases

PAS III exact prevalence is unknown, but it is more common in women than in men and it is typically observed in middle-aged women, but can occur in people of any age. PAS III mortality and morbidityare determined by the individual components of the syndrome.

MATERIAL AND METHOD

Investigated population

The group of children was represented by 83 subjects aged between 7-17 years.All children from the study group had Type 1 diabetes.In the studied group, the gender distribution of the children was 5.9/1, represented by 71 girls (85.54%) and 12 boys (14.45%).

The methods of investigation were represented by clinical data - case history, current status, imagistic- thyroid ultrasound, biochemical - for glycemic balance: fasting blood glucose, glycosylated hemoglobin, investigation of the thyroid gland: TSH, FT_4, FT_3, thyroid antibodies, investigation of the adrenal gland: cortisol, 21-hydroxylase antibodies, gonadotropins: FSH, LH and appropriate sex hormones (testosterone, estradiol), investigation of celiac disease: anti-tissue-transglutaminase antibodies, investigation of pernicious anemia: complete blood count with mean cell volume and vitamin B_{12} levels.

Determination of plasma glucose was performed by enzyme technique with glucosooxidasis. Normal values were taken between 70 - 110 mg%; diabetes mellitus - values equal or over 126 mg%, impaired glucose tolerance - values between 110 - 125 mg% and the OGTT at2 h between 140 - 200 mg% and impaired fasting glucose - values between 110 - 125 mg% and OGTT at 2 h under 140 mg%.

Determination of HbA1c was achieved through the DiaStat for measuring HbA1c reported to the total HbA.

To determine **the TSH level in plasma, the free fraction of triiodotironin (FT_3), and the plasma free fraction of thyroxin (FT_4)**were performed a quantitative method ARCHITECT; witch is an immunological method,Chemilum nescentMicroparticle Immunoassay (CMIA). Normal values were following: TSH = 0.465 - 4.68 Miu/ml, FT_3 = 3.69 -10.4 pmol/l, FT_4 = 10 - 28.2 pmol/l.

To obtain ***the level of cortisol*** was performed the technique IMMULITE / IMMULITE 1000, an immunometric method, in solid phase, competitive, of chemiluminescent, Immuno-Chemilumino-Enzymometric assay (ICEM). It was considered normal: a.m. 5 - 25 microgram/dl.

FSH level was measured quantitatively by the ARCHITECT method; a ChemilumnescentMicroparticle Immunoassay. Reference values: determined with ARCHITECT test (Table I).

Table I. The reference values for FSH

Population field	mIU/ml
Women:	
- Follicular phase	3.35 – 21.63
- Ovulating phase	4.97 – 20.82
- Luteal phase	1.11 – 13.99
- Postmenopausal	2.58 – 150.53
Men	1.37 – 13.58

LH level was measured quantitatively by the ARCHITECT method; a ChemilumnescentMicroparticle Immunoassay. Reference values: determined with ARCHITECT test (Table II).

Table II. The reference values for LH

Population field	mIU/ml
Women:	1.26 – 10.05
- Follicular phase	2.57 – 26.53
- Ovulating phase	18.06 – 90.23
- Luteal phase	0.67 – 23.75
- Postmenopausal	
Men	1.09 – 92.45

Testosterone was determinate by ELISA method. The references values are depending by age and gender:
Adults:

- men: 0.019 - 0.145 nmol/L;
- women in fertile period: < 0.014 nmol/L;
- pills: 0.001 - 0.0069 nmol/L;
- postmenopausal: 0.0003 - 0.0058 nmol/L.

Estradiolwas determinate by immunochemical with electrochemiluminiscent detection method (ECLIA). The references values are depending by age and gender, and at women also with the menstrual cycle period and pregnancy (Table III).

Table III. The reference values for estradiol

Age and gender	***References values (pmol/L)***
Adults – ***Women*** • Follicular phase	46.0 - 607
• Ovulating phase	315 - 1828
• Luteal phase	161 - 774
• Postmenopausal	<18.4 - 201
– ***Men***	28.0 - 156
Pregnancy (first quarter)	789 – 15781
Children (1-10 years) • girls	22.0 - 99.1
• boys	<18.4 - 99.1

The immunological parameters were represented by autoimmune thyroid markers - antibodies(antiTPO and antiTg antibodies).

To determine **serum levels of antiTPO antibodies** it was used the kit AxSYMantiTPO, an immunological method (Microparticle Enzyme Immunoassay) (MEIA). Normal values: antiTPO antibodies <35 IU/ml.

To determine **serum levels of antiTg antibodies** it was used the kit AxSYMantiTg, a MEIA method as well (Microparticle Enzyme Immunoassay). Normal values: antiTg antibodies <55 IU/ml.

To determine **21-hydroxylase (anti 21-OH antibodies) antibodies level** it was used the radioimunodetermination method combined with a technique of imunoprecipitation, based on human 21-OH marked with I 125 reacting with the antibodies anti 21-OH from the samples test and forming immune complexes that precipitated with the solid-phase of protein A. Normal range: <1 IU/ml

Antitissuetransglutaminase antibodies were determinate by ELISA method.

Referencesvalues: IgA, IgG :<10 U/mL: negative; ≥10 U/mL: positive.

Vitamin B_{12} levels were determinate by immunochemical with electrochemiluminiscent detection method (ECLIA). References values: 191 - 663pmol/L (for European population).

Determination of complete blood count was achieved with automatic method: electric impedance method. Normal values (for children): erythrocytes = 4-5.5 mil/mm^3, leukocytes = 4500-11000 mil/mm^3, plateletes = 150000-450 000/mm^3, hematocrit (Ht): 32-44 %, hemoglobin (Hb): 9.5-15.5 g/dl.

Constantsandred cellindicesare calculatedautomatically, depending on the values of Hb, Ht and red blood cells (RBC) count. Normal values**:** mean corpuscular volume(MCV) = 80 - 100 fl, mean corpuscular hemoglobin concentration **(**MCHC) = 32-36 g Hb/100 ml erythrocytes, mean corpuscular hemoglobin(MCH) = 27-32 pg.

Thyroid ultrasound was performed in all cases and allowed us to measure thyroid volume, thyroid study and the changes in parenchyma's density.

An increased density, uniform, characterizes normal thyroid parenchyma easily distinguished from the neck muscles that are hypo dens.

Inflammatory processes and autoimmune pathology appearshypo dens**.** The scale was assessed as being discreet +, moderate ++ and marked +++.

In the autoimmune thyroid disease the parenchyma of the gland appears hypo dens.

Chronic autoimmune thyroid disorder appears with a hypoecogenity of the parenchyma and normal or increased thyroid volume.

STATISTICAL ANALYSIS

For statistical analysis we used Microsoft Excel and POP Tools from Microsoft Office 2003 and EPI 2000 program. To measure the quantitative variables were determined average (A) and standard deviation (SD), and to assess the gender differences we used the unpaired t test and ANOVA test, considering statistically significant a $p < 0.05$.

RESULTS AND DISCUSSION

The prevalence of PAS III in the study group was 59.03 % (85.71% girls and 14.29% boys, $p < 0.001$, $X^2 = 50$).

The main endocrine autoimmune association was represented by autoimmune chronic thyroiditis and DM type 1. In 4.82% cases was associated Graves' disease to DM type 1. Other non endocrine disease association was represented by vitiligo and decalvandpelada. (Table IV).

Table IV. Prevalence of endocrine and non – endocrine disorders in the studied group

Associations	**Subjects group**	
	No	%
DM type 1	83	
DM type 1 + ACT	47	56.63%
DM type 1 + ACT + vitiligo	1	1.2 %
DM type 1 + ACT + decalvantpelada	1	1.2%
DM type 1 + Graves – Basedow disease	4	4.82%

From the 83 cases of children and adolescents, in 47 cases were associated with two autoimmune endocrine disorders, and in 2 cases three disorders. Type 1 diabetes was present in all 53 cases. In 49 cases with type 1 diabetes was associated ACT, and in 4 cases Graves' disease.

The most frequently detected PAS was PAS III containing after Blizzard's and Neufeld classification, association of autoimmune thyroid disease (Hashimoto's chronic thyroiditis, idiopathic myxedema, asymptomatic autoimmune thyroiditis, Graves' disease, endocrine ophthalmopathy) with:

- one or more autoimmune diseases - diabetes type 1 - type III a
- association with gastric atrophy, pernicious anemia - type III b
- association with vitiligo, alopecia, myasthenia gravis - type III c

Another classification of autoimmune polyendocrinopathies and of associated non endocrine systemic imunopathies was proposed by Volpe (Table V):

Table V. Autoimmune endocrino pathies and system icimunopathies commonly associated (R. Volpe, 1985 modify.) (10)

Autoimmuneendocrinopathies	**Non endocrine disorders**
GravesBasedowdisease	Perniciousanemia
Hashimoto's thyroiditis	Vitiligo
IdiopathicAddison's disease	Myastheniagravis
Insulin dependent diabetes mellitus	Sjogrensyndrome
Autoimmunegonadalinsufficiency	Rheumatoid arthritis
Autoimmune hypoparathyroidism	Thrombocytopenicidiopathicpurpura
Autoimmune pituitary disease	Chronic activehepatitis
Autoimmune sterility(through antisperm antibodies)	Primary biliary cirrhosis

In the case of PAS III, its worldwide prevalence is not known.

It turned out that occurs more frequently in women than in men and at middle age but can occur at any age. The most common PAS III is the type 3a (DM type 1 association with chronic autoimmune thyroiditis). DM type 1 can be consecutive or may precede thyroiditis (10).

In the children and adolescents group of, in all cases of diabetes type 1/ACT association, the first immunopathy was DM type 1, followed by ACT appearance at a variable period of time (10 cases after 10-15 years, 34 cases after 6 – 10years and 5 cases after 1-5 years of development of DM type 1) (Figure 1). In the case of association DM type 1/Graves-Basedow disease, the latter appeared before type 1 diabetes with 1 year in all 4 cases.

The medium interval between onset age of type 1 diabetes and the occurrence of ACT was 8.59 ± 3.24 years.

No case presented the first endocrine immunopathy ACT, followed by type 1 diabetes.

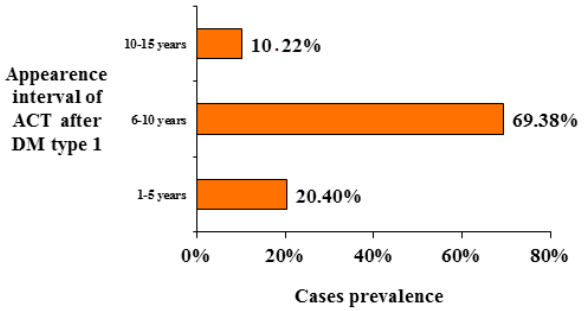

Fig. 1. Appearence interval of ACT after DM type 1

In the study group was a net predominance of females (85.71% girls and 14.29% boys, $p < 0.001$, $X^2 = 50$).

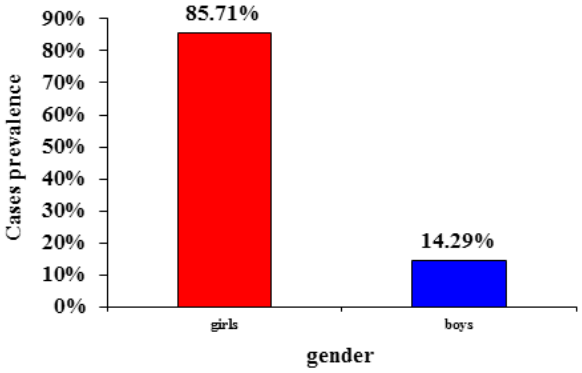

Fig. 2. Gender distribution of PAS III in the study group

Two endocrine immunopathies may be associated with a variable incidence no endocrineorgan-specific systemic diseases.In our studied group, the patients presented no endocrineorgan-specific autoimmune diseases. They were represented by vitiligo met at 1.2% cases and by the decalvantpelada found in 1.2% of cases. In 1 case was associated vitiligo, characterized by skin pigmentation due to autoimmune destruction of melanocytes. It occurred before the onset of endocrine immunopathies. Decalvantpelada was met in 1 case and also precede the onset of autoimmune endocrinopathies.

In literature, a case of PAS III c in 12-year-old boy with generalized vitiligo, alopecia universalis, and Hashimoto thyroiditis was recently reported from Turkey, and the patient was the youngest of previously reported cases (8).

In 4 cases we found the initial presence of Graves' disease, followed by an interval of 1 year by the occurrence of DM type 1.

So, in our study, the prevalence of PAS type III was 59.03 % (85.71% girls and 14.29% boys, $p < 0.001$, $X^2 = 50$). The subtype was III a (autoimmune thyroiditis + DM type 1), and prevailed in girls. In 1.2% were associated non endocrine disease as vitiligo and decalvantpelada, and in 4.82% cases we have association between DM type 1 and Graves' disease.

CONCLUSION

In the children group we have PAS III a, which prevailed in girls. Because the frequent association was between autoimmune thyroiditis and DM type 1, if we have a child with an autoimmune disease is better to investigate him for another autoimmune disease, because these can coexist and be asymptomatic.

Many disorders involved in PAS present a long prodromal phase, characterized by the presence of characteristics antibodies for each disorder in part, before the clinical manifestations.

Because the second disease appears after 6 – 10 years after the first and in this interval can be present, but asymptomatic, and because the first was DM type 1 follow by ACT after a variable period, and this can evolve with hypothyroidism it is better if we have an autoimmune disease to investigate it for another autoimmune disease.

Because the frequent association was between DM type 1 and ACT, it is better to determinate in the case of a patient with DM type 1 TSH and specific antibodies, and in the case of ACT fasting glucose annual.

The PAS (and mostly PAS III) classification is not final. This may change over time, with the onset of new endocrine disorders or associations with new autoimmune determination.

REFERENCES

1. Aung K, Salmon M. Polyglandular Autoimmune Syndrome, Type III, In *Endocrinology* (electronic book), 2006, 1-18.
2. Maclaren N. Autoimmune polyglandular syndromes. *Endocrinology*, Fifth Edition, 2009, Chapter 149.
3. Maclaren NK, Riley WJ. Thyroid, gastric, and adrenal autoimmunities associated with insulin-dependent diabetes mellitus. *Diabetes Care*, 1985, 1:34.
4. Neufeld M,Maclaren NK, BlizzardRM. Two types of autoimmune Addison's disease associated with different polyglandular autoimmune (PGA) syndromes. *Medicine* (Baltimore), 1981; 60:355.
5. Osserman KE. Muscels (myasthenia gravis). *Grune and Stratton*, New York,1969.
6. Song YH, Connor EL, Muir A, She JX, Zorovich B, Derovanesian D, Maclaren N. Autoantibody epitope mapping of the 21-hydroxylase antigen in autoimmune Addison's disease. *J Clin Endocrinol Metab*,1994; 78:1108.

7. Song YH, Connor E, LiY, ZorovichB, BalducciP, Maclaren N.The role of tyrosinase in autoimmune vitiligo. *Lancet*, 1994; 344:1049.
8. Turkoglu Z, Kavala M, Kolcak O, Zindanci I, Can B. Autoimmune polyglandular syndrome-3C in a child. *Dermatol Online J*, 2010;16(3):8.
9. Valerio G, Maiuri L, Troncone R. Severe clinical onset of diabetes and increased prevalence of other autoimmune diseases in children with coeliac disease diagnosed before diabetes mellitus. *Diabetologia*, 2002; 45: 1719-22.
10. Zosin I. Curs de endocrinologieclinică, *Lito UMF*, 1997; 59-107.

PREVALENȚA SINDROMULUI POLIGLANDULAR AUTOIMUN TIP III ÎNTR-UN GRUP DE COPII CU BOALĂ TIROIDIANĂ ŞI DIABET ZAHARAT

REZUMAT

Introducere: Sindromul poliglandular autoimun (SPA) (cunoscut şi ca sindromul insuficienţelor poliglandulare) reprezintă o asociere a mai multor insuficienţe a glandelor endocrine. Sindromul poliglandular autoimun tip III (SPA III) cuprinde tiroidita autoimună, diabetul zaharat mediat imun, boala celiacă, hipogonadismul, miastenia gravis, sarcoidoza, sindromul Sjogren, artrita reumatoidă, neoplazia gatrică, şi malabsorbţia. Scopul acestui studiu este de a determina prevalenţa PAS III într-un grup de copii cu boală tiroidiană şi diabet zaharat (DZ) tip 1. Metode: Grupul de copii a cuprins 83 de copii cu DZ tip 1 (71 fete şi 12 băieţi), cu vârsta între 7 şi 17 ani. Metodele de investigaţie au fost reprezentate de determinarea unor parametrii clinici, imagistici, biochimici, hormonali şi imunologici. Rezultate: Prevalenţa SPA III în grupul de studiu a fost de 59,03% (85,71% fete şi 14,29% băieţi, $p < 0.001$, $X^2 = 50$). De asemenea, subtipul SPA a fost tip III a (tiroidită cronică autoimună + DZ tip 1) şi a predominat la fete. În 1,2 % cazuri s-au asociat şi afecţiuni neendocrine ca vitiligo şi pelada decalvantă. Concluzie: În grupul de copii s-a întâlnit SPA III a, care a predominat la fete. Deoarece cea mai frecventă asociere a fost cea dintre tiroidita cronică autoimună şi DZ tip 1, în cazul în care avem un copil cu o boală autoimună este bine să-l investigăm şi pentru alte boli autoimune, deoarece acestea pot coexista şi pot fi asimptomatice.
Cuvinte cheie: diabet zaharat, boală autoimună tiroidiană, sindrom poliglandular autoimun tip III, copii

IN VITRO TOXICITY STUDIES ON BREAST CANCER CELL LINE SK-BR3 AND TUMOR-ASSOCIATED FIBROBLASTS (TAF)

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ABSTRACT
Solid tumors are formed of tumor cells and stroma. Within the tumor stroma, one of the most important component are the tumor-associated fibroblasts, which provide support for tumor cells development, growth, and metastasis. The purpose of this study was to investigate the effects of metformin, a well-known anti-diabetic drug, on breast cancer cell line (SK-BR3) and tumor-associated fibroblasts (TAFs) and to reveal the possible role of this drug as anti-tumor adjuvant treatment. We used two comparative methods, Alamar Blue assay which is an indicator of cellular metabolic activity, and xCELLigence system, which quantifies proliferation, viability and adherence of tested cells. We concluded that metformin induced a blockage of TAFs proliferation ability, but did not influence the viability of these cells.
Key words: Alamar Blue, tumor-associated fibroblasts, xCELLigence system, breast cancer

INTRODUCTION

Tumors are formed of two compartments: tumor cells and a variety of untransformed cells within a specialized extracellular matrix – stroma. Auxiliary cells found within the tumor stroma can be: fibroblasts, myofibroblasts, leukocytes, endothelial cells, and bone marrow-originating cells.

Recent studies showed that tumorigenesis depends upon signals which are received by tumor cells from peri-tumoral stroma. Due to diverse pathophysiological processes, the stroma is remodeling tumor progression. In normal tissues it has a barrier role against tumorigenesis, by blocking proliferation of tumor cells. However, during the tumorigenesis process it was proven that stroma helps tumor growth as a response to signals received from tumor cells.

Tumor development and progression towards advanced stages of the disease require co-evolution of both tumor and stromal cells. In order to facilitate this process, diverse changes take place at stromal level. Stromal fibroblasts are an important component of stroma, which suffer genetic and epigenetic transformations, thus modulating tumor progression. These cells are also known as myofibroblasts, tumor-associated fibroblasts or reactive fibroblasts.

The first hypothesis about inflammation role in tumorigenesis was elaborated by Virchow, who noticed that inflammation induced by certain irritant factors can enhance cellular proliferation (1). Several studies showed that fibroblasts are endowed with important role in immune response due to chemokines and cytokines production (2). It was supposed that chronic inflammation is strongly connected with abnormal fibroblasts behavior, which is not interrupting the inflammatory program and lead to to unsuitable survival signals and retention of leukocytes within inflamed tissue (3). From the clinical point of view, chronic inflammation and cancer are strongly related, cancers being considered as a wound that never heals. Tumor cells are secreting pro-inflammatory cytokines, while tumor-associated fibroblasts (TAFs) attract immune cells towards tumor tissue. Thus, macrophages, neutrophils, and lymphocytes are recruited by tumor stroma by diverse factors secreted by fibroblasts. Macrophages, once reaching tumor stroma, begin to differentiate into tumor-associated macrophages and help development of tumor and metastasis. By secretion of different factors, such as VEGF, HGF, MMP2, and IL-8, they can influence behavior of endothelial cells, which begin to be actively involved into the angiogenesis process (4).

Tumor invasion or metastasis is a complex process in which tumor cells of primary tumor tissue reach and colonize other tissues and organs. For a long time it was considered that metastasis is strictly influenced by genetic and epigenetic changes of tumor cells, but latest studies showed that interaction between tumor cells and stroma facilitate tumor invasion.

Different studies demonstrated that presence of a large population of myofibroblasts within the stroma of different tumor types is associated with an increased metastasis risk and poor prognosis. Cross-signaling between tumor cells and tumor-associated fibroblasts contribute to changes of extracellular matrix and basal membrane. It is know that basal membrane breakage is the first step towards intravasation of tumor cells within systemic circulation. On the other hand, extracellular matrix remodeling can alter expression of genes which are essential in cytoskeleton organization (5). Actually, tumor-associated fibroblasts have an

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invasive potential, demonstrated by in vivo studies. Using transgenic GFP (green fluorescent protein) mice, under the VEGF promoter control, Fukumura discovered that tumor-associated fibroblasts positive for GFP invade tumor sites (6). Another role of the fibroblasts is to direct tumor invasion towards a certain site. The results of in vitro experiments on co-cultures showed that cells guiding tumor invasion are of stromal origin and facilitate tumor cells migration due to degradation of extracellular matrix and basal membrane, thus inducing structural and proteolytic changes (7).

The purpose of this study was to investigate the effects of metformin, a well-known anti-diabetic drug, on breast cancer cell line (SK-BR3) and tumor-associated fibroblasts (TAFs) and to reveal the possible role of this drug as anti-tumor adjuvant treatment.

MATERIALS AND METHODS

xCELLigence system

We used RTCA DP Analyzer (ACEA Biosciences.Inc, San Diego, USA) for assessment of proliferation rate of breast cancer cell line SK-BR3 and tumor-associated fibroblasts (TAF). The cells were detached from the culture plate using Trypsin, and cellular suspension was replated in specific 16-well culture plate (E-plate 16), in humid atmosphere, with 5% CO_2, at 37° C. For each well we added 5000 cells in 150 μl of specific media. 24 hours after cell addition, we added metformin (Sigma-Aldrich Company, Germany) in 2 concentrations, of 5 mM and 10 mM. The results were interpreted before metformin addition, and 48 hours after addition of the chemical substance. The experiment lasted for 144 hours, and results interpretation was performed using RTCA Software (ACEA Biosciences.Inc).

xCELLigence system is based on using gold biosensors for detecting impedance change as a result of cellular interaction and the adherent surface of each well. Each sample is analyzed at least as duplicate. On the base of each well a microelectrode is placed to detect changes in cellular number, cellular morphology, cell adhesion and cell membrane integrity for specified time duration.

Alamar Blue assay

The proliferation rate was tested in parallel using AlamarBlue® (Invitrogen). AlamarBlue® is a non-toxic reagent which is used for evaluation of metabolic activity of live cells, which will reduce resazurin (dark blue colored compound) with intrinsic fluorescence, to resorufin, a pink-colored compound, highly fluorescent (extinction 579 nm, emission 584 nm). Maximum absorbance of resazurin is at 605 nm, while for resorufin it is 573 nm.

For AlamarBlue® assay the cells were cultured in 96-well plates, at a cellular density of 7000 cells/well, in quadruplicates. 24 hours after incubation, metformin was added in concentrations of 5 and 10 mM in specific media, while AlamarBlue® was added after 48 hours. The values of sample extinctions were read at 570 and 600 nm using Bio Rad Microplate Reader spectrophotometer (Benchmark). Interpretation of the AlamarBlue® test results uses the following calculation formula, which provides the level of reagent reduction due to cellular metabolic activity:

$$AB\%N = \frac{(O2 \times A1) - (O1 \times A2)}{(R1 \times N2) - (R2 \times N1)} \times 100$$

Where:
- AB%N-percentage value of Alamar Blue reagent reduction compared to negative control (culture media without cells)
- O1- molar extinction coefficient (E) for oxidized Alamar Blue at 570 nm
- O2- E for Alamar Blue oxidized at 600 nm
- R1- E for Alamar Blue reduced at 570 nm
- R2- E for Alamar Blue reduced at 600 nm
- A1- absorbance of tested cells at 570 nm
- A2- absorbance of tested cells at 600 nm
- N1- absorbance of negative control at 570 nm
- N2- absorbance of negative control at 600 nm

Molar extinction coefficients for Alamar Blue are:

Wave length	Reduced form (R)	Oxidized form (O)
570 nm	155677	80586
600 nm	14652	117216

Increased percent of reduced Alamar Blue is indicative of intense cellular metabolic activity, and high viability and cellular proliferation, because proliferating viable cells induce a chemical reduction of Alamar Blue reagent, while dead cells or non-proliferating cells maintain an oxidized form of the reagent.

RESULTS

xCelligence System assays

Analysis of cellular proliferation rate was performed in three stages: before metformin addition, 48 hours after metformin addition, and the following 48 hours. Considering the positive control proliferation rate (cells with specific medium), we noticed that 72 hours after plating (3 days) the proliferation rate begins to decrease. This meant that without any intervention, cellular viability is impaired. So, this was the reason why we considered the 48 hours time interval after addition of metformin (72 hours from cellular plating) as the relevant time interval for analysis of metformin effect on cellular proliferation.

Proliferation graph given by xCelligence system suggest that SK-BR3 cells proliferation increases with increase of metformin concentration (Figure 1). The graphs depicting time and proliferation velocity (doubling time and slope) support previous results. Three stages analysis showed that 48 hours after metformin addition in culture medium, proliferation velocity of SK-BR3 cells is more increased for 5 mM metformin concentration than for 10 mM metformin concentration (Figures 2 and 3).

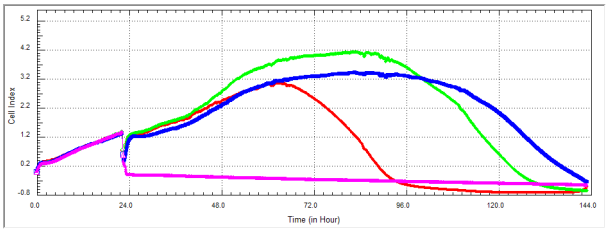
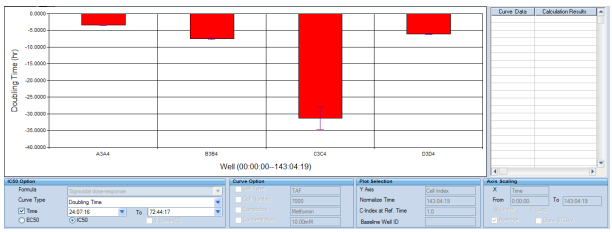


Fig. 1. Proliferation rate expressed by Cell index for SK-BR3 breast cancer cells during the entire experiment (from 0 to 144 hours).



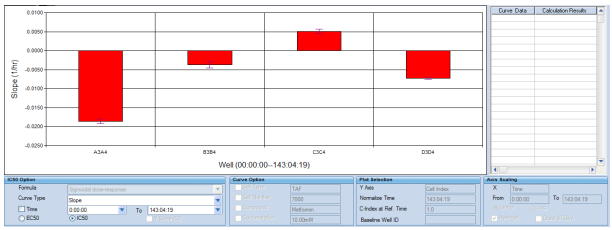
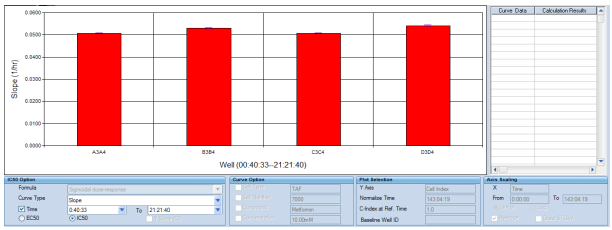
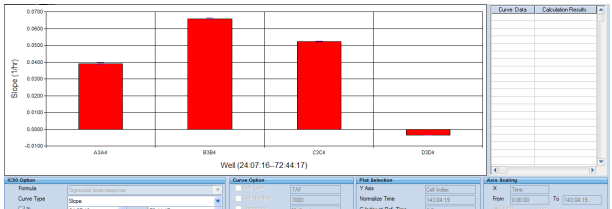


Fig. 2. Upper panel – Doubling time; lower panel – Slope (proliferation velocity) for SK-BR3 cells during the entire experiment (0-144 hours)





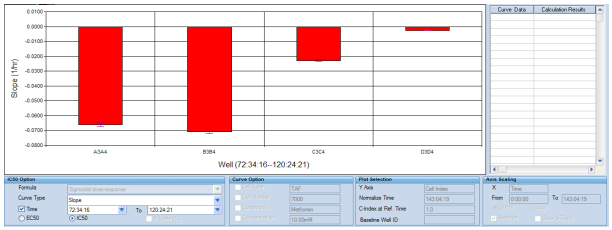


Fig. 3. Proliferation velocity (slope) for SK-BR3 cells. Upper panel – before metformin addition; middle panel – first 48 hours after metformin addition; lower panel – after addition of 5 mM and 10 mM metformin

Proliferation diagram given by xCelligence system suggest that TAFs proliferation decreases when cells are treated with metformin (Figure 4). The graphs representing doubling time and proliferation velocity (slope) are also supporting this result. Three stages analysis showed that 48 hours after metformin addition in culture medium, proliferation velocity of TAFs decreased proportional with metformin concentration, being lower for 10 mM than 5 mM metfomin concentration (Figures 5 and 6).

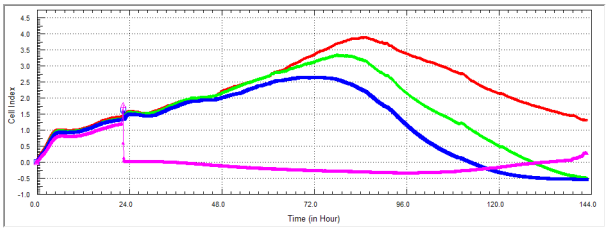
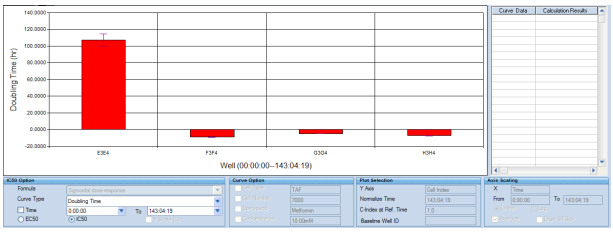


Fig. 4. Proliferation rate expressed by Cell index for TAFs during the entire experiment (from 0 to 144 hours).



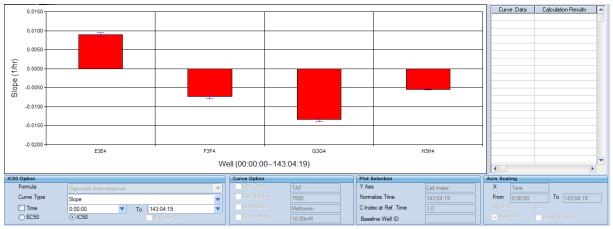
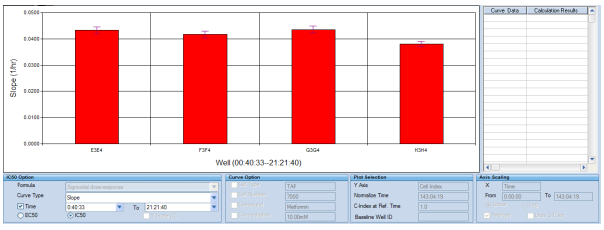
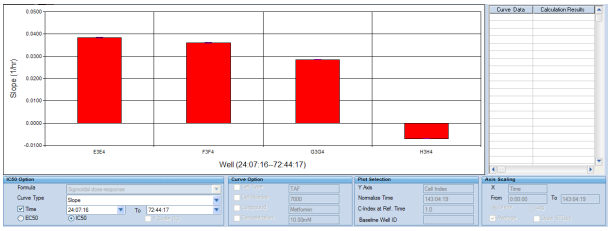


Fig. 5. Upper panel – Doubling time; lower panel – Slope (proliferation velocity) for TAFs during the entire experiment (0-144 hours)





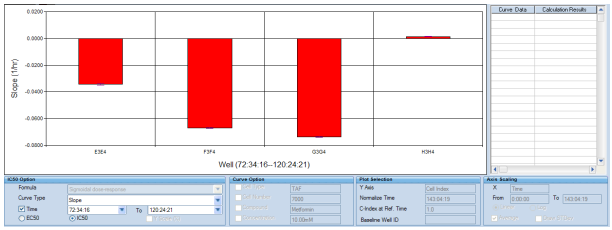


Fig. 6. Proliferation velocity (slope) for TAFs. Upper panel – before metformin addition; middle panel – first 48 hours after metformin addition; lower panel – after addition of 5 mM and 10 mM metformin

Alamar Blue assay results

Alamar Blue assay was performed during three days, and daily we tested the metabolic activity of SK-BR3 cells and TAFs, untreated, and treated with metformin in 5 mM and 10 mM concentration.

Within the analysis we monitored time-dependent and metformin concentration-dependent metabolic activity of the cells. For SK-BR3 we noticed a decrease in metabolic activity in the first day, followed by an increase in the following two days (day 2 and 3). We can notice a higher decrease of metabolic activity for cells treated with lower metfromin concentration (Figure 7). For TAFs, we were not able to detect any change in metabolic activity, for any of metformin concentrations. Moreover, related to time, metabolic activity remains constant (Figure 8). The results suggest that 5 mM metformin concentration negatively influences metabolic activity of SK-BR3 cells, more than higher metformin concentration (10 mM). Cellular metabolic activity of TAFs is not influenced by metformin.

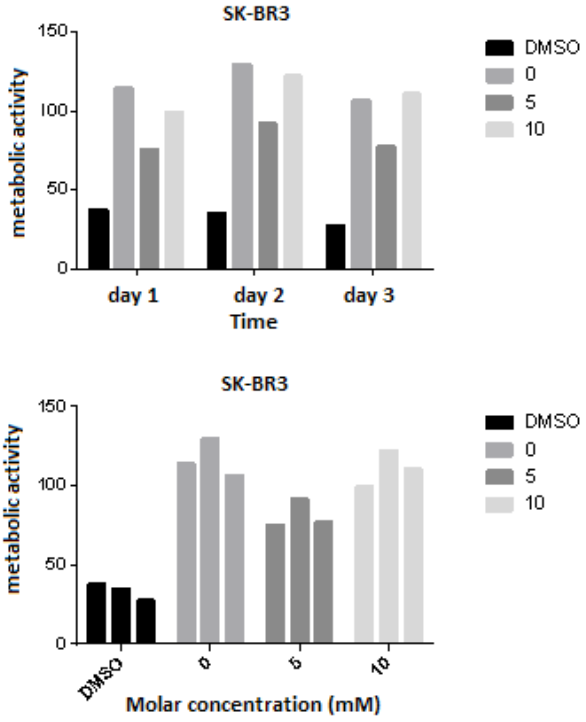


Fig. 7. Alamar Blue assay results for SK-BR3 during 3 days of experiment after addition of 5 mM and 10 mM, comparative to positive control cells (untreated) and negative control cells (DMSO-treated).

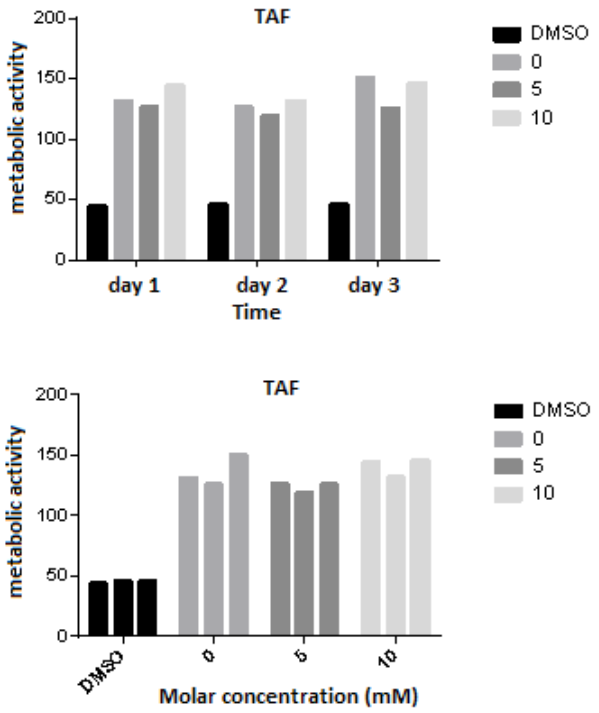


Fig. 8. Alamar Blue assay results for TAFs during 3 days of experiment after addition of 5 mM and 10 mM, comparative to positive control cells (untreated) and negative control cells (DMSO-treated).

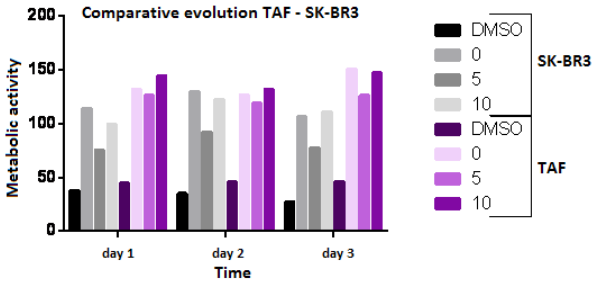


Fig. 9. Comparative analysis of metabolic activity for TAFs and SK-BR3 when treated with 5 mM and 10 mM metformin, during 3 days of experiment.

The results of *in vitro* toxicity studies performed with xCelligence system showed that SK-BR3 proliferation rare increases when metformin is added into culture media. Although proliferation velocity is increased in presence of metformin, cellular metabolic activity of SK-BR3 cells is negatively influenced. Toxicity studies employing Alamar Blue reagent suggest that 5 mM metformin concentration induced more important changes in metabolic activity than 10 mM concentration. Interestingly, following the diagram of proliferation velocity within 48 hours after metformin addition, we noticed that proliferation velocity (slope) increases for 5 mM metformin canocentration, more than for 10 mM metformin concentration, with no statistic significance. So, we may conclude that metformin effect on cellular proliferation and metabolic activity is augmented for 5 mM compared to 10

mM. Metformin does not stop SK-BR3 cells proliferation, but stimulates it, inducing in the same time changes in cellular metabolism. In order to establish the connection between increased proliferation velocity and decreased cellular metabolism, many elaborated cellular metabolism tests are required.

In contrast with results obtained for SK-BR3 cells, metformin decreases TAFs proliferation, without affecting cellular metabolism. Proliferation diagram obtained using the xCelligence system demonstrates that TAFs proliferation decreases when metformin is added into the culture medium, anti-proliferative effect being stronger for 5 mM metformin concentration. According to Alamar Blue tests, metabolic activity of TAFs is not influenced by metformin. So, we may conclude that metformin decrease the proliferation rate, without affecting the cellular viability results. Metformin is not inducing cellular death, but probably blocks the cells in a certain phase of cellular cycle.

DISCUSSION AND CONCLUSION

There are numerous studies related to metformin action on breast cancer cells. Most studies are performed on MCF-7 cell line, and only few studies included SK-BR3 cells. Vasquez-Martin et al. studied the influence of metformin on Her2 expression in SK-BR3 cells (8). In this study, SK-BR3 were treated with diverse metformin concentrations and they noticed a decreased cellular metabolic activity and viability, directly proportional with concentration increase. Our results also showed that metabolic activity decreases with metformin addition, but we were not able to demonstrate the dose-effect relationship (lower concentration induced a more decreased viability compared to higher metformin concentration).

Metformin action was tested on different tumor cells, but there was no study published regarding metformin effects on tumor-associated fibroblasts. There are many studies supporting the importance of fibroblasts for tumor growth and development. Considering all the above, we investigated metformin effects on tumor-associated fibroblasts. As a result of our experiments, we concluded that metformin induces a decrease of TAFs proliferation capacity, but is not influencing cellular metabolism and viability. Thus, we concluded that further analyses are required, including cell cycle assays, to investigate whether metformin blocks the cell cycle, and to show in which of its phase. There are studies showing that metformin blocks cell cycle in pancreatic tumor cells (9), and triple negative breast cancer cells (10), while metformin blocks prostate cancer cells in G(0)/G(1) phase (11).

As a conclusion, we may say that metformin can perform its anti-tumor action on tumor-associated fibroblasts and can be considered as adjuvant treatment to anti-tumor chemotherapy.

Acknowledgements

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REFERENCES

1. Balkwill F, Mantovani A. Inflammation and cancer: back to Virchow? *The Lancet*, 2001; 357(9255): 539-45.
2. Buckley C, Filer A, Haworth O, Parsonage G, Salmon M. Defining a role for fibroblasts in the persistence of chronic inflammatory joint disease. *Ann Rheum Dis*, 2004; 63: ii92-ii95.
3. Buckley CD, Pilling D, Lord JM, Akbar AN, Scheel-Toellner D, Salmon M. Fibroblasts regulate the switch from acute resolving to chronic persistent inflammation. *Trends Immunol.*, 2001; 22(4): 199-204.
4. Leek RD, Harris Al. Tumor-associated macrophages in breast cancer. *J Mammary Gland Biol Neoplasia*, 2002; 7(2): 177-189.
5. Lukashev ME, Werb Z. ECM signalling: orchestrating cell behaviour and misbehaviour. *Trends Cell Biol.*, 1998; 8(11): 437-441.
6. Fukumura D, Xavier R, Sugiura T, Chen Y, Park EC, et al. Tumor induction of VEGF promoter activity in stromal cells. *Cell*, 1998; 94(6): 715-25.
7. Gaggioli C, Hooper S, Hidalgo-Carcedo C, Grosse R, Marshall JF, Harrington K, Sahai E. Fibroblast-led collective invasion of carcinoma cells with differing roles for Rho-GTPases in leading and following cells. *Nat. Cell Biol.*, 2007; 9(12): 1392-1400.
8. Vazquez-Martin A, Oliveras-Ferraros C, Menendez JA. The antidiabetic drug metformin suppresses HER2 (erbB-2) oncoprotein overexpression via inhibition of the mTOR effector p70S6K1 in human breast carcinoma cells. *Cell Cycle*, 2009; 8(1): 88-96.
9. Wang LW, Li ZS, Zou DW, Jin ZD, Gao J, Xu GM. Metformin induces apoptosis of pancreatic cancer cells. *World J. Gastroenterol.*, 2008; 14(47): 7192-98.
10. Liu B, Fan Z, Edgerton SM, Deng XS, Alimova IN, Lind SE, Thor AD. Metformin induces unique biological and molecular responses in triple negative breast cancer cells. *Cell Cycle*, 2009; 8(13): 2031-2040.
11. Sahra IB, Laurent K, Loubat A, Giorgetti-Peraldi S, Colosetti P, et al. The antidiabetic drug metformin exerts an antitumoral effect in vitro and in vivo through a decrease of cyclin D1 level. *Oncogene*, 2008; 27(25): 3576-86.

STUDII DE TOXICITATE *IN VITRO* EFECTUATE PE LINIA CELULARA DE CANCER DE SAN SK-BR3 SI FIBROBLASTELE PERI-TUMORALE (TAF)

REZUMAT

Tumorile solide sunt formate din celule tumorale si stroma. La nivelul stromei tumorale, una dintre cele mai importante componente sunt fibroblastele peri-tumorale, care asigura suport pentru dezvoltarea tumorala, crestere si metastazare. Scopul acestui studiu a fost investigarea efectelor metforminului, un anti-diabetic oral frecvent utilizat, asupra celulelor liniei de cancer mamar (SK-BR3) si la nivelul fibroblastelor peri-tumorale (TAFs), pentru a releva un posibil rol al acestui medicament in terapia adjuvanta anti-tumorala. Am utilizat doua metode comparative, testul Alamar Blue, care este un indicator al activitatii metabolice celulare si sistemul xCELLigence, care poate oferi informatii referitoare la proliferarea, viabilitatea si aderenta celulara. In urma analizei rezultatelor obtinute putem concluziona ca metforminul induce blocarea capacitatii de proliferare a TAFs, dar nu influenteaza viabilitatea acestor celule.
Cuvinte cheie: Alamar Blue, fibroblaste peri-tumorale, sistem xCELLigence, celule tumorale mamare

LYMPHOCYTES SUBSETS INVOLVED IN ALZHEIMER DISEASE-ASSOCIATED IMMUNOSENESCENCE

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ABSTRACT
Immunosenescence is defined as progressive alteration of morphological and functional characteristics, at the level of immune system, during the aging process. The purpose of this study was to define the immunosenescence status, to characterize proliferative potential and apoptosis intensity in T lymphocyte subpopulations, as well as to define the Alzheimer disease (AD)-associated immunosenescence in clinically diagnosed dementia, as well as prodromal stages (pre-dementia). The expected impact of this study is related to significant development of state of the art in the field, related to mechanisms and clinical symptoms occurring in immunosenescence and AD-related immunosenescence.
Key words: immunosenescence, Alzheimer disease, pre-dementia

INTRODUCTION
Alzheimer's disease (AD) is one of the most common forms of dementing disorder, characterized by deterioration of functional and cognitive ability. From the neurological point of view, Alzheimer disease (AD) patients present loss of synaptic and neuronal capacity, and extracellular plaques, mainly consisting of amyloid-β (aβ), and intracellular neurofibrillary tangles of the microtubule-associated protein Tau. The amyloid hypothesis is based on increased production of aβ, cytoskeletal changes, and neuronal loss. These aβ changes are associated with disease-causing mutations in genes related to the generation of aβ.

The pathology typically starts in the structures of the medial temporal lobe, and extends to the other cortical areas in a hierarchical manner (1,2), leading to vascular alterations with deposition of aβ in the vessel walls, especially in carriers of the apolipoprotein (apoE) ε4 allele (3), as well as various inflammatory reactions (4).

The possible influence of viral infections on AD development has been investigated (5), such as herpes simplex virus andhuman cytomegalovirus (CMV). These viruses cause chronically persistent infection that in the immunocompetent adult rarely escapes immune surveillance, but can causesevere disease in patients with suppressed immune function (6,7). The CMV hasbeen shown to inflict a deep imprint in the host T-cell compartment that is characterized by an age-related oligoclonal expansion of differentiated CD8 (CD27-CD28-) cellsand a corresponding decrease inproportion of naïve cells (8-11). Also, the degree of differentiation in the CD4compartments has been shown to correlate with levels of CMVIgG(12). Alterations in systemic immunity have been shown to occurin the elderly, and the term immunosenescence is used to describe the age-related decline in capacity and regulatorybalance of both innate and adaptive immune responses (13,14). Deregulation of immunoactive cells could also explainthe progression of baseline systemic inflammation called inflammaging, which is considered a risk factor for several age-relateddiseases and where the role of CMV has beeninvestigated (15). Clinically important immunosenescence signs include an impaired vaccine response and an increased incidence of severe bacterial and viralinfections in the elderly. In Swedish octogenarian andnonagenarian cohorts (16,17) a defined immune risk profile(IRP) consisting of a shift in CD4/CD8 ratio was associatedwith increased morbidity and mortality (18). Alzheimer's disease has previously been associated withshifts in CD4 as well as CD8 T-cell subsets (19-21).

The aim of this study was to establish the correlation between AD-associated immunosenescence and T cell subpopulation characteristic markers, in different stages of AD, comparatively with control, healthy patients.

MATERIALS AND METHODS
In order to establish the study groups for patients with AD in different stages, we evaluated 24 patients, age between 57-84 years old. After signing the Informed Consent form (elaborated based on a well-established protocol, approved by the Ethics Committee of "Victor Babes" University of Medicine and Pharmacy Timisoara), two tests were applied to these subjects: Standardized Mini-Mental State Examination and Clock drawing test.

As a result of this evaluation, correlated with other clinical symptoms, the subjects were divided in two groups: early stage dementia (10 patients) and severe dementia (14 patients). As control group, we investigated 20 elderly patients without dementia, with similar age range.

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Blood harvesting and cell processing

10 ml peripheral blood was harvested on anticoagulant (Heparin) from both study and control groups. Blood was diluted with Dulbecco's Phosphate Buffered Saline (PBS; Sigma-Aldrich Company, St. Louis, USA) with a dilution ratio of 1:1, and then we used gradient centrifugation (Ficoll-Hipaque, GE Healthcare Bio-Sciences AB) for 25 minutes at 2500 rpm for peripheral blood mononuclear cells (PBMCs) separation. All PBMCs samples were cryopreserved until further use.

Cell cycle analysis

PBMCs were submitted to cell cycle analysis, performed using CycleTest™ Plus, DNA Reagent Kit (Becton-Dickinson, San Jose, CA, USA). This method involves dissolving cell membrane lipids, eliminating cellular cytoskeleton and nuclear proteins, digestion of cell RNA, and stabilization of nuclear chromatin, followed by propidium iodide (PI) binding to the clean, isolated nuclei. All procedures were according to the manufacturer protocols and included a first step of addition of 250 µL of Solution A (trypsin buffer) which was left to interact for 10 minutes at room temperature. A second step of 200 µL of Solution B (trypsin inhibitor and RNase buffer) addition and 10 minutes resting at room temperature was followed by the last step of 200 µL of cold (2° to 8°C) Solution C (propidium iodide stain solution) addition in the same tube, gentle mixture and incubation for 10 minutes in the dark in the refrigerator.

Annexin V/PI viability assay

Annexin V-FITC (MiltenyiBiotec, Gladbach, Germany) was used in cell death flowcytometric studies (apoptosis) combined with Propidium Iodide Staining Solution (BD Biosciences, San Jose, CA, USA) following the manufacturer protocol. Shortly, 10^6 cells were washed in 1 x Annexin V Binding Buffer (BD Pharmigen) and centrifuged at 300 x g for 10 minutes, resuspended in the same solution and incubated with 10 µl of Annexin V-FITC for 15 minutes in the dark. After washing the cells with 1 ml specific binding buffer and centrifugation, the cell pellet was resuspended in 500 µl binding buffer and 1 µg/ml of PI solution was added immediately prior to analysis by flow cytometry.

Data acquisition for Annexin V/PI assay and Cell cycle flowcytometric procedures was performed on a four-color capable FACSCalibur (Becton-Dickinson) flow cytometer, while data analysis employed CellQuest Pro software (Becton-Dickinson).

Immunophenotypical analysis of PBMC

For flowcytometric analysis of surface and intracellular markers, PBMCs were washed twice in PBS and resuspended in 100 µL PBS for a concentration of 10^5 cells/mL. Cells were incubated at room temperature, in the dark for 30 minutes with mouse anti-human antibodies, conjugated with specific fluorochromes, at a dilution specified by the producer. Cells were then washed twice with 1 mL of cell wash solution (Cell Wash Solution - BD Biosciences, San Jose, CA, USA) and resuspended in 500 µL of the same solution for further acquisition and analysis with FACSCalibur flowcytometer (Becton-Dickinson), which can detect 4 colors simultaneously. PBMCs were analyzed for presence of the following markers: CD3 (FITC)/CD16+56 (PE)/CD45 (PerCP)/CD19 (APC); CD3 (FITC)/CD8 (PE)/CD45 (PerCP)/CD4 (APC); CD45RA (FITC)/CD45RO (PE)/CD3 (PerCP)/CD4 (APC); CD25 (FITC)/CD154 (PE); CD28 (FITC)/CD184 (PE); Bcl2 (FITC)/TCR γδ (PE). Conjugated antibodies used were purchased from BD Pharmingen™. Sample acquisition and analysis was performed using CellQuest Pro software (Becton-Dickinson), and Flowing Software 2.5, respectively.

RESULTS AND DISCUSSION

Statistical analysis of PBMCs in different phases of cell cycle showed that AD patients present a decrease of cells in DNA synthesis and replication phase (S) or mitosis preparation phase (G2/M), which demonstrates that proliferation rate of these cells is much decreased. Similar results can be observed in case of patients with pre-Alzheimer, where there is an increase of cells in growth and DNA synthesis preparation phase (G0/G1), but this increase ratio is not found in other phases of cell cycle, demonstrating blockage of cell proliferation (Figures 1, 2 and Table I).

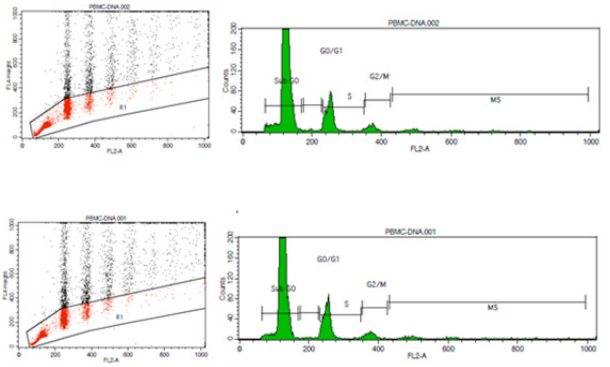

Fig. 1. Graphic results for cell cycle analysis in young subjects/older subjects groups

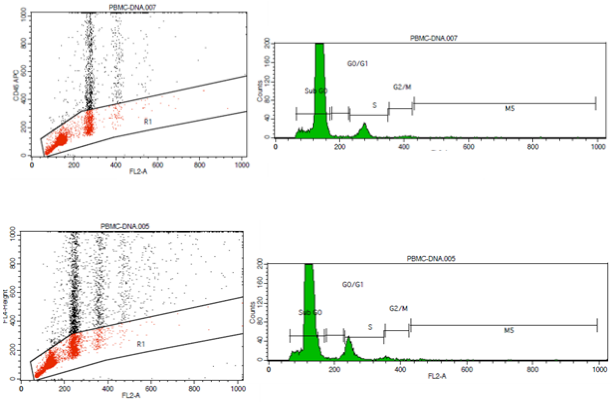

Fig.2. Graphic results for cell cycle analysis in pre-Alzheimer/Alzheimer patients group

Table I. Statistic analysis of PBMC in different phases of cell cycle

Sample	Sub-G0 (%)	G0/G1 (%)	S (%)	G2/M (%)
Control	88.12±2.24	0.32±0.05	8.9±1.03	1.53±0.24
Pre-Alzheimer	91.56±1.85	1.13±0.08	6.09±0.89	0.63±0.12
Alzheimer	94.23±1.79	0.49±0.04	4.37±0.76	0.39±0.13

Statistical analysis of apoptosis propensity in different study groups (Figure 3) shows that, compared to control subjects, intensity of apoptotic processes in pre-Alzheimer patients and AD patients, respectively, is significantly increased compared to control group (Table II). There are no statistical significant differences between pre-Alzheimer and AD patients regarding the total apoptotic processes. Moreover, cell death is similar between different study groups, significant differences being in case of early apoptosis (36% compared to 5%) and late apoptosis (8% compared to 2%), respectively (data not shown).

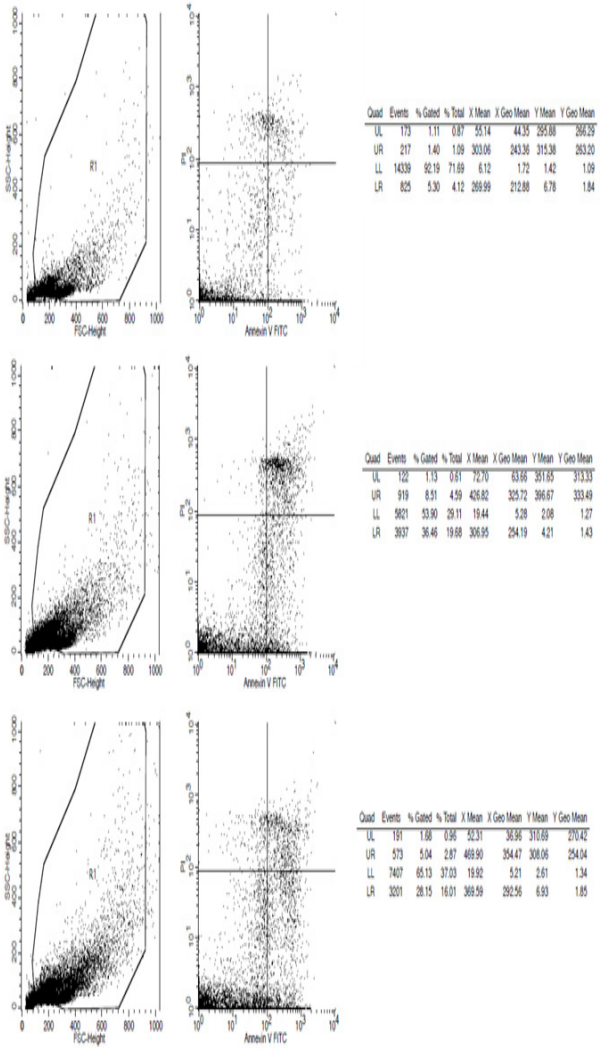

Fig. 3. Graphic aspects of Annexin V/PI viability assay in control/pre-Alzheimer/Alzheimer patients groups

Table II. Statistical analysis of PBMC in different apoptosis phases in control, pre-Alzheimer, and Alzheimer subjects

Sample	Un-functional cells (dead cells, late/ early apoptotic cells)	Viable cells
Control	15.13±1.2%	84.87±1.3%
Pre-Alzheimer	62.09±0.98%	37.91±1.02%
Alzheimer	67.7±0.95%	32.3±1.04%

Flowcytometric analysis of T cells subpopulations showed normal distributions of cytotoxic T cells ($CD4^+$), with significant proportion of activated cells ($CD25^+$), less naïve T cells ($CD45RA^+$), and increased number of memory T cells ($CD45RO^+$). The ratio between Th/CTL is maintained at 2/1.

For intracellular staining of Bcl2 we used monoclonal antibody mouse anti-human Bcl2 oncoprotein/FITC (DakoCytomation) and DakoCytomation Intrastain – intracellular staining kit, in accordance with producer recommendation. Bcl2oncoprotein is associated to mitochondria, smooth endoplasmic reticulum, and peri-nuclear membrane, being endowed with central role in apoptosis inhibition (programmed cell death). Expression of this protein in T cells from the control group was evaluated at 10% from total T lymphocytes (Figure 4).

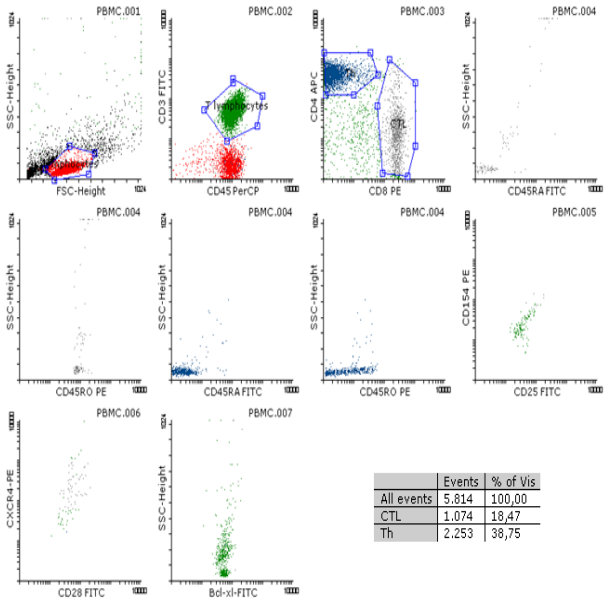

Fig. 4. Flowcytometric analysis of T cells subpopulations for subjects in control group. Dotplot graphs for markers representative to studied lymphocytic subpopulations.

In case of patients from the pre-Alzheimer and AD groups, respectively, we used the same panel for acquisition of analysis of T cells subpopulation. We revealed a change in Th/CTL ratio from 2/1 to less than 1, with increased proliferation of cytotoxic $CD8^+$ T cells. However, CTLs are naïve cells ($CD8^+CD45RA^+$), wich are not activated at global level ($CD25^-$). Bcl2 oncoprotein expression level was evaluated at 8% from total T lymphocytes,

significantly decreased compared to control groups, both young and elders (Figure 5).

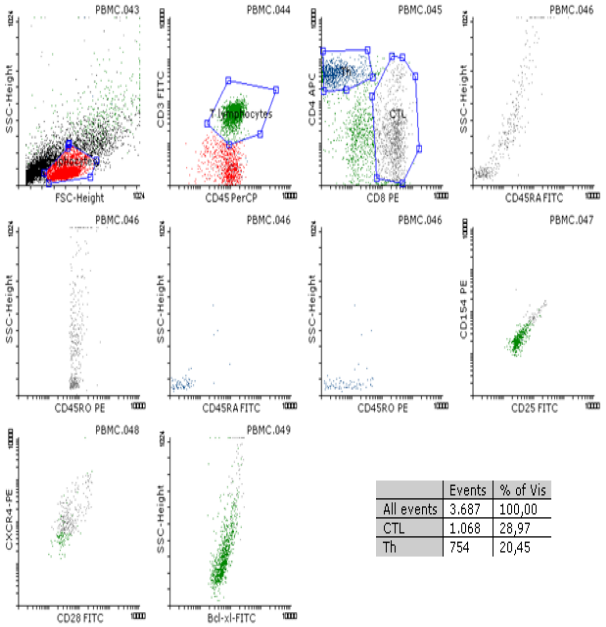

Fig. 5. Flowcytometric analysis of T cells subpopulations for subjects in pre-Alzheimer and AD groups. Dotplot graphs for markers representative to studied lymphocytic subpopulations.

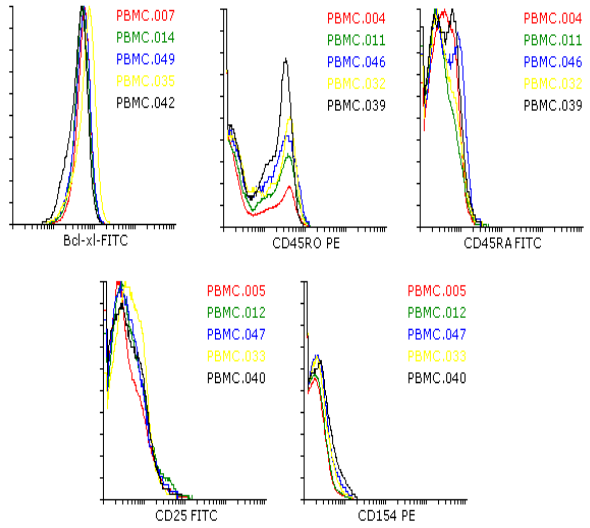

Fig. 6. Flowcytometric analysis of T cells subpopulations, comparatively for all studied groups. Histogram graphs for markers representative to studied lymphocytic subpopulations (analysis was performed using Flowing Software 2.5)

As the individuals are growing older, there is an important decrease in immune response. This leads in most of the cases to occurrence of severe infections and decreased protective effect of vaccination (22). Thymus involution and decreased production of T lymphocytes are the most frequent changes which occur in elders (23). This is the reason why in elders there is an increase proportion of highly differentiated T cells, which exhibits surface phenotypic markers and present specific functional characteristics (22,24). Additionally, alteration in cytokines production or in recognition of these cytokines by the cells of immune system, are responsible of changes observed during the immunosenescence process, especially associated with AD (25).

Expression of co-stimulatory molecules CD27 and CD28 was used for study of differentiation phenotype of antigen-specific cells, during chronic phase of persistent infection (5). According to T lymphocytes differentiation pattern, $CD27^{+}CD28^{+}$ T cells represent population of naïve cells or cells in early stages of differentiation, which progresses towards $CD27^{-}CD28^{-}$ phenotype, which represent finally differentiated T cells (26). Due to association of CD27 and CD28 expression with different differentiation stages of both CD4+ and CD8+ T cells (5), this classification is used for comparing the subsets of CD4+ T cells in young persons, elders, and neurodegenerative disorders patients (AD). For studying differentiation status of CD4+ T cells, some other markers may be used, such as CD45RA, CD11a, CCR7, perforines production, and telomere length.

Although cytokines production is controversial in elders, it is well known that senescence is associated with increased global production of INFγ (22). INFγproduction by $CD8^{+}$ T cells was investigated and correlated with accumulation of effector T cells $CD28^{-}$, terminally differentiated, while their number increases dramatically in neurodegenerative disorders. However, there are few data related to cytokines profile of $CD4^{+}$ T cells subpopulations, correlated with senescence process, especially in AD-associated immunosenescence. Another unsolved aspect is the correlation between cytokines production related to T cells shift, from naïve $CD4^{+}$ cells to effector, memory cells. $CD4^{+}$ T cells are essential for induction of immunity as a result of vaccination. Due to decreased vaccination protection in elders, further directions of research would be to monitor and compare proliferation capacity of $CD4^{+}$ T cells in healthy youngers and elders, to AD patients in different disease stages.

Acknowledgements

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REFERENCES

1. 1Hyman BT, Van Hoesen GW, Damasio AR, Barnes CL. Alzheimer's disease: cell-specific pathology isolates the hippocampal formation. *Science*, 1984; 225: 1168-1170.
2. Braak H, Braak E. Neuropathologicalstageing of Alzheimer-related changes. *ActaNeuropathol*, 1991; 82: 239-259.
3. Premkumar DR, Cohen DL, Hedera P, Friedland RP, Kalaria RN. Apolipoprotein E-epsilon4 alleles in cerebral amyloid angiopathy and cerebrovascular pathology associated with Alzheimer's disease. *Am J Pathol*, 1996; 148: 2083-2095.
4. Nilsson L, Rogers J, Potter H. The essential role of inflammation and induced gene expression in the pathogenic pathway of Alzheimer's disease. *Front Biosci*, 1998; 3: d436-d446.
5. Marques AR, Straus SE, Fahle G, Weir S, Csako G, et al.Lack of association between HSV-1 DNA in the brain, Alzheimer's disease and

apolipoprotein E4. *J Neurovirol*, 2001; 7: 82-83.
6. Boeckh M, Nichols WG. The impact of cytomegalovirus serostatus of donor and recipient before hematopoietic stem cell transplantation in the era of antiviral prophylaxis and preemptive therapy. *Blood*, 2004; 103: 2003-2008.
7. Sund F, Lidehäll AK, Claesson K, Foss A, Tötterman TH, et al. CMV-specific T-cell immunity, viral load, and clinical outcome in seropositive renal transplant recipients: a pilot study. *Clin Transplant*, 2010; 24: 401-409.
8. Khan N, Shariff N, Cobbold M, Bruton R, Ainsworth JA, et al. Cytomegalovirus seropositivity drives the CD8 T cell repertoire toward greater clonality in healthy elderly individuals. *J Immunol*, 2002; 169: 1984-1992.
9. Vescovini R, Biasini C, Fagnoni FF, Telera AR, Zanlari L, et al.Massive load of functional effector CD4+ and CD8+ T cells against cytomegalovirus in very old subjects. *J Immunol*, 2007; 179: 4283-4291.
10. Vescovini R, Telera A, Fagnoni FF, Biasini C, Medici MC, et al.Different contribution of EBV and CMV infections in very long-term carriers to age-related alterations of CD8+ T cells. *ExpGerontol*, 2004; 39: 1233-1243.
11. Derhovanessian E, Maier AB, Hähnel K, Beck R, de Craen AJ, et al. Infection with cytomegalovirus but not herpes simplex virus induces the accumulation of late-differentiated CD4+ and CD8+ T-cells in humans. *J Gen Virol*, 2011; 92: 2746-2756.
12. Alonso Arias R, Moro-García MA, Echeverría A, Solano-Jaurrieta JJ, Suárez-García FM, et al. Intensity of the humoral response to cytomegalovirus is associated with the phenotypic and functional status of the immune system. *J Virol*, 2013; 87: 4486-4495.
13. Solana R, TarazonaR, Aiello AE, Akbar AN, Appay V, et al. CMV and Immunosenescence: from basics to clinics. *Immun Ageing*, 2012; 9: 23.
14. Herndler-Brandstetter D, Landgraf K, Tzankov A, Jenewein B, Brunauer R, et al. The impact of aging on memory T cell phenotype and function in the human bone marrow. *J LeukocBiol*, 2012; 91: 197-205.
15. Bartlett DB, Firth CM, Phillips AC, Moss P, Baylis D, et al. The age-31: 685-90.
related increase in low-grade systemic inflammation (Inflammaging) is not driven by cytomegalovirus infection. *Aging Cell*, 2012; 11: 912-915.
16. Wikby A, Johansson B, Olsson J, Löfgren S, Nilsson BO, et al. Expansions of peripheral blood CD8 T-lymphocyte subpopulations andan association with cytomegalovirus seropositivity in the elderly: theSwedish NONA immune study. *ExpGerontol*, 2002; 37: 445-453.
17. Wikby A, Maxson P, Olsson J, Johansson B, Ferguson FG. Changes in CD8 and CD4 lymphocyte subsets, T cell proliferationresponses and non-survival in the very old: the Swedish longitudinalOCTO-immune study. *Mech Ageing Dev*, 1998; 102: 187-198.
18. Colonna-Romano G, Akbar AN, Aquino A, Bulati M, Candore G, et al. Impact of CMV and EBV seropositivity on CD8 T lymphocytes inan old population from West-Sicily. *ExpGerontol*, 2007; 42: 995-1002.
19. Pellicanò M, Larbi A, Goldeck D, Colonna-Romano G, Buffa S, et al.Immune profiling of Alzheimer patients. *J Neuroimmunol*, 2012; 242:52-59.
20. Larbi A, Pawelec G, Witkowski JM, Schipper HM, Derhovanessian E, et al. Dramatic shifts in circulating CD4 but not CD8 T cell subsetsin mild Alzheimer's disease. *J Alzheimers Dis*, 2009; 17: 91-103.
21. Pirttilä T, Mattinen S, Frey H. The decrease of CD8-positivelymphocytes in Alzheimer's disease.*J NeurolSci*, 1992; 107: 160-165.
22. Gruebeck-Loebenstein B, Wick G. The aging of the immune system. *Adv. Immunol*., 2002; 80:243.
23. Gardner EM, Murasko DM. Age-related changes in type 1 and type 2 cytokine production in humans. *Biogerontology*, 2002; 3:271.
24. Banderes E, Merino J, Vazquez B, et al. The increase INF-gamma production through aging correlates with the expanded CD8(+high) CD28(-)CD57(+) subpopulation. *Clin.Immunol.*, 2000; 96:230.
25. Fulop T, Larbi A, Pawelec G. Human T cell aging and the impact of persistent viral infections. *Front Immunol*, 2013; 4:27
26. Derhovanessian E, Theeten H, Hahnel K, et al. Cytomegalovirus associated accumulation of late-differentiated CD4 T cells correlates with poor humoral response to influenza vaccination. *Vaccine*, 2013;

SUBPOPULAȚII LIMFOCITARE IMPLICATE ÎN IMUNOSENESCENȚA ASOCIATĂ BOLII ALZHEIMER

REZUMAT

Imunosenescenta este definita ca alterarea progresiva a aspectului morphologic si functional, care apare la nivelul sistemului imun in timpul procesului de imbatranire. Scopul acestui studiu a fost definirea statusului de imunosenescenta, caracterizarea potentialului proliferativ si a gradului de apoptoza a subpopulatiei de limfocite T si definirea imunosenescentei asociate bolii Alzheimer (AD) in dementa clinic diagnosticata si in stadiile prodromale (predementa). Impactul asteptat al acestui studiu este legat de dezvoltarea semnificativa a nivelului cunoasterii in acest domeniu, in ceea ce priveste mecanismele si manifestarile imunosenescentei si imunosenescentei asociata bolii Alzheimer.

Cuvinte cheie: imunosenescenta, boala Alzheimer, predementa

METHYLENETETRAHYDROFOLATE REDUCTASE GENE MUTATIONS AND OPPORTUNITY OF ANTICOAGULANT TREATMENT

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ABSTRACT
Considering that hypercoagulable status means accurate diagnostic, starting from clinical signs, most of them unspecific, along with expensive lab work, we considered useful studying thrombotic events in patients with modified molecular genetic analysis and establishing a connection between the existence of these mutations and the risk for thrombotic events. Also, we considered the opportunity of anticoagulant treatment to decrease this pathology. Existing studies and guidelines concluded that testing for hereditary thrombophilia is not recommended in first degree relatives of the patients who had at least once in their life a thrombotic manifestation. Prophylaxis against thrombosis in patients with gene mutations with a predictable trigger factor for this pathology is indicated.
Keywords: thrombophilia, molecular genetic analysis, anticoagulant.

INTRODUCTION

Methylenetetrahydrofolate reductase (MTHFR) is an essential enzyme in folate metabolism. MTHFR reduces 5,10 methylenetetrahydrofolate to 5-methyltetrahydrofolate, which represents a cofactor for homocysteine remethylation to methionine. The involvement of MTHFR in pathology was first described in a patient presenting homocystinuria, with a severe defeciency in the enzyme activity (1).

In 1988, a 'thermolabile' enzyme with reduced activity was described, associated with high risk of coronary disease. After 1994, MTHFR analysis became more intense and mild or severe deficiencies of this enzyme were identified (2).

The thermolabile variant was associated with mutation in nucleotide 677, where cytosine (C) is substituted with thymine (T), with high risk of coronary disease and elevated level of homocysteine in the blood in patients with folate deficiency. The mechanism based on which MTHFR mutations are involved in vascular pathology it is not well established. One hypothesis stands for associated high levels of homocysteine in the blood, which may determine vascular endothelial injury with secondary venous thrombembolism (3).

MTHFR gene mutations were connected to various pathology, including colon cancer, acute leukemia, vascular diseases, depression, schizophrenia, migraine, glaucoma, Down syndrome, neural tube defects.

The most frequent MTHFR mutations are C677T and A1298C. The first one makes MTHFR 20% less active in metabolizing homocysteine. Studies report an incidence of 11% in Caucasian population for the homozygous state of this mutation, associated with risk of elevated levels for homocysteine in the blood and several pregnancy complications – chromosomal anomalies, congenital malformations, pregnancy loss, pathology of placenta and preeclampsia, including thrombotic events late in pregnancy and also in the postpartum period (4).

Heterozygous patients for this mutation have normal levels of homocysteine in the blood and low risk for thrombosis.

A1298C mutation is not associated with hyperhomocysteinemia, no matter the hetero- or homozygous state (5).

The combined heterozygous state for both MTHFR mutations can generate clinical manifestations similar to the homozygous state for C677T; this association is recognized as a risk factor for neural tube defects (6).

MATERIALS AND METHOD

The present study was performed on 16 patients admitted in the Onco-Hemathology Department, Paediatric Clinic of the County Clinical Hospital of Constanta from January 2012 to January 2013 for thrombotic events or representing first degree relatives of children with thrombotic events. They all have modified genetic test already known to increase the risk for thrombosis.

Following data was charted for these patients:
– age;
– gender;
– basic coagulation tests, like PT, APTT, INR, number of platelets;
– clinical manifestations at the onset of the acute event;
– thrombophilia profile: C protein, S protein, antithrombin, Leiden factor mutation, lupic anticoagulant;
– mutation for prothrombin gene;
– anticardiolipin antibodies;
– homcysteine in blood and urine;
– MTHFR gene mutations.

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The data obtained were analyzed and presented as graphics and tables.

RESULTS

From the total of 16 patients, 25% of them are adults and 75% children under 18 years of age. The results show that the hypercoagulable state is more frequently seen at early age. 62% of the persons in the study are female.

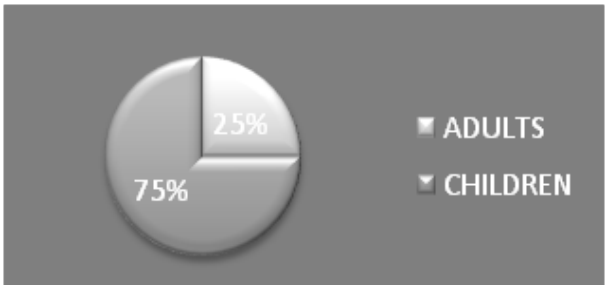

Fig. 1. Age distribution in the study group

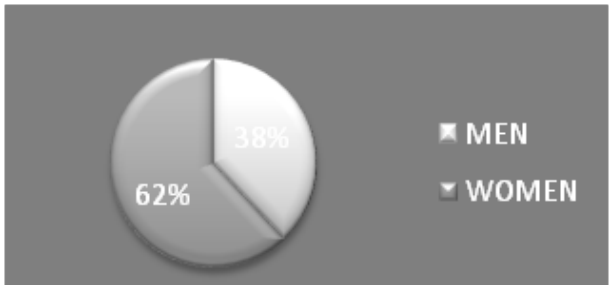

Fig.2. Gender distribution of the patients in the study group

For all the patients with MTHFR gene mutations, the incidence for thrombotic events was 62% in the studied group. The most frequent affected areas were represented by cerebral pathology-strokes, followed by thrombotic events regarding pregnancy.

Also, from the 62% with clinical manifestations, none is homozygous for MTHFR gene mutations.

In the study group, 25% presented C677Thomozygous state, 37% are C677T heterozygous, 25% have heterozygous A1298C mutation and only 13% are double heterozygous for both mutations.

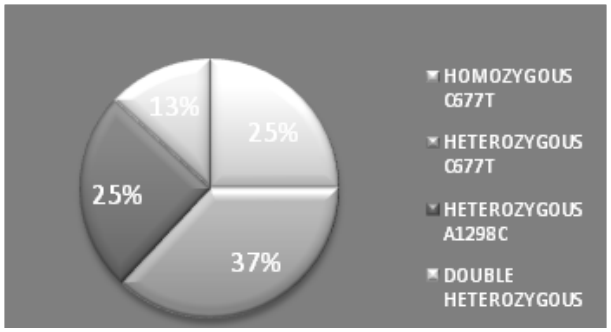

Fig.3. Distribution of MTHFR gene mutations in the study group

The levels for homocysteine in the blood were normal for all patients. We can think that homocysteine did not represent a trigger or even a predictable factor for thrombosis.

Table I. Homocysteine levels in the blood

No.	Homocysteine (µmol/l)	No.	Homocysteine (µmol/l)
1.	4.29	9.	6.1
2.	2.25	10.	7.05
3.	6.79	11.	6.98
4.	5.25	12.	5.8
5.	6.22	13.	5.95
6.	7.1	14.	5.7
7.	6.09	15.	7.01
8.	5.1	16.	6.29

Patients with homozygous mutations in the study group haven't had any clinical thrombotic event, at least not yet.

DISCUSSION

Based on existing literature, we can say that not the gene mutations represented the trigger factor for thrombosis in this study; not even the homocysteine level, but other causes must be considered in the presence of a thrombotic manifestation.

The authors highlight a particular case report of a nine year old boy who was admitted to hospital for stroke. The trigger factors considered for the thrombotic event appears to be a trip in the mountains, standing still for several hours and also a certain degree of dehydration. All these factors could represent trigger factors in a patient with later discovered MTHFR gene mutations, heterozygous state.

After molecular analyses, the authors noticed that the boy's father and sister carry together homozygous MTHFR mutations and they haven't had thrombotic events until the moment of determinations. This idea stands for the fact that genetic mutations are not enough for thrombotic pathology and we must consider the trigger factors which should be well known and avoided in these patients.

Thrombosis with normal levels for homocysteine enhances the idea that its elevation must be just a secondary effect after the already produced vascular damage.

We considered the opportunity of guidelines in testing hereditary thrombophilia. Many international studies for hypercoagulable state mention the utility of guidelines in testing hereditary thrombophilia; they also offer information for prophylaxis of thrombotic events.

CONCLUSION

Primary hypercoagulable state remains a diagnostic based on very sensitive lab work, with high specialization for the people involved in this work, including high costs.

The MEGA study concludes that it is not justified testing for genetic defects in first degree relatives of the patients with

thromboembolic events in early childhood, even if hereditary thrombophilia is proved – "testing does not reduce the recurrence of thrombosis".

We have to take into consideration the factors that could become trigger for thrombosis: large varices, oncologic and hematologic pathology, with high viscosity, severe infections, neprotic syndrome, preeclampsia, congestive heart failure, important hemorrhage, dehydration.

Prophylaxis against thrombosis must be initiated knowing all the possible trigger factors and, of course, we should take into account the risk for adverse reactions.

Homocysteine levels weren't relevant in monitoring the risk for thromboembolic events, probably because this determination was always made after the acute event; homocysteine remains a parameter for secondary hypercoagulable state.

REFERENCES

1. Montgomery RR, Scott JP. Hemoragic and thrombotic disease. In Nelson textbook of Pediatrics, ed. 17, 2004.
2. Rey E, Kahn SR, David M, Shrier I. Thrombophilic disorders and fetal loss: a meta-analysis. *Lancet* 2003*;* 361*:* 901-*908.*
3. Serban M, Schramm W. Hemostazeologie clinica, Ed. Brumar, Timisoara, 2001.
4. Steegers-Theunissen RP, Van Iersel CA, Peer PG, et al. Hyperhomocysteinemia, pregnancy complications, and the timing of investigation. *Obstet Gynecol* 2004; 104*:* 336-343.
5. http://www.synevo.ro/profil-trombofilie-2/
http://www.synevo.ro/gena-mthfr-mutatii-c677t-a1298c-risc-trombofilie/

MUTATIILE GENEI MTHFR SI OPORTUNITATEA TRATAMENTULUI ANTICOAGULANT

REZUMAT

Tinand cont de faptul ca statusul hipercoagulabil are la baza un diagnostic elaborat, incepand de la manifestarile clinice, multe dintre ele nespecifice, impreuna cu analize de laborator cu cost crescut, am considerat util studiul manifestarilor trombotice la pacientii cu analize modificate de genetica moleculara si stabilirea unei legaturi intre existenta acestor mutatii si riscul de evenimente trombotice. De asemenea, am luat in discutie oportunitatea tratamentului anticoagulant pentru scaderea incidentei acestei patologiii. Toate studiile si ghidurile pana in acest moment au concluzionat ca testarea pentru trombofilia ereditara nu este recomandata la rudele de gradul I ale pacientilor care au suferit cel putin o data in viata lor o manifestare trombotica. Profilaxia trombozei la pacientii cu mutatii genetice cu un factor declansator predicitibil pentru aceasta patologie este indicata.

Cuvinte cheie: trombofilie, analize de genetic moleculara, anticoagulant.

CORRELATION BETWEEN EOSINOPHILIC CATIONIC PROTEIN WITH OTHER LABORATORY TESTS AND ATOPIC ENVIRONMENT IN CHILDREN WITH BRONCHIAL HYPERREACTIVITY

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ABSTRACT

Asthma is one of the most common chronic diseases in children, which causes a significant number of emergency hospitalizations and has a great impact on the lives of children and their family. In most children with asthma, airway hyperreactivity, manifested as an exaggerated bronchoconstrictor response, is interrelated with bronchial inflammation and responsible for the clinical manifestations.The aim of the study is to determine the value of new methods of non-invasive investigations of bronchial inflammation. It represents a prospective study conducted on a sample of 199 patients with obstructive respiratory disease. The sample lot of 199 patients was observed for a period of 4 years or until the diagnosis of asthma was established. By the end of the period of the study, we identified a number of 126 cases of children with asthma and 73 cases without asthma. We determined the serum eosinophil cationic protein (ECP) levesl and assessed the relationship between the values of eosinophilic cationic protein and IgE values which shows an important positive correlation, statistically significant between the values of the two tests ($p < 0.001$). Comparison of the values of eosinophilic cationic protein with the result of the Phadiatop Infant test showed no statistically significant difference, the same result being obtained in the case of comparing the values of eosinophilic cationic protein with the evaluation of the presence of atopic environment.

Keywords: asthma, Ig E, eosinophilic cationic protein

INTRODUCTION

In recent years the focus is increasingly higher on non-invasive explorations of bronchial inflammation, thus, in 2005 a new field called "inflammamometry" was considered at European Respiratory Society congress in 2005, highlighting the importance of pathophysiologic substrate in the diagnosis, treatment and follow-up of patients with obstructive pulmonary diseases.

Asthma is one of the most common chronic diseases in children and causes a significant number of hospitalizations and emergencies. It has a significant impact on the daily life of the child, family and entourage where the child with asthma lives (4,5).

Asthma is a chronic disease, and for many patients has unpredictable evolution or progression, which may affect physical activity, social activities and quality of life. Conventional measurements as spirometry or bronchial hyperreactivity bring important information about airways clinical conditions, but do not offer information about local bronchial inflammation. Lately, the focus is increasingly higher on exploring non-invasive bronchial inflammation (6).

High serum ECP level may be a predictor and a risk factor for asthma exacerbation and may be used for guiding treatment (7).

OBJECTIVES

This study aims to determine the value of the new methods of non-invasive investigation of inflammatory bronchial eosinophilic cationic protein, the correlation that exists with the IgE levels, eosinophils level and the differences in patients with a positive Phadiatop Infant test and those with a negative Phadiatop Infant test.

MATERIAL AND METHODS

The study is a prospective study, conducted on a sample of 199 cases of respiratory disease cases, which were followed for a maximum period of 4 years, from 2009 to 2013. The patients were consulted yearly for a reevaluation of their medical status, and once it was possible, spirometry was conducted in order to certify the diagnosis of bronchial obstruction. Patients were followed until the diagnosis of asthma was established, or until the end of the study period.

The inclusion criteria for the study were the presence of wheezing in children with ages between 2 and 5 years old. Exclusion criteria are represented by previous diagnosis of chronic obstructive pulmonary disease.

We measured the Eosinophilic Cationic Protein (ECP), IgE values, eosinophils values and we performed Phadiatop Infant testing at admittance, using standard procedures recommended by the laboratory.

Eosinophilic Cationic Protein (ECP) was determined using immunoenzymatic with detection by fluorescence (FEIA)

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method. IgE was determined with a COBAS 6000 device produced by Hitachi using immunochemistry with detection by electrochemiluminescence (ECLIA) method. Phadiatop Infant was determined with UNICAP 250 device using imunoenzymatic with detection by fluorescence (FEIA) method. Blood eosinophils were determined with SYSMEX XT 1800i device using fluorescent flow cytometry, using semiconductor lasers and focusing hydrodynamic technologies (8). The presence of atopic environment was assessed during the initial consult using a questionnaire.

For the analysis of the data we used descriptive statistics. Shapiro-Wilk test was used to determine the normality of the distribution of the values, and according to the result obtained we used parametric or nonparametric methods. For parametric methods of testing the null hypothesis we used t test and Pearson correlation, while for nonparametric testing we used Mann-Whitney U test and Spearman rho in case of correlations. The p value to consider the result as being statistically significant is less than 0.05.

From the total of 199 patients, by the end of the study, 126 patients were diagnosed with asthma (63.3%) and 73 cases without asthma, which remained assigned to the group called wheezing (36.7%) (Figure 1)

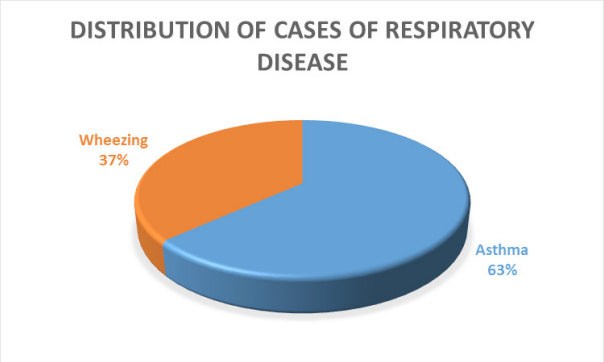

Fig.1. Distribution of cases by respiratory disease

At the beginning of the study,the patients from the study sample were distributed equally according to their age (Figure 2).

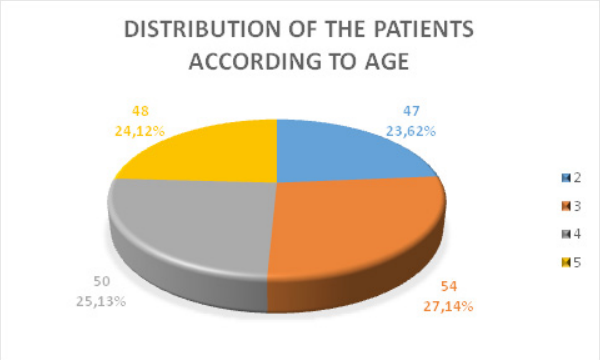

Fig. 2. Distribution of the patients included in the study according to age

The distribution according to sex for the whole study group reveals a higher proportion of males, 118 cases (59.3%) compared to females, 81 cases (40.7%) (Figure 3).

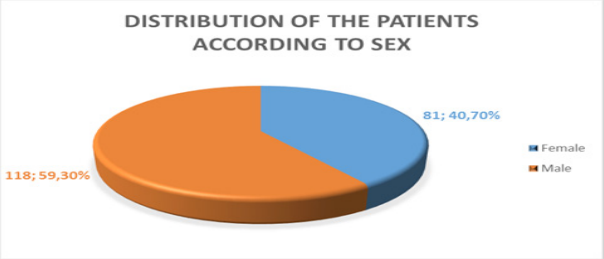

Fig.3. Distribution of cases included in the study according to gender

RESULTS AND DISCUSSION

Our study investigated ECP due to eosinophils important role in the pathophysiology of asthma. ECP is stored in the granules of eosinophil granulocytes together with the major basic protein (MBP), eosinophil peroxidase (EPO) and eosinophil - derived neurotoxin / eosinophil protein X (EDN/EPX) \ which have been demonstrated to cause airway damage (10,11). During eosinophil's degranulation ECP is released in the tissues upon exposures to surfaces coated with immunoglobulins and complement with the escape of some ECP to circulation (12). Serum ECP can also be used to assess the exposure to environmental allergens (13) and as a measure of allergen provocation causing increased eosinophil activity (14).

Recently, serum ECP is studied to conclude if it represents a valuable marker for the diagnosis and prognosis of bronchial asthma (9).

We measured serum eosinophil cationic protein and compared the results with those of the tests exploring bronchial hyperactivity. We evaluated the relationship between the values of eosinophilic cationic protein and IgE values in all patients in the study, patients that have bronchial hyperreactivity. The mean value measured for eosinophil cationic protein is 21.228 mg/dl, with standard deviation of 12.37 mg/dl. The mean value for IgE is 648.89 IU / ml, with standard deviation of 918.68 IU/ml (Table I)

Table I. Descriptive analysis of eosinophilic cationic protein and IgE values in all patients with bronchial hyperreactivity

		Eosinophil Cationic Protein	IgE
N	Valid	199	196
	Missing	0	3
Mean		21.228	648.8995
Median		17.400	315.0000
Mode		32.0	45.00
Std. Deviation		12.3776	918.68976
Variance		153.204	843990.872
Skewness		1.034	3.553

Std. Error of Skewness	.172	.174
Kurtosis	.773	18.135
Std. Error of Kurtosis	.343	.346
Range	66.9	7354.50
Minimum	3.1	3.50
Maximum	70.0	7358.00

The result of statistical analysis (Figure 4) shows a major positive correlation, statistically significant between the values of the two tests (r = 0.537, p <0.001). Therefore, we conclude that in the case of patients with bronchial reactivity considered for this study, there is an important relationship between the values of the ECP and the values of the IgE. This data is in accordance to other studies (15,16).

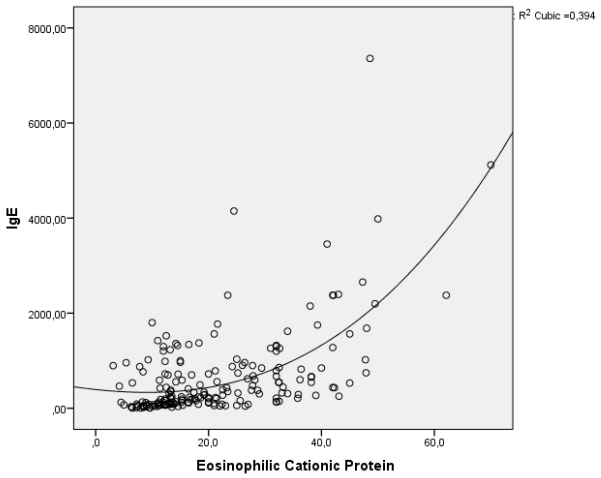

Fig.4. Correlation between eosinophil cationic protein and IgE values in all patients with bronchial hyperreactivity

Table II. Descriptive analysis of eosinophilic cationic protein and IgE values in patients in the wheezing group

	Eosinophil Cationic Protein	IgE
N	73	71
Range	66.9	5115.50
Minimum	3.1	3.50
Maximum	70.0	5119.00
Mean	17.148	413.2283
Std. Deviation	11.6145	743.66841
Variance	134.897	553042.711
Skewness	1.989	4.278
Std. Error of Kurtosis	.281	.285
Kurtosis	5.442	23.449
Std. Error of Kurtosis	.555	.563

The result of statistical analysis (Figure 5) show a major positive correlation, statistically significant between the values of the two tests (r = 0.542, p <0.001). Therefore, we conclude that in the case of patients from the wheezing group considered for this study, there is an important relationship between the values of the ECP and the values of the IgE.

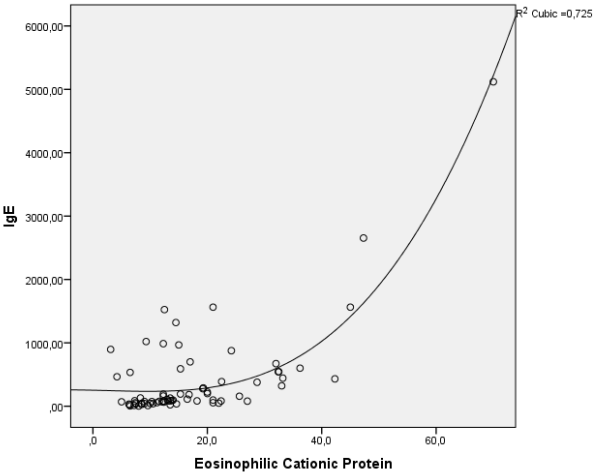

Fig. 5. Correlation between eosinophil cationic protein and IgE values for the wheezing group

The descriptive analysis for the patients that were diagnosed with asthma during the period of the study, is available in Table III.

Table III. Descriptive analysis of eosinophilic cationic protein and IgE values in patients diagnosed with asthma

	Eosinophil Cationic Protein	IgE
N	73	71
Range	66.9	5115.50
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Skewness	1.989	4.278
Std. Error of Kurtosis	.281	.285
Kurtosis	5.442	23.449
Std. Error of Kurtosis	.555	.563

The result of statistical analysis (Figure 6) shows a moderate positive correlation, statistically significant between the values of the two tests (r = 0.465, p <0.001). Therefore, we conclude that in the case of patients from the asthma group considered for this study, there is a moderate relationship between the values of the ECP and the values of the IgE.

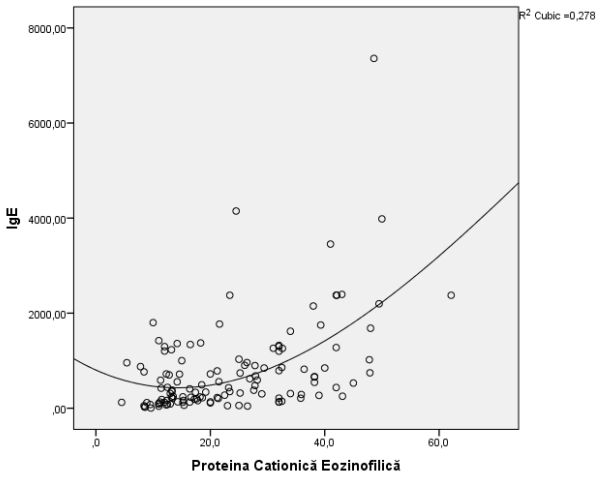

Fig.6. Correlation between eosinophil cationic protein and IgE values for the asthma group

A comparison was also followed with eosinophilic cationic protein test values and Phadiatop Infant. From the total of 199 cases, this testing was conducted in 185 of them. From these, 57 had a negative result, while the other 128 had a positive result.

Table IV. Phadiatop Infant distribution of the patients according to the result

		Frequency	Percent	Valid Percent	Cumulative Percent
Valid	Negative	57	28.6	30.8	30.8
	Pozitive	128	64.3	69.2	100.0
	Total	185	93.0	100.0	
Missing	System	14	7.0		
Total		199	100.0		

Mean eosinophilic cationic protein value in patients with negative Phadiatop infant test is 18.684, with standard deviation of 9.85. For those with Phadiatop infant positive test result, mean eosinophil cationic protein is 22.623 with standard deviation of 13.39 (Table V).

Table V. Comparisson of eosinophilic cationic protein values between groups with positive and negative result in Phadiatop Infant test

Phadiatop Infant	N	Mean	Std. Deviation	Median
Negative	57	18.684	9.8559	17.400
Pozitive	128	22.623	13.3950	18.250
Total	185	21.410	12.5193	17.600

Following statistical analysis, using Mann-Whitney U test, as the distribution of the values differs significantly from a normal distribution (Shapiro-Wilk test rejects the null hypothesis with a p value less than 0.001 for cases with positive Phadiatop Infant test and with p=0.001 for cases with Phadiatop Infant negative test), we observe that there is no statistically significant difference between the two groups (U= 3125, z=1.554, p=0.120)

Exposure to atopic environment particularly in early life may be an important factor in the pathogenesis of atopic and asthmatic symptoms (17). The most important inhaled allergen frequently associated with asthma is house dust mite and maybe prevention is needed to minimization the potential allergens and pollutants from the house (17). In our study, 63.3% of the patients were not exposed to an atopic environment, while the other 73 were considered to live in an atopic environment (Figure 7).

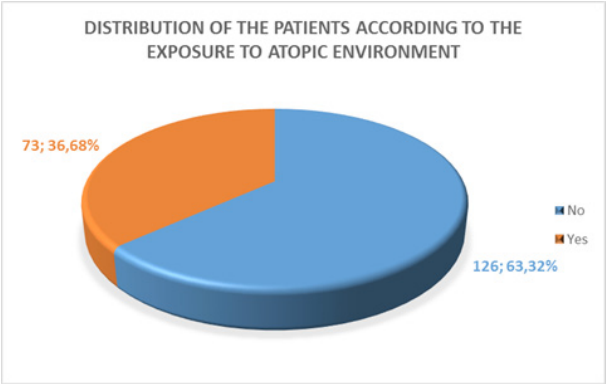

Fig. 7. Distribution of all patients according to the exposure to atopic environment

Eosinophilic cationic protein assessment accordingly to environmental presence of atopic factors show that the average values of eosinophilic cationic protein in patients not exposed to atopic environment is 19.881 with standard deviation of 10.49. Patients exposed to an atopic environment have average value of 23.553 for ECP with a standard deviation of 14.88. The data is available in Table VI.

Table VI. Evaluation of eosinophil cationic protein according to the presence of atopic environment

Exposure to atopic environment	N	Mean	Std. Deviation	Median
Yes	73	23.553	14.8866	19.300
No	126	19.881	10.4900	16.650
Total	199	21.228	12.3776	17.400

We observed in patients exposed to atopic environment wider range of values for eosinophilic cationic protein (Figure 8). Test for normality reveals that distributions of eosinophilic cationic protein values are significantly different from a normal distribution in both groups (p <0.001 respectively p = 0.004).

Despite these differences, the statistical testing, done using Mann-Whitney U test shows no statistically significant difference between the two groups (U=4150, z=1.147, p=0.251).

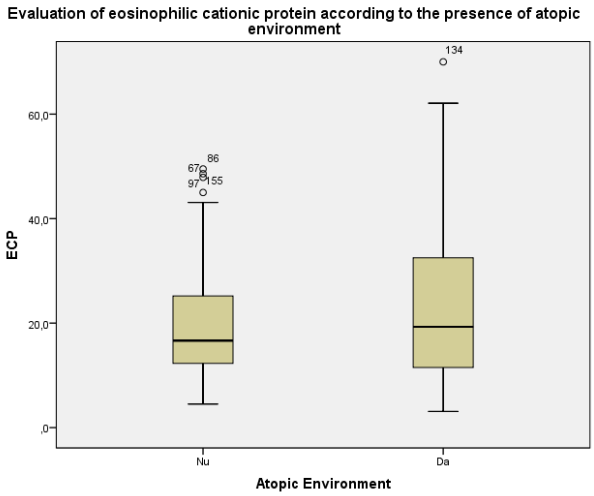

Fig.8. Evaluation of eosinophil cationic protein according to the presence of atopic environment

Eosinophils are major inflammatory cells present in bronchial asthma patient's respiratory tract mucosa and submucosa (18). A subpopulation of low density eosinophils with increased cytotoxic activity and increased numbers of activated blood T cells represent part of the pathophysiological processes of asthma (19,20).

We considered the correlation between eosinophilic cationic protein and blood eosinophilia but it has shown a negative weak correlation (r=-0.197), which is not statistically significant (p=0.081) (Table VII).

Table VII. Correlation between eosinophilic cationic protein and blood eosinophilia

Correlations

			ECP	Eozinophils
Spearman's rho	ECP	Correlation Coefficient	1.000	-.197
		Sig. (2-tailed)	.	.081
		N	199	79
	Eosinophils	Correlation Coefficient	-.197	1.000
		Sig. (2-tailed)	.081	.
		N	79	79

CONCLUSION

In our study, ECP serum concentration, blood eosinophil number and total IgE in serum are significantly higher in asthmatic children demonstrating the role of eosinophilic inflammation in the pathogenesis of asthma.

Evaluation of the relationship between the values of eosinophil cationic protein and IgE values in patients with bronchial abnormal reactivity show a positive correlation major statistically significant between the two analysis values (r = 0.537, p <0.001).

For the population in our study, there was no statistically significant difference between patients with a positive Phadiatop Infant test and the ones with a negative one (U= 3125, z=1.554, p=0.120), even though the values observed in the group with a negative test have a lower average value compared to the values observed in the group with a positive result.

There is no statistically significant difference for the values of the ECP levels between patients that come from an atopic environment and the ones that live in an atopic-free environment (U=4150, z=1.147, p=0.251).

The values of the ECP do not correlate with the values of eosinophils number. (r=-0.197, p=0.081).

ECP levels in serum reflect better than IgE allergic inflammatory process, ie the activation of eosinophils, correlate well with disease activity, so the severity of atopy. This laboratory parameter is useful for objectification and surveillance therapy in asthma and atopic dermatitis.

Direct measurement of airways inflammation using biological markers like ECP, could potentially enhance asthma management. This explains the interest in measuring the levels of eosinophil granule proteins especially in asthma.

REFERENCES

1. Sterk PJ, Fabbri LM, Quanjer P, et al. Airway responsiveness: standardized challenge testing with pharmacological, physical and sensitizing stimuli in adults. *Eur Respir J*, 1993; 6(16): 53-83.
2. Bierman CW, Shapiro GG. Clinical expression of bronchial hyperreactivity in children. *Clin. Rev Allergy*, 1989; 7(3): 301-20.
3. Francis JG, Warren L. The burden of paediatric asthma: economic and familiar. *European Monograph*, 2012; 56: 71.
4. Juniper EF, Guyatt GH, Feeny DH, et al. Measuring quality of life in the parents of children with asthma, *Quality of Life Research*, 1996; 5: 27-34.
5. Ion I, Badiu G. Fiziologie-curs, Ed Ex Ponto, Constanţa, 2000
6. Restrepo RD, Peters J. Near-Fatal Asthma: Recognition and Management. *Curr Opin Pulm Med*, 2008; 14(1): 13-23.
7. Oddera S, Silvestri M, Penna R, et al. Airway eosinophilic inflammation and bronchial hyperresponsiveness after allergen inhalation challenge in asthma. *Lung*, 1998; 176: 237-247.
8. Laborator Synevo. Referintele specifice tehnologiei de lucru utilizate 2010. Ref Type: Catalog.
9. Koh GCH, Shek LPC, Goh DYT, et al. Eosinophilic cationic protein: is it useful in asthma? A systemic review. *Respiratory Medicine*, 2007; 101: 696-705.
10. Byström J, Garcia RC, Håkansson L, et al. Eosinophil cationic protein is stored in, but not produced by peripheral blood neutrophils. *Clin Exp Allergy*, 2002; 32:1082-91.
11. Bousquet, Chanez P, Lacoste Y, et al. Eosinophilic inflammation in asthma. *New Eng J Med*, 1990; 323: 1033-9.
12. Lee BioSolutions, Inc. http://www.leebio.com/eosinophil-cationic-protein-human-P359.html
13. Nieto A. What role does ECP have in the evaluation of asthma severity. *Allergol Immunopathol*, 2000; 28:119-124.
14. Oddera S, Silvestri M, Penna R, et al. Airway eosinophilic inflammation and BHR after allergen inhalation challenge in asthma. *Lung*, 1998; 176: 237-247.
15. Esengül M, Hamza Y, Arzu G. To evaluate serum eosinophil cationic protein and total IgE concomitantly may predict the persistence of wheezing in young children. ISRN Pediatrics International Scholarly

Research Network, 2010.
16. Mohammad RK, Majid M, Mojtaba S, et al. Is serum or sputum eosinophil cationic protein level adequate for diagnosis of mild asthma? *Iran J Allergy Asthma Immunology*, 2009; 8(3):155-160.
17. Sporik R, Chapman MD, Platts-Mills TA. House dust mite exposure as a cause of asthma. *Clin Exp Allergy,* 1992; 22: 897-906.
18. Koh Y, Kang H, Kim CK. Ratio of serum eosinophil cationic protein/blood eosinophil counts in children with asthma: comparison between acute exacerbation and clinical remission. *Allergy Asthma Proc*, 2003; 24(4): 269-274.
19. Koller DY, Herouy Y, Götz M, et al. Clinical value of monitoring eosinophil activity in asthma. *Arch Dis Child* 1995; 73:413-417.
20. Seroogy CM, Gern J. The role of T regulatory cells in asthma. *J Allergy Clin Immunol*, 2005; 116:996-9.

CORELATIILE PROTEINEI CATIONICE EOZINOFILICE CU IG E TOTAL, PHADIATOP INFANT, NUMARUL TOTAL DE EOZINOFILE SI MEDIUL ATOPIC LA COPIII CU HIPERREACTIVITATE BRONSICA

REZUMAT
Astmul este una dintre cele mai comune boli cronice la copii, care provoacă un număr semnificativ de spitalizări de urgență și are un impact mare asupra vieții copiilor si familiilor lor. Scopul acestui studiu este de a determina valoarea unor noi metode de investigație non-invazive a inflamației bronșice. Studiul este un studiu prospectiv realizat pe un eșantion de 199 de pacienți cu boli respiratorii obstructive. Am determinat nivelul seric al proteinei cationice eozinofilice (ECP). Studiul a cuprins un număr de 199 de pacienți, urmăriți o perioadă de 4 ani sau până ce diagnosticul de astm a fost stabilit. Până la sfârșitul perioadei de studiu, am identificat un număr de 126 de cazuri de copii cu astm bronsic și 73 de cazuri fără astm. Evaluarea relației dintre valorile ECP și valorile IgE arată o corelație pozitivă importantă, semnificativă statistic între valorile celor două teste (r=0,537 p <0,001). Compararea valorilor ECP în funcție de rezultatul testului Phadiatop Infant a arătat lipsa unor diferențe semnificativ statistic între cei cu test pozitiv și cei cu test negativ, același rezultat fiind obținut în cazul comparării valorilor ECP bazate pe evaluarea prezenței mediului de viață atopic.
Cuvinte cheie: proteina cationică eozinofilica, IgE, astm bronşic.

BARIATRIC SURGERY IN OBESE PATIENTS WITH INCISIONAL HERNIA – A NEW METHOD TO REDUCE THE HERNIA RECURRENCE RATES

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ABSTRACT
Post-incisional hernia occurs in approximately 10% of cases of abdominal surgery and it is the most common long-term complication, one of the main risks for developing a post-incisional hernia is obesity. Laparoscopic incisional ventral hernia repair (LIVHR) became increasingly popular in the last years due to faster recovery and lower rate of wound infection. We used laparoscopic greater curvature plication (LGCP) of the stomach for treating obese patients. The present study was a prospective case series that aimed to evaluate the short and the long term outcomes of combined LIVHR and LGCP of the obese patients with incisional hernia operated in 2012 in Surgical 2 Clinic of County Emergency Hospital Timisoara. 6 patients were enrolled, all females. 4 patients had 24 months follow-up, the others two had 20 and 16 months follow-up. Median age was 42, median wall defect was 56 cm^2, and median hospital stay was 5 days, complications rates were 83%. Complications were nausea in 5 patients, vomiting in 2 cases, seroma formation in one. Median body mass index dropped from 36.7 kg/m^2 to 29.85 kg/m^2. Excess weight loss was 52.6% at two years, no recurrence of the hernia occurs during follow-up. LIVHR repair is a safe method in obese patients, if LGCP is added in the same operative time the rate of short term minor complication is increased, but long term recurrence is decreased due to weight loss. Larger studies are required to drown a final conclusion upon our combined method.
Keywords: bariatric surgery, LIVHR, gastric plication

INTRODUCTION

Post-incisional hernia occurs in approximately 10% of cases of abdominal surgery and it is the most common long-term complication (1), one of the main risks for developing a post-incisional hernia is obesity (2). Laparoscopic incisional ventral hernia repair (LIVHR) became increasingly popular in the last years due to faster recovery and lower rate of wound infection. Also for obese patients bariatric surgery seems to have the highest rate of success in reducing the excess weight. One of the methods used for treating obese patients is laparoscopic greater curvature plication (LGCP) of the stomach; some of the advantages of this method are the low rate of post-operative complications, lower cost and the digestive tube is not opened. We decide to use a combined operation for the obese patients with incisional abdominal hernia – LGCP and LIVHR in the same operating time.

METHODS

The present study was a prospective case series that aimed to evaluate the short and the long term outcomes of combined LIVHR and LGCP. The study begun in 2012, morbidly obese subjects referred to our surgery clinic with incisional ventral hernia (IVH) were offered simultaneous LIVHR and LGCP. During 2012 the patients were enrolled, follow-up was planned at 1, 3, 6, 12 and 24 months.

Inclusion criteria were the presence of an IVH, obesity and the eligibility of the patient to undergo laparoscopic surgery.

The maximum size of the IVH was 10 cm diameter; only patients with reducible hernias and undergoing elective surgery were included in the study.

Patients required a body mass index (BMI) between 30-40 kg/m^2 and an absence ofpsychological conditions that might influence postoperative evaluations and recommendations.All recruited patients underwent a pre-surgicalevaluation, including consultations with a cardiologist, psychologist, and nutritionist plus routine laboratory test. They must have a strong motivation to lose weight and the potential for continuous diet and exercise after operation.

Patients with multiple abdominal surgeries, irreducible hernias and with no will to lose weight were not offered the combined procedure.

During the surgical procedure first step was to dissect and reduce the content of incisional hernia. Pneumoperitoneum was created inserting the Veress' needle at the Palmer's point, left mid clavicular line below the last rib either using the open Hasson technique for the insertion of the first trocar on the left flank. Another two trocars are inserted in the left flank. The adhesions in the abdomen were lysed with or without the use of an energy source. This choice was dictated by the proximity of the bowel and the surgeon preference. No energy was applied adjacent to any structure that might result in an injury. Missed enterotomy is one of the most feared laparoscopic complications of hernia

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repair. Once the hernia defect or defects were revealed we determined their dimensions. In two cases additional three trocars were inserted in the right flank for proper hernia dissection.

Then the patient was placed 30° reverse Trendelenburg position, with the operating surgeon standing between patient legs. Dissection started at the greater curvature of the stomach from the middle of the antrum and continued to the left diaphragmatic crus, and downwards to the level of the pyllor. Communicating vessels were ligated by LigaSure (LigaSure™ Covidien, USA), Ultracision (Harmonic® Ultrasonic, Ethicon, USA) or clips. Continuous suturing from 2 cm below the cardia through the antrum, 4 cm distance above the pyllorus, was performed in this stage, making one and then two layers of plicated stomach from the anterior wall of the stomach to the posterior wall. For suturing 2-0 prolene (2-0 PROLENE™ Polypropylene Suture, Ethicon, USA) was used, and the bulk of each stitch was 1 cm with a 1-cm interval. For gastric tube calibration we used a 36 Fr tube. Sutures were extramucosal, preventing absorption by gastric acid (3).

Next step was to place the mesh in order to cover the parietal defect. After we determined the defect size we used a mesh at least 8 cm larger which provided an overlap over the fascial edges of the hernia of at least 4 cm on both sides. Then we placed four transfascial sutures using fascia closure. Then the mesh final fixation was done using titanium either absorbable tacks. The use of a single row of tacks either the double-crown technique was left at the surgeon preference.

Drainage was used for 24 hours in order to see if there was any bleeding.

Post-operative we administrated to all the patients proton pump inhibitors 2x40 mg/day, anti-inflammatory and anti-nausea/vomiting drugs. Low molecular heparins were administrated for up to three weeks to prevent deep vein thrombosis and superior mesenteric vein thrombosis.

Postoperative diet was resumed 24 hours after surgery and consisted only in fluids up to the 9th POD. Next three weeks all the food will be semi-liquid.

The demographics and the comorbidities of the patients were assessed. The peri-operative data gathered regarded operating time, blood loss, parietal defect size, wound infection, seroma formation, nausea and vomiting, re-admission, hospital stay, first bowel movement, morbidity and mortality in the first 30 post-operative days.

Follow-up regarded recurrence of the hernia, percentage of the excess weight loss, BMI, comorbidities resolution, mortality.

RESULTS

6 patients were enrolled, all females. 4 patients had 24 months follow-up, the others two had 20 and 16 months follow-up. The primary surgery was open cholecystectomy in three cases: one perforated appendicitis with generalized peritonitis, and two hysterectomies for benign lesion of the uterus. Demographics of the patients are showed in Table I. None of the patients had diabetes mellitus. Peri-operative data are shown in Table II.

Table I. Demographics of the patients

Median age	42
Median BMI	36.70 kg/m^2
Comorbidities	83%
ASA I	2
ASA II	4

BMI – body mass index

Table II. Peri-operative data of the patients

OR time (median)	105 min (75;135)
Blood loss (median)	90 ml (70;130)
Defect size (median)	56 cm^2 (24;82)
First bowel movement	POD 1
Hospital stay (median)	5 days (3;10)
Complications	83%

POD – post-operative day

All the surgery were started and finished laparoscopically. No perforation of the stomach either of the bowel happened. For the first three days IV fluids were administrated. The patients were recommended to walk in the same day with the surgery and oral diet was resumed first POD.

Complications were nausea in 5 cases and vomiting in two out of these five. Seroma was search with the ultrasound and all the patients developed seroma, but only one needed puncture aspiration in order to evacuate the fluid. Mortality was null.

Follow-up data (Table III) – no recurrence of the hernia or mortality was recorded. No bulging at the hernia site was observed. No regain happened in the follow-up period.

Table III. BMI Follow-up

	preoperative	1 month	3 months
BMI	36.7	33.82	30.1
EWL %	-	23.61	52.59
	6 months	12 months	24 months
BMI	29.51	28.9	29.85
EWL %	54.5	55.1	52.6

EWL - excess weight loss; BMI - Body mass index

CONCLUSION

LIVHR repair is a safe method in obese patients, if LGCP is added in the same operative time the rate of short term minor complication is increased, but long term recurrence is decreased due to weight loss. Larger studies are required to drown a final conclusion upon our combined method.

DISCUSSION

LIVHR is becoming increasingly popular; one of the reasons is the spread of the laparoscopic technique and the consecutive raise in laparoscopic skills of the surgeons, the other is the lower

complications rates of the minimally invasive method. A meta-analysis done by Goodney et al. (4) identified eight studies for a total of 712 patients which have two arms comparing LIVHR and open incisional hernia repairs. Patients undergoing a LIVHR in these studies were 58% less likely to develop a perioperative complication compared to those undergoing an open repair. The new technologies advance – dual mesh, biologique mesh, absorbable tackers, glue – make LIVHR looking more attractive than ever. Another benefit of the laparoscopic approach is identifying small fascial defects, known as ''Swiss cheese'' defects, which may be missed during an open repair. These small fascial defects are a source of incisional hernia recurrence; therefore identification is important for a successful hernia repair (5).

The laparoscopic approach of incisional hernia is ideal in the obese patient, due to the smaller wounds and theoretically, decreased wound complications (6). Sugermanet al. (7) reported that obesity grade II or more (BMI >35 kg/m^2) was a greater risk factor for incisional hernia and recurrence than chronic steroid use. Same group has shown that morbid obese patients, and especially those with central obesity, have increased basal intra-abdominal pressure (8). This higher pressure creates more strain on the mesh placed for the hernia repair and reduces the blood flow in the abdominal wall, which are probably responsible for the higher rate of recurrence following repair.

Adding a restrictive procedure to the hernia repair we try to decrease one of the most important risk factor for the recurrence - obesity. In our pilot study of six patients we didn't had any recurrence in the follow-up period of two years, one of the explanation might be the use of laparoscopy, and the bariatric procedure that allowed our patients to lose more than 50% of the excess body weight. We choose to follow-up our patients for two years because most of the recurrences occur in this period; approximately 50% of all incisional hernias develop within the first 2 years after surgery (9).

We chose to associate LGCP to LIVHR because of the unique feature of this procedure among bariatric procedure – the digestive tube stays closed during procedure, there is no digestive spillage in contact with the mesh lowering the risk of mesh infection. Also LGCP has a low rate of gastric fistula and complications as Talebpour et al reported in the largest series of LGCP that 8 patients out of 800 cases (1%) required reoperation due to complications like: micro perforation, obstruction and vomiting following adhesion of His angle (10).

The results of LGCP are satisfying with obese persons with a BMI lower than 40 kg/m^2 and a strong motivation to lose weight (10). Long-term preservation of weight reduction is a challenge in patients with greater BMI. There is only one report regarding long-term outcome of LGP that reported weight regain of 15 % (11 of 75), 30 % (10 of 35), and 50 % (5 of10) after 3, 7, and 10 years, respectively (11).

There is no bulging at the hernia repair site; although we thought that reducing the volume of the abdomen will lead to bulging. Seroma near to mesh implant was present, but in only one case required drainage by repeated puncture.

LGCP provide a good loss of the excess body weight, 41% to 62% at 24 months, but the overall excess weight loss was lower at 24 months than at 12 months, these patients are watched carefully and other restrictive bariatric surgery like laparoscopic gastric by-pass or duodenal switch might be more effective on the long term.

5 patients had nausea after surgery, and 2 out five vomited up to 7 days post-operatively. In order to prevent dehydration we administrated IV fluids up to 4 liters/day until the vomiting stops. Great care was taken to prevent electrolyte imbalance. Atlas et al had 79.5% nausea and vomiting in their 44 patients' series up to ten days (12), and generally it's considered that nausea and vomiting appears because of the gastric wall edema.

Our study had only six patients due to difficulty to enrolled more patients, most of the obese patients grade I and II with abdominal incisional hernia did not want a gastric restrictiveprocedure same operative time with the LIVHR. But our results are encouraging us to propose our combined method to new patients.

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REFERENCES

1. Santora TA, Roslyn JJ. Incisional hernia. *Surg Clin North Am*, 1993; 73: 557-570.
2. Yahchouchy-Chouillard E, Aura T, Picone O, Etienne JC, Fingerhut A. (2003) Incisional hernias and related risk factors. *Digest Surg*, 2003; 20: 3-9.
3. Copaescu C. Plicaturarea marii curburi gastrice pe cale laparoscopicã (pentru tratamentul obezitãtii morbide). *Chirurgia*, 2011; 106: 91-97.
4. Goodney PP, Birkmeyer CM, Birkmeyer JD. Short-term outcomes of laparoscopic and open ventral hernia repair: a meta-analysis. *Arch Surg*, 2002; 137:1161-65.
5. Rudmik L R, Schieman C, Dixon E, Debru E. Laparoscopic incisional hernia repair: a review of the literature. *Hernia*, 2006; 10: 110-119.
6. Ramshaw BJ, Esartia P, Schwab J, et al. Comparison of laparoscopic and open ventral herniorrhaphy. *Am Surg*, 1999; 65(9): 827-31.
7. Sugerman HJ, Kellum JM Jr, Reines HD, et al. Greater risk of incisional hernia with morbidly obese than steroid-dependent patients and low recurrence with prefascial polypropylene mesh. *Am J Surg*, 1996; 171(1):80-4.
8. Sugerman H, Windsor A, Bessos M, et al. Effects of surgically induced weight loss on urinary bladder pressure, sagittal abdominal diameter and obesity co-morbidity. *Int J Obes Relat Metab Disord*, 1998; 22(3): 230-5.
9. Anthony T, Bergen PC, Kim LT, et al Factors affecting recurrence following incisional herniorrhaphy. *World J Surg*, 2000; 24: 95-100.
10. Talebpour M, Motamedi SM, Talebpour A, Vahidi H. Twelve year experience of laparoscopic gastric plication in morbid obesity: development of the technique and patient outcomes. *Ann Surg Innov Res,* 2012; 22; 6(1): 7.
11. Talebpour M, Vahidi H, Talebpour A. Eleven years experience about the new technique "laparoscopic vertical gastric plication" in morbid obesity introduced the first time in the world. In: Proceeding of the XVI Congress of the International Federation for the Surgery of Obesity and Metabolic Disorders (IFSO); Hamburg, Germany, 2011: 1017.
12. Atlas H, Yazbek T, Garneau PY, Safa N, Denis R. Is there a future for laparoscopic gastric greater curvature plication (LGGCP)? A review of 44 patients. *Obes Surg*, 2013; 23(9):1397-403.

CHIRURGIA BARIATRICA LA PACIENTII OBEZI CU HERNIE INCIZIONALA – O NOUA METODA DE REDUCERE A RATEI DE RECURENTA A HERNIEI

REZUMAT

Hernia post-incizionala apare in aproximativ 10% dintre cazurile de interventii chirurgicale abdominale si este cea mai comuna complicatie pe termen lung, unul dintre riscurile majore pentru aparitia herniei post-incizionale fiind obezitatea. Repararea laparoscopica a herniei ventrale incizionale (LIVHR) a devenit o metoda foarte populara in ultimii ani datorita recuperarii mai rapide si a ratei scazute de infectie. Am utilizat plicaturarea laparoscopica a marii curburi (LGCP) a stomacului pentru tratarea pacientilor obezi. Studiul de fata este un studiu prospectiv al unei serii de cazuri clinice, care si-a propus evaluarea rezultatelor pe termen lung si scurt ale combinatiei LIVHR si LGCP pentru pacientii obezi cu hernie incizionala operati in anul 2012 in Clinica de Chirurgie 2 a Spitalului Judetean de Urgente Timisoara. Au fost inrolati in acest studiu 6 pacienti de sex feminin. 4 dintre pacienti au fost monitorizati 24 de luni, ceilalti 2 fiind monitorizati pe o perioada de 20 si respectiv 16 luni. Varsta medie a pacientilor a fost de 42 de ani, iar dimensiunea medie a defectului parietal a fost de 56 cm^2, durata medie de spitalizare a fost de 5 zile, iar rata complicatiilor de 83%. Complicatiile intalnite au fost: greturi la 5 dintre pacienti, sindrom vomitiv la 2 dintre pacienti si formarea seromului la unul dintre pacienti. Indicele de masa corporala a scazut de la 36,7 kg/m^2 la 29,85 kg/m^2. Pierderea de greutate excesiva a fost de 52,6% la doi ani, fara recurenta a herniei in acest interval de timp. Repararea prin LIVHR este o metoda sigura pentru pacientii obezi, iar daca este adaugata si LGCP in aceasi sedinta chirurgicala, rata complicatiilor minore pe termen scurt este crescuta, dar pe termen lung recurenta este scazuta datorita scaderii in greutate. Sunt necesare studii mai ample pentru a avea o concluzie finala asupra metodei noastre combinate.

Cuvinte cheie: chirurgie bariatrica, LIVHR, plicaturare gastrica

THE USE OF PERIOPERATIVE INTRAPERITONEAL CHEMOTHERAPY ASSOCIATED WITH NEOADJUVANT THERAPY FOR ADVANCED OVARIAN CANCER AS CONSOLIDATION THERAPY

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ABSTRACT

Objectives: To determine the effectiveness of perioperative intraperitoneal chemotherapy and the safety of this method for patients with ovarian neoplasm who have high residual volume after primary surgery. Methods: A retrospective clinical study in which 24 female patients with ovarian neoplasm, stage FIGO IIIC, operated in the Surgical Oncology Clinic of the Municipal Hospital Timisoara over a period ofsix years (2003-2009), were evaluated. Results: The female patients studied were diagnosed after surgical staging +/- suboptimal tumor cytoreduction, had voluminous residual tumor after surgery, have been given cisplatin and perioperatively endoxan, followed by six cycles of neoadjuvant systemic chemotherapy. Among the side effects of the chemotherapy administration into the peritoneal cavity, pain was present in 18 patients, ileus in 5 patients, leukopenia in 2 patients and renal toxicity in 2 patients. The treatment response evaluated after a further surgery in 20 patients and by computed tomography in 4 patients was complete in 9 cases (37.5%), partial in 10 patients (41.66%) and negative in 5 patients (20 83%), the disease being stagnant or in evolution. The median survival of patients was 30.9 months, with a median of 24.3 months. Conclusions: Intraperitoneal chemotherapy used as consolidation treatment in patients with high residual volume after primary surgery is devoid of major complications and in combination with systemic chemotherapy and cytoreductive surgery may improve long-term prognosis.

Keywords: intraperitoneal chemotherapy, ovarian cancer, cytoreductive surgery.

INTRODUCTION

The epithelial ovarian cancer is an important cause of death among gynecological cancers in women, with an incidence in the European Union by 18/100.000 women/year and a mortality of 12/100.000 women/year (1). The gloomy prognosis is because of the fact that approximately 75% are diagnosed in advanced stages (FIGO III-IV), when there are usually about peritoneal, hematogenous or lymphatic disseminated metastasis (2,3).

The primary route of dissemination of ovarian cancer is the peritoneal by implanting intraperitoneal cancer cells in different locations, following the peritoneal fluid hydrodynamicsthrough the spaces parieto-colice to diaphragm and back to pelvis (4,5). This hypothesis has captured the attention of studies in recent decades, directing therapeutic protocols toward intraperitoneal administration of cytostatic agents.

For advanced ovarian neoplasm the basic principle of treatment is the combination of primary cytoreductive surgery with the remnants of tumor volume as low as possible and adjuvant chemotherapy, six cycle of platinum-based regimens (cisplatin/carboplatin) and taxanes (6,7,8).For female patients with unresectable tumors is recommended between 3 and 6 cycles of neoadjuvant chemotherapy with the same platinum-based regimens and taxanes followed by "surgery interval" with cytoreductive purposes.

Multiple studies have reported benefits in terms of survival and disease-free period for intraperitoneal administration of cytotoxic agents (9,10). The probable mechanisms by which the use of cytostatics directly into the peritoneal cavity bring this benefit are: getting an increased levels of cytotoxic substance in contact with the peritoneal serous, having a low clearance from the systemic circulation; stimulation of local immunological mechanisms of the peritoneal cavity with increasing cytotoxicity antineoplastic agents; conversion from "chemoresistant" tumors to "chemosensitive" tumors; direct penetrating of chemotherapy in tumor tissue (11).

Although current indications of intraperitoneal cytotoxic drugs in ovarian cancer are the small residual volume after primary cytoreductive surgery or recurrent disease, in our study we aimed to highlight the possible advantages by using this method also to the female patients that present high residual volume after primary surgery.

METHODS

The study was conducted on a group of 24 patients diagnosed with advanced ovarian cancer who were hospitalized in the General Surgery II and Surgical Oncology Clinic of the Emergency Municipal Hospital Timisoara, between 2003-2009. Patients were selected from a total of 236 sick subjects, diagnosed and treated

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in our clinic during that time.

The inclusion criteria of the patients in the study were represented by: histopathologic diagnosis of epithelial ovarian cancer, locally advanced disease, residual tumor volume increased after staging laparotomy or cytoreductive surgery performed as the first therapeutic act, patients who had performance index increased, following postoperatively intraperitoneal chemotherapy. Were excluded from the study patients who had significant comorbidities (other neoplastic diseases associated with heart, kidney or liver failure), who showed a lower performance index.

All patients were diagnosed by staging laparotomy +/- tumor cytoreduction, which was the first therapeutic approach.Only 5 patients underwent cytoreductive tumor, but did not reach optimal, they presented postoperatively increased residual volume (> 2 cm) and in the remaining 19 patients, the surgery was limited only to the exploratory laparotomy, considering abdominal tumors as unresectable. Postoperatively, all the patients received intraperitoneally chemotherapy by normothermia treatment. Subsequently, patients who survived the postoperative period received adjuvant chemotherapy or neoadjuvant systemic including regimens platinum agents (cisplatin/carboplatin) according to the treatment guidelines, 20 patients were subjected to a new surgery with cyto-reductive purposes.

Intraperitoneal chemotherapy technique: intra-operatively a catheter is placed pelvic which externalizes through counter-incision in the right flank for administration and a drainage tube also placed pelvic for disposal. During the first 3-4 hours after surgery is administered50 mg Cisplatin and 1000 mg Endoxan added to 1000 ml of normal saline heated to 37 ° for one hour and evacuated after 12 hours. The procedure repeats itself in the next two days with only 50 mg Cisplatin diluted in 1000 ml saline under the same conditions. Throughout the treatment is being maintained a diuresis of 100 ml/h and a maximum creatinine 2 mg/dl.

Postoperatively, the following parameters were monitored: blood counts, urea, creatinine, transaminases, coagulation times, diuresis, resumption of intestinal transit time, postoperative complications. After the completion of systemic chemotherapy sessions it was appreciated the response to treatment with a new cyto-reductive surgery in 20 patients, and the remaining 4 patients by computed tomography.

The study is based on retrospective analysis of survey sheets, discharge registers, records of outpatient consultation, operators protocols, histopathological bulletins and oncology records.

RESULTS

The study followed 24 patients hospitalized and treated in the Surgical Oncology Clinic, with an average age of 58.49 years with a minimum value of 30 and maximum of 77 years. The peak incidence was recorded in the age group 50-65 years was 62.5%.

After the staging laparatomy, 24 patients have been in the stage FIGO IIIC, 22 patients presented voluminous neoplastic ascites and all the patients presented generalized peritoneal carcinomatosis.

Regarding histopathology (Figure 1), papillary adenocarcinoma was the most common result (83.33%); histopathological diagnosis showed mucinous adenocarcinoma in two cases (8.33%); in only a case was present adenocarcinoma with clear cell (4.16%), and in other case was present undifferentiated carcinoma (4.16%). The G2/G3 tumor grading report ratio was 16/8.

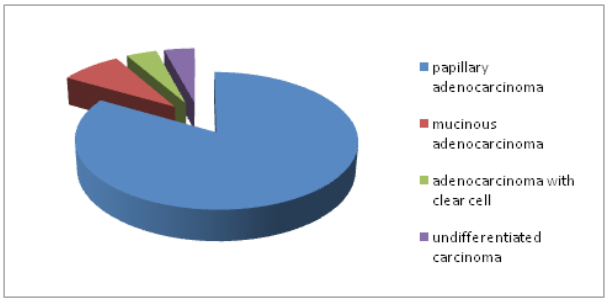

Fig.1. Statistical histopathological results regarding the incidence of different carcinoma types

Postoperatively patients received intraperitoneal chemotherapy by normothermia treatment and subsequent neoadjuvant systemic chemotherapy. The side effects related to the intraperitoneal administration of anti-neoplastic agents were: 18 patients presented abdominal pain during administration, 2 patients experienced leukopenia (WBC < 4,000/mm^3), 5 patients had prolonged postoperative ileus (over 4 days), 2 patients had renal toxicity (creatinine > 3 mg/dl), and 2 patients had postoperative wound infections.

Of the 24 patients only 20 patients were subjected to a new purpose cytoreductive surgery, 4 patients being diagnosed with progressive disease, therefore surgery was considered inadvisable. After the second surgery 9 patients were in complete remission, showing parts of sterile resection; 10 patients had partial remission, in 6 patients we could perform optimal cytoreductive (residual tumor <1 cm) and in 4 patients we could perform suboptimal cytoreduction to a residue tumor postoperatively between 1 and 2 cm; in one patient surgery was limited to exploratory laparotomy, presenting voluminous tumor masses un-resectable.

The short-term result in terms of response to neoadjuvant chemotherapy and systemic intraperitoneal postoperatively, after surgery evaluation by clinical examination and by clinical and imaging examination (Figure 2) was favorable to 79.16%, the remaining 20.83% showing chemoresistance.

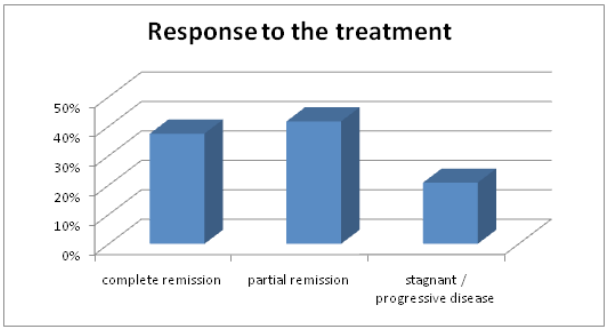

Fig.2. Statistical representation of treatment outcomes in cancer patients

The median survival of patients was 30.9 months, with a median of 24.3 months. For the patients who had a favorable response after neoadjuvant therapy there was a median survival of 35.62 months, with a median of 29.16 month. The survival rate for the same group of patients who received neoadjuvant therapy and cyto-reductive surgery was 12 months to 95.85%, 24 months to 50%, 36 months to 29.16% and 5 years to 16.66%.

DISCUSSION

Ovarian neoplasm is an important cause of mortality and morbidity in the malignancy. Optimal tumor cytoreduction performed both as a first therapeutic act as well as after neoadjuvant chemotherapy is considered a favorable prognostic factor in terms of survival for patients with advanced ovarian neoplesm (12,13). Residual tumor volume after primary surgery is closely related to survival, such a study on 3,126 patients where du Bois et al. (14) found an average survival of only 29.6 months in patients who had residual disease after primary surgery > 1 cm. Considering 75% (2) of ovarian neoplasm cases are diagnosed in advanced stages, being almost impossible an oncologic surgery, the analysis and evaluation of adjuvant or neoadjuvant opportunities that would make possible the application of a radical onco-surgical protocol requires searching for new methods for these cases.

One such method is the intraperitoneal chemotherapy applied according to numerous studies (GOG-114, GOG-158, GOG-172) at patients that could get a small residual tumor after primary surgery, the survival rate and disease-free period being significantly elevated for patients with intraperitoneal chemotherapy. The problem of this method occurs in patients whose primary tumor cytoreduction cannot achieve the optimal threshold. In our study we analyzed the data and the results of applying this method to patients without optimal cytoreduction who undergo neoadjuvant systemic chemotherapy.

Intraperitoneal administration of cytostatic agents is associated with greater toxicity than intravenous administration. Of the side effects related to intraperitoneal administration of cytostatic agents remember abdominal pain during the treatment, marrow toxicity, renal toxicity, infectious complications, neuropathy and even intestinal fistulas. In the present study we observed that the abdominal pain was the most frequently encountered side effect, followed by delaying intestinal transit, the remaining reactions being negligible due to the relatively low percentage.

There are some studies in the literature showing the effectiveness and safety of intraperitoneal chemotherapy in patients who have voluminous disease after staging surgery (16, 17). Thus S. Nagao et al. in a study of 44 patients after a combination therapy with intraperitoneal carboplatin and paclitaxel intravenously had a favorable answer to ~ 80% of patients, 16 patients were in complete remission, 19 had a partial response and 9 had stagnant or progressive disease and the median survival was 31 months. In another study conducted by Zylberberg B et al. on 13 patients with ovarian cancer stage IIIC and who had high intra-abdominal tumor volume received a chemotherapy regimen based on cisplatin and immunotherapy between 4 and 10 cycles, 6 patients had complete remission (18). U Beller et al, using a system of consolidation intraperitoneal chemotherapy with cisplatin and cyclophosphamide intravenously achieved complete remission in 32 patients (65%) with advanced ovarian cancer from a total of 49 patients, but the median survival for patients who had residual volume increased after primary surgery was only 24 months (19).

Analyzing the results of our study and comparing them with those in the literature we can notice the fact that there are insufficient data in order to demonstrate the superiority for the use of cytotoxic agents in patients with voluminous residual disease after primary surgery. In our study, the results obtained are in a reasonable cited in the literature.

CONCLUSIONS

The perioperative administration of cytostatic agents into the peritoneal cavity is not associated with major complications, but patients require additional follow up.

The use of perioperative intraperitoneal chemotherapy as consolidation treatment in neoadjuvant systemic chemotherapy made possible the tumor cytoreduction to an increased number of patients.

Patients with advanced ovarian cancer, who cannot make optimal primary cytoreductive surgery, the prognosis can be improved by using peritoneal and systemic cytotoxic agents in combination with secondary cytoreductive surgery.

REFERENCES

1. Aebi S, Castiglione M. On Behalf of the ESMO Guidelines Working Group. Newly and relapsed epithelial ovarian carcinoma: Esmo Clinical Recommendations for diagnosis, treatment and follow-up. *Annals of Oncology*, 2009; 20(4): iv21-iv23.
2. Ozols RF. Update on gynecologic Oncology Group (GOG) trials in ovarian cancer. *Cancer Invest*. 2004; 22(2):11-20.
3. Thigpen T. First-line therapy for ovarian carcinoma: what's next? *Cancer Invest.* 2004; 22(2):21-28.
4. Markman M. Intraperitoneal chemotherapy in the management of ovarian cancer: rationale and results. In Abstract book of 20th International Congress on Anticancer Treatment. Paris, February 3-6, 2009: 140-142.
5. Vincent T, DeVita Jr, Hellman S, Steven A, Rosenberg. Cancer: Principles and Practice of Oncology, 7th ed., 2005.
6. Ledermann JA, Raja FA, Fotopoulou C, Gonzalez-Martin A, Colombo N, Sessa C. Newly diagnosed and relapsed epithelial ovarian carcinoma: ESMO Clinical Practice Guidelines for diagnosis, treatment and follow-up. *Annals of Oncology*, 2013; 24(6): vi24-vi32.
7. Hoskins.WJ. Surgical staging and cytoreductive surgery of epithelial ovarian cancer. *Cancer*, 1993; 71:1534-40.
8. Vergote I, Tropé CG, Amant F, Kristensen GB, Ehlen T, Johnson N, Verheijen RHM, et al. European Organization for Research and Treatment of Cancer–Gynaecological Cancer Group and the NCIC Clinical Trials Group-a Gynecologic Cancer Intergroup Collaboration, Neoadjuvant Chemotherapy or Primary Surgery in Stage IIIC or IV Ovarian Cancer. *N Engl J Med*, 2010; 363: 943-953.
9. Markman M, Bundy BN, Alberts DS, et al. Phase III trial of standard-dose intravenous cisplatin plus paclitaxel versus moderately high-dose carboplatin followed by intravenous paclitaxel and intraperitoneal cisplatin in small-volume stage III ovarian carcinoma: an intergroup

study of the Gunecologic Oncology Group, Southwestern Oncology Group, and Eastern Cooperative Oncology Group. *J Clin Oncol*, 2001;19(4):1001-1007.
10. Armstrong DK, Bundy B, Wenzel L, et al. Intraperitoneal cisplatin and paclitaxel in ovarian cancer. *N Engl J Med*, 2006; 354(1):34-43.
11. Helm CW, Edwards RP. Intraperitoneal Cancer Therapy; Ed. Humana Press Totowa, New Jersey 2007.
12. Hoskins WJ, McGuire WP, Brady MF, Homesley HD, Creasman WT, Berman M, et al. The effect of diameter of largest residual disease on survival after primary cytoreductive surgery in patients with suboptimal residual epithelial ovarian carcinoma. *Am J Obstet Gynecol*, 1994; 170:974-80.
13. Bristow RE, Tomacruz RS, Armstrong DK, Trimble EL, Montz FL. Survival effect of maximal cytoreductive surgery for advanced ovarian carcinoma during the platinum era: A meta-analysis. *J Clin Oncol*, 2002; 20: 1248-59.
14. du Bois A, Reuss A, Pujade-Lauraine E, et al. Role of surgical outcome as prognostic factor in advanced epithelial ovarian cancer: a combined exploratory analysis of prospectively randomized phase 3 multicenter trials: by the Arbeitsgemeinschaft Gynaekologische Onkologie Studiengruppe Ovarialkarzinom (AGO-OVAR) and the grouped' Investigateurs nationaux our les Etudes des cancers de l'Ovaire (GINECO). *Cancer.* 2009; 115(6):1234-1244.
15. Markman M, Reichman B, Hakes T, Rubi S, Lewis JL Jr, Jones W, Barakat R, Curtin J, Almadrones L, Hoskins W. Evidence supporting the superiority of intraperitoneal cisplatin compared to intraperitoneal carboplatin for salvage therapy of small volume residual ovarian cancer. *Gynecol Oncol.* 1993: 50(1): 100-104.
16. Nagao S, Fujiwara K, Ohishi R, Nakanishi Y, Iwasa N, Shimizu M, Goto T, Shimoya K. Combination chemotherapy of intraperitoneal carboplatin and intravenous paclitaxel in suboptimally debulked epithelial ovarian cancer. *Int J Gynecol Cancer.*2008; 18: 1210-1214.
17. Fujiwara K, Nagao S, Kigawa J, Noma J, Akamatsu N, Miyagi Y, Numa F, Okada M, Aotani E. Phase II study of intraperitoneal carboplatin with intravenous paclitaxel in patient with suboptimal residual epithelial ovarian or primary peritoneal cancer: a Sankai Gyecologic cancer group study. *Int J Gynecol Cancer*. 2009; 19: 834-837.
18. Zylberberg B, Dormont D, Janklewicz S, Darai E, Bretel JJ, Poncelet C, Guillet JL, Madelenat P. Response to neo-adjuvant intraperitoneal and intravenous immunochemotherapy followed by interval secondary cytoreduction in stage IIIc ovarian cancer. *Eur J Gynaecol Oncol.* 2001; 22(1):40-5.
19. Beller U, Speyer J, Colombo N, Sorich J, Wernz J, Hochster H, Zeleniuch-Jacquotte A, Porges R, Beckman EM. Consolidation with Intraperitonealcisplatin in first-line therapy of advanced ovarian cancer. *J Clin Oncol.* 1991; 9(5): 809-17.

UTILIZAREA CHIMIOTERAPIEI INTRAPERITONEALE PERI-OPERATIVE ASOCIATA CU TERAPIA NEOADJUVANTA PENTRU CANCERUL OVARIAN AVANSAT CA TERAPIE DE CONSOLIDARE

REZUMAT

Obiective: Determinarea eficacitatii chimioterapiei peri-operative intraperitoneale si a sigurantei acestei metode la pacientii cu cancer ovarian care prezinta volum rezidual crescut dupa chirurgia primara. Metode: Studiu clinic retrospectiv in care au fost incluse 24 de paciente cu cancer ovarian, stadiul FIGO IIIC, care au suferit intervetie chirurgicala in Clinica de Oncologie a Spitalului Municipal Timisoara pe o perioada de 6 ani (2003-2009). Rezultate: Pacientele cuprinse in studiu au fost diagnosticate dupa stadializarea chirurgicala +/- citoreductia tumorala suboptimala, prezentand tumora reziduala voluminoasa dupa interventia chirurgicala; a fost administrat cisplatin si endoxan perioperator, urmat de 6 cicluri de chimioterapie sistemica neoadjuvanta. Printre efectele adverse ale administrarii chimioterapiei la nivelul cavitatii peritoneale s-au numarat: durere la 18 paciente, ileus la 5 paciente, leucopenie la 2 paciente si toxicitate renala la 2 paciente. Raspunsul la tratament a fost evaluat dupa interventia chirurgicala ulterioara la 20 paciente prin tomografie computerizata; la 9 paciente raspunsul a fost complet (37,5%), partial la 10 paciente (41,66%) si negativ la 5 paciente (20,83%), afectiunea fiind stagnanta sau in evolutie. Rata de supravietuire a pacientelor a fost de 30,9 luni, cu o medie de 24,3 luni. Concluzie: Chimioterapia intraperitoneala folosita ca tratament de consolidare la pacientii cu volum rezidual crescut dupa chirurgia primara este lipsita de complicatii majore, iar in combinatie cu chimioterapia sistemica si chirurgia citoreductiva poate imbunatatii prognosticul pe termen lung.

Cuvinte cheie: chimioterapie intraperitoneala, cancer ovarian, chirurgie citoreductiva

CHANGES INDUCED BY ADDITION OF METFORMIN IN CULTURE MEDIA OF TUMOR-ASSOCIATED FIBROBLASTS (TAF)

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ABSTRACT

Considering the important role played by tumor-associated fibroblasts (TAFs) in tumor development and progression, we tried to investigate in this study the influence of metformin on TAFs' phenotype. We isolated TAFs from surgical resection pieces obtained from patients with diagnosis of infiltrative ductal mammary carcinoma. Cultured cells were treated with metformin in two concentrations (5 mM and 10 mM) for 72 hours, and then the cells were flowcytometricaly analyzed for expression of surface markers CD44 and TGFβ-RII. Additional flowcytometric procedures included viability assay using Annexin-V/PI. The results showed that metformin-treated TAFs present an increased expression of CD44 and a decrease in TGFβ-RII expression on cellular surface. We concluded that metformin can induce changes in TAFs phenotype, which could switch their behavior towards a less aggressive phenotype, which will not provide support for tumor cells.

Key words: tumor-associated fibroblasts (TAF), metformin, surface markers, apoptosis

INTRODUCTION

Origin of tumor-associated fibroblasts (TAFs)

Within tumor tissues, myofibroblasts originate from diverse cellular types: pre-existent fibroblasts, adipocytes and pre-adipocytes, smooth muscle cells, endothelial cells, and bone marrow-derived cells (Figure 1). From all these cellular types, bone marrow-derived cells are a representative source of myofibroblasts within tumor stroma. Recent studies showed that 28 days after implantation of tumor pancreatic cells within a mouse model, approximately 40% of total myofibroblasts from tumor stroma derive from bone marrow cells (1).

Although during the normal fibrosis process, epithelial cells are the ones transforming into activated fibroblasts, there are not sufficient data demonstrating whether normal or malignant epithelial cells are transforming into myofibroblasts within tumor tissue.

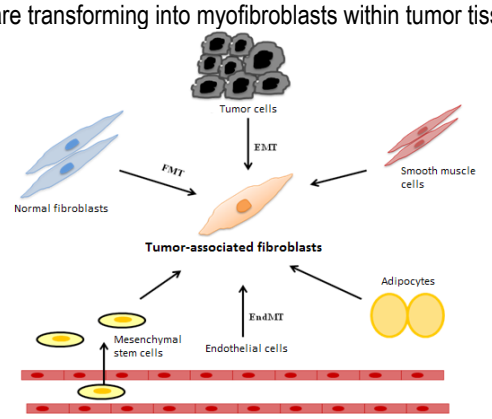

Fig. 1. Origin of tumor-associated fibroblasts (TAF). Mesenchymal stem cells (MSCs) transmigrate and can differentiate into TAFs at the level of tumor tissue. Other cells can also transform into TAFs: normal fibroblasts (fibroblast-mesenchymal transition-FMT), tumor cells (epithelial-mesenchymal transition-EMT), smooth muscle cells, adipocytes and endothelial cells (endothelial-mesenchymal transition-EndMT)

Tumor-associated fibroblasts role in tumorigenesis and metastasis

Proliferation of tumor cells is an important step in invasion and metastasis processes. In order to ensure an optimal environment for proliferation and development, tumor cells are recruiting stromal cells, which will support tumor development through secretion of growth factors and cytokines.

FSP-1 (fibroblast secreted protein-1) secreted by tumor-associated fibroblasts is an important factor for tumor cells development. FSP-1 knockout mice presented a decreased incidence in tumor formation and did not developed metastases when tumor cells were injected, while co-injection of FSP-1 overexpressing fibroblasts induced tumor formation (2). The results of this experiment showed that tumor-associated fibroblasts have an important influence on tumor microenvironment, stimulating tumor development. SDF-1 (stromal derived factor 1) is a cytokine secreted by tumor-associated fibroblasts, with a role in recruiting endothelial cells precursors, which will further be involved in angiogenesis (3). SDF-1 is also known as CXCL12, ligand for CXCR4, overexpressed in tumor cells. CXCR4 activation can stimulate tumor cells proliferation (4).

In some of the malignant tumors, such as breast cancer (5) and colon cancer (6), an increased expression level for TGFβ was reported, TGFβ being a growth factor which stimulates normal fibroblasts differentiation into tumor-associated fibroblasts and myofibroblasts.

Considering the important role played by tumor-associated fibroblasts (TAFs) in tumor development and progression, we tried to investigate in this study the influence of metformin on TAFs' phenotype. Changes induced in TAFs could switch their behavior towards a less aggressive phenotype, which will not provide support for tumor cells.

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MATERIALS AND METHODS

Isolation and culture of tumor-associated fibroblasts

Human tumor-associated fibroblasts were isolated using both explant method and enzymatic digestion with Collagenase type IV-S obtained from *Clostridium histolyticum* (Sigma-Aldrich Company, St. Louis, MO, USA). Surgical rezection pieces were obtained from patients with diagnosis of infiltrative ductal mammary carcinoma. Isolated cells were washed with saline solution (PBS, Phosphate Buffer Saline, Sigma-Aldrich) and successively filtered through 0.7 and 0.4 µm cell strainers. Cellular suspension was centrifuged (1500 rpm, 10 min), and cells were resuspended in culture medium - Dulbecco's Modified Eagle Medium (DMEM, Sigma), supplemented with 10% Fetal Calf Serum (FCS; PromoCell, Heidelberg, Germany) and 1% antibiotic solution containing Penicillin/Streptomycin (Pen/Strep, 10,000 IU/mL, PromoCell). TAFs were cultured in adherent culture flasks (T75 culture flasks), at a cellular density of 10,000 cells/cm^2, and expanded at 37°C, in 5% CO_2 atmosphere.

All tissue samples were obtained from human subjects after signing the Informed Consent Form elaborated by Ethics Committee of "Victor Babes" University of Medicine and Pharmacy Timisoara, according to Helsinki declaration of World health Organization.

Immunophenotypical analysis

TAFs were cultures in 6-well adherent culture plates. 24 hours after plating, metformin was added in 2 concentrations – 5 mM and 10 mM – in culture medium, and after 4 days of culture, the cells were detached from the culture plates using 0.25% Trypsin-EDTA (Sigma-Aldrich Company), centrifuged for 10 minutes at 1500 rpm, and washed twice with PBS. Cells were further resuspended in 100 µl PBS for each tube of flowcytometric analysis, together with 4 µl of fluorochrome-conjugated mouse anti-human antibodies. Cells are vortexed together with the antibodies and left to incubate for 30 minutes, in the dark, at room temperature. After incubation, cells are washed with 2 ml wash solution (Cell Wash Solution, BD Biosciences), centrifuged 10 minutes at 1500 rpm, the supernatant is discarded, and the pellet is resuspended in 500 µl wash solution and are ready for flowcytometric acquisition and analysis. Flowcytometric acquisition was performed using FACSCalibur flowcytometer (Becton-Dickinson), endowed with 2 lasers and capable to differentiate 4 distinct colors. The fluorochrome-conjugated antibodies were: PE - TGFβ-RII, FITC - CD44.

Viability assay Annexin-V/PI

In order to determine metformin capacity to induce cellular apoptosis, we performed Annexin-V/PI flowcytometric assay. Annexin-V binds phosphatidylserine on injured cell membrane. For Annexin-V assay, the cells were washed with 1 ml Annexin-V Binding Buffer and centrifuged 10 minutes at 1500 rpm. The supernatant is discarded and cells are resuspended in 100 µl de Annexin-V Binding Buffer, and 10 µl of FITC-conjugated Annexin-V are added. After 15 minutes of incubation at room temperature, in the dark, cells are washed again, and resuspended in 500 µl of the same buffer, while PI solution (1 µg/ml) is added just before the flowcytometric acquisition procedure.

Analysis of flowcytometric graphs was performed using FCS Express 4 Flow Research Edition software (De Novo Software), while for statistic analysis we used GraphPad Prism 6 (GraphPad Software).

RESULTS

Annexin V-FITC / PI assay

Flowcytometric analysis of Annexin-V/PI assay performed on TAFs revealed a decrease of live cells, after addition in culture medium of metformin in concentrations of 5 mM and 10 mM, for 72 hours. Compared to control cells (untreated TAFs), this assay showed that a larger cell number are entering in early apoptotic stage, when treated with metformin 5 mM; in case of using higher metformin concentrations (10 mM), the cells found in late apoptotic stages are in higher amount than for lower metformin concentrations (5 mM) (Figures 2 and 3).

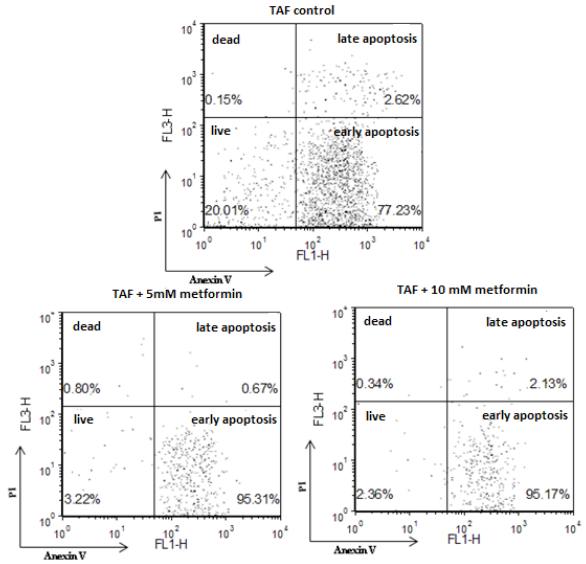

Fig. 2. Dot plot graphs showing distribution of metformin-treated TAFs in different viability stages: live cells, early or late apoptotic cells. Only few cells are dead after metformin treatment, for both concentrations used.

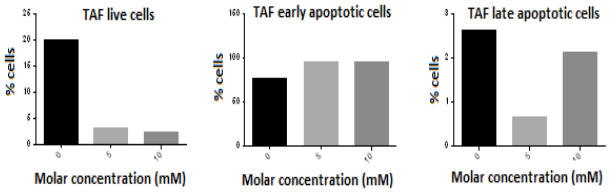

Fig. 3. Annexin-V/PI assay performed on tumor-associated fibroblasts showed a decrease of live cells when treated with metformin in different concentrations. The remaining cells are in early or ate apoptotic stages, correlated with metformin concentration

Flowcytometric analysis of CD44 and TGFβ RII markers

Flowcytometric analysis of CD44 and TGFβ RII on TAFs sur-

face showed that the cells treated with metformin in cancentration of 5 mM and 10 mM present an increased expression of CD44. For TGFβ RII, the analysis revealed an increased expression when 5 mM metformin was added and a significant decrease of its expression when the cells were treated with 10 mM metformin (Figures 4, 5, 6, and 7).

CD44 is a transmembrane glycoprotein which functions as ligand for hyaluronic acid, but also for other ligands, such as matrix metalloproteinases, involved in tumor metastasis. CD44 is involved in cellular interactions, cellular adhesion and migration. Moreover, this protein participates in lymphocytes activaton, recirculation, homing, hematopoiesis, and tumor metastasis. CD44 interact with osteopontin and leads to initiation of tumor process (7). This glycoprotein interacts with laminin, collagen, and fibronectin, but the results of their physiological interactions are not completely understood (8). CD44 expressed on surface of tumor cells is involved in systemic migration of tumor cells, through interactions with selectins L and (9). This molecule is also involved in signaling pathways of tumorigenesis initiation through interaction with tyrosine kinase receptors (10). CD44 is largely used as surface marker for isolation of cancer stem cells in breast cancer, prostate, ovary, and colon cancer (11). Using other additional surface markers, we can differentiate different tumor types (12). There are still contradictions related to correlation between CD44 and cancer prognostic. In many cancer types, CD44 present a major role in initiation of tumorigenesis and metastasis. Other studies performed on prostate tumor cells (13) and breast cancer cells (14), CD44 overexpression is not correlated with carcinogenesis.

TGFβ RII is part of TGFβ receptors family. Dependant on structure and properties, there are three types of receptors. Type I and type II receptors have similar affinity for ligands, higher in case TGF-β1, and decreased for TGF-β2. Type III receptors have increased affinity, both for TGF-β1, as well as for TGF-β2.

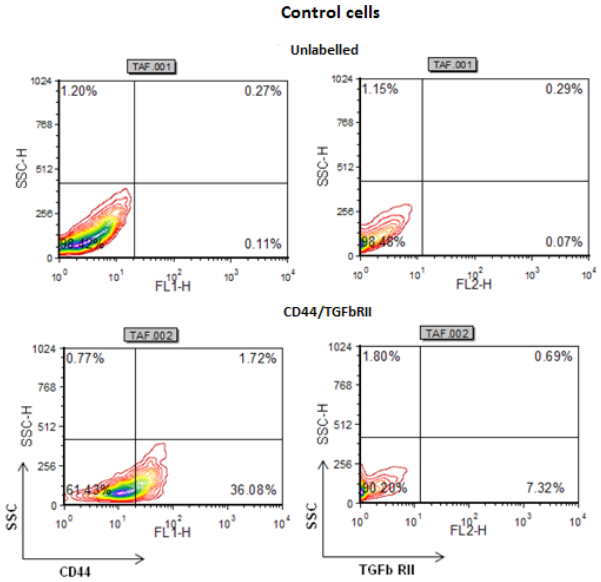

Fig. 4. Contour plot of TAFs control (untreated cells)

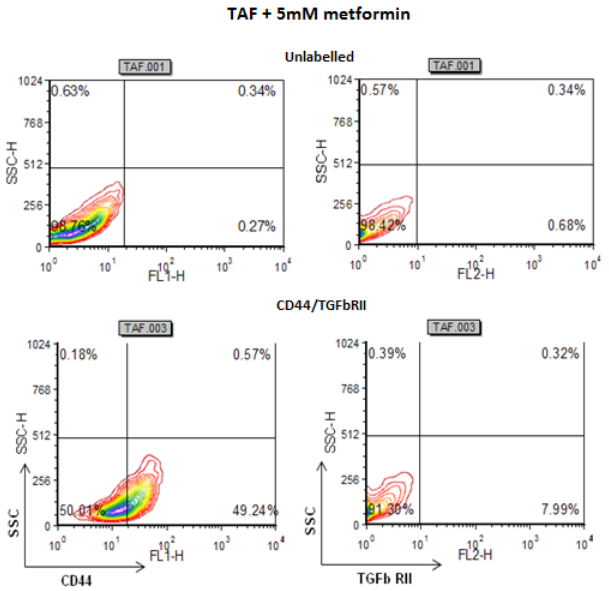

Fig. 5. Contour plot of 5 mM metformin-treated TAFs

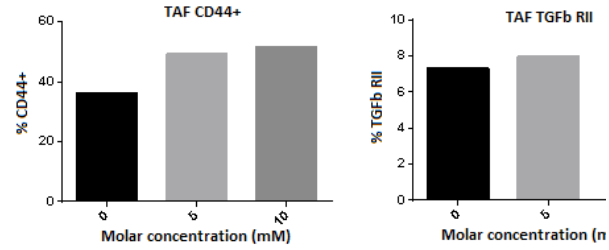

Fig.6. Contour plot of 10 mM metformin-treated TAFs

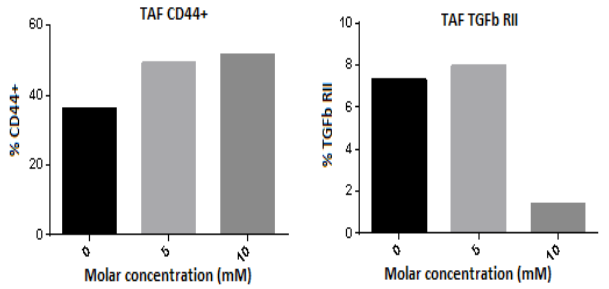

Fig. 7. Comparative graphs depicting expression of CD44 and TGFβ RII in control TAFs (untreated cells) and metformin-treated TAFs

DISCUSSION AND CONCLUSION

The results of flowcytometric assay using Annexin V/PI employed to investigate metformin influence on cellular apoptosis showed that TAFs present a decreased in live cell population, while large number of cells are in different apoptotic stages, thus indicating that metformin is capable of activating apoptotic pathways in TAFs.

In order to better understand metformin effect on TAF, we used immunophenotypical assays. We investigated expression of CD44 and TGFβ RII surface markers on TAFs. Interestingly, we showed a significant decrease of TGFβ RII on TAF surface when 10 mM metformin was added in the culture medium.

There are several studies sustaining that metformin is able to induce apoptosis, but other studies are supporting the opposite idea. It was shown that metformin has the capacity ot induce apoptosis in lung cancer cells by activating MAPK pathway (15), in ovary tumor cells by activating Bcl-2 protein family (16), while in mammary tumor cells (MCF-7) on the ERK signaling pathway (17). Contradicting these studies, Alimova et al. showed that metformin is not inducing apoptosis in several cell lines, including breast cancer cells MCF7, MCF7/713, BT474 and SK-BR3 (18). In our experiments we support the hypothesis that metformin has the capacity to induce apoptosis in tumor-associated fibroblasts (TAF).

Although some studies suggest that metformin reduces CD44+ CD24- cell population in tumor cells, in case of tumor-associated fibroblasts we noticed an increased in CD44 expression and maintenance of vimentin expression (data not shown). We may conclude that TAFs maintained mesenchymal characteristics as a result of metformin treatment. Interestingly, metformin induces a decrease in TGFβ-RII expression on TAF surfce. In other studies performed on breast cancer cells, TGFβ transcription factor was suppressed, which was correlated with epithelial-mesenchymal transition. So far there are not enough data to associate TGFβ-RII inhibition with a certain biological activity, but further studies will include more thorough analysis of metformin effects on TGFβ activity at the level of tumor-associated fibroblasts, performing co-cultures of TAFs and breast cancer cells.

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REFERENCES

1. Ishii G, Sangai T, Oda T, Aoyagi Y, Hasebe T, et al. Bone-marrow-derived myofibroblasts contribute to the cancer-induced stromal reaction. *Biochem. Biophys. Res. Commun.*, 2003; 309(1): 232-240.
2. Grum-Schwensen B, Klingelhofer J, Berg CH, El-Naaman C, Grigorian M, Lukanidin E, Ambartsumian N. Suppression of tumor development and metastasis formation in mice lacking the S100A4(mts1) gene. *Cancer Res.*, 2005; 65(9): 3772-80.
3. Goh PP, Sze DM, Roufogalis BD. Molecular and cellular regulators of cancer angiogenesis. *Curr Cancer Drug Targets*, 2007; 7(8): 743-758.
4. Orimo A, Gupta PB, Sgroi DC, Arenzana-Seisdedos F, Delaunay T, et al. Stromal fibroblasts present in invasive human breast carcinomas promote tumor growth and angiogenesis through elevated SDF-1/CXCL12 secretion. *Cell*, 2005; 121(3): 335-348.
5. SerraR, Crowley MR. TGF-beta in mammary gland development and breast cancer. *Breast Dis*, 2003; 18: 61-73.
6. Tsushima H, Kawata S, Tamura S, Ito N, Shirai Y, et al. High levels of transforming growth factor beta 1 in patients with colorectal cancer: association with disease progression. *Gastroenterology*, 1996; 110 (2): 375-382.
7. Rangaswami H, Bulbule A, Kundu GC. Osteopontin: role in cell signaling and cancer progression. *Trends in Cell Biology*, 2006; 16(2): 79-87.
8. Ponta H, Sherman L, Herrlich PA. CD44: From adhesion molecules to signalling regulators. *Nat Rev Mol Cell Biol*, 2003; 4(1): 33-45.
9. Napier SL, Healy ZR, Schnaar RL, Konstantopoulos K. Selectin ligand expression regulates the initial vascular interactions of colon carcinoma cells. *J. Biol. Chem.*, 2007; 282 (6): 3433-41.
10. Bourguignon LYW. CD44-mediated oncogenic signaling and cytoskeleton activation during mammary tumor progression. *J Mammary Gland Biol Neoplasia*, 2001; 6(3): 287-297.
11. Bapat SA. Human ovarian cancer stem cells. *Reproduction*, 2010; 140(1): 33-41.
12. Strauss R, Li ZY, Liu Y, Beyer I, Persson J, et al. Analysis of epithelial and mesenchymal markers in ovarian cancer reveals phenotypic heterogeneity and plasticity. *PLoS ONE*, 2011; 6(1): e16186.
13. Gao AC, Lou W, Dong JT, Isaacs JT. CD44 is a metastasis suppressor gene for prostatic cancer located on human chromosome 11p13. *Cancer Res*, 1997; 57(5): 846-849.
14. Lopez JI, Camenisch TD, Stevens MV, Sands BJ, McDonald J, Schroeder JA. CD44 attenuates metastatic invasion during breast cancer progression. *Cancer Res*, 2005; 65(15): 6755-63.
15. Wu N, Gu C, Gu H, Hu H, Han Y, Li Q. Metformin induces apoptosis of lung cancer cells through activating JNK/p38 MAPK pathway and GADD153. *Neoplasma*, 2011; 58(6): 482-490.
16. Yasmeen A, Beauchamp MC, Piura E, E. Segal, Pollak M, Gotlieb WH. Induction of apoptosis by metformin in epithelial ovarian cancer: involvement of the Bcl-2 family proteins. *Gynecol. Oncol.*, 2011; 121(3): 492-498.
17. Malki A, Youssef A. Antidiabetic drug metformin induces apoptosis in human MCF breast cancer via targeting ERK signaling. *Oncol. Res.*, 2011; 19(6): 275-285.
18. Alimova IN, Liu B, Fan Z, Edgerton SM, Dillon T, Lind SE, Thor AD. Metformin inhibits breast cancer cell growth, colony formation and induces cell cycle arrest in vitro. *Cell Cycle*, 2009; 8(6): 909-915.

MODIFICARI INDUSE DE ADAUGAREA METFORMINULUI IN MEDIUL DE CULTURA AL FIBROBLASTELOR PERI-TUMORALE (TAF)

REZUMAT

Considerand rolul important pe care il au fibroblastele peri-tumorale (TAF) in dezvoltarea si progresia tumorala, am investigat in acest studiu influenta metforminului asupra fenotipului acestor celule. TAF au fost izolate din piese de rezectie chirurgicala de la paciente diagnosticate cu carcinom mamar ductal infiltrativ. Celulele cultivate au fost tratate cu metformin in doua concentratii (5 mM si 10 mM) timp de 72 de ore, celulele fiind ulterir analizate flowcitometric pentru expresia markerilor de suprafata CD44 si TGFβ-RII. Proceduri flowcitometrice aditionale au inclus testul de viabilitate care utilizeaza Annexin-V/PI. Rezultatele au aratat ca fibroblastele peri-tumorale tratate cu metformin au prezentat expresie crescuta a CD44 si o scadere a expresiei TGFβ-RII la nivelul suprafetei celulare. Se poate astfel concluziona ca metfromin poate induce modificari fenotipice la nivelul TAF, care ar putea modifica comportamentul acestor celule spre un fenotip mai putin agresiv, care sa nu ofere suport pentru dezvoltarea tumorala.

Cuvinte cheie: fibroblaste peri-tumorale, metformin, markeri de suprafata, apoptoza