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### CONTENTS

## CUPRINS

# ROLE OF PNPLA3 I148M VARIANT IN CHRONIC LIVER DISEASE

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**ABSTRACT**

Patatin-like phospholipase domain containing 3 (PNPLA3) is an enzyme preferentially expressed in the liver, with dual anabolic and catabolic roles in lipid metabolism. A single nucleotide polymorphism in *PNPLA3* gene (rs738409 C>G), leading to the substitution of isoleucine with methionine in position 148 of the protein, was recently described as a key determinant of liver fat content. Variant I148M was associated with the development of the full spectrum of liver injury related to lipid accumulation, from simple steatosis to steatohepatitis. Subsequent studies established a strong link between PNPLA3 I148M variant and liver fibrosis progression towards advanced stages, cirrhosis development, clinical outcome in chronic hepatitis C, and hepatic carcinogenesis. PNPLA3 rs738409 polymorphism represents a valuable genetic predictor and a potential therapeutic target in liver disease. In this review, we briefly summarize the main hypotheses on the cellular functions of PNPLA3 and the role of its I148M variant as a risk factor in the progression of major forms of liver disease.

**Keywords:** Patatin-like phospholipase domain containing 3 (PNPLA3), liver disease, fibrosis, fatty liver disease, hepatocellular carcinoma, genetic risk factor

## INTRODUCTION

Patatin-like phospholipase domain containing 3 (PNPLA3), also known as adiponutrin, is a member of the patatin-like phospholipase family. It shares a common domain with patatin, a major protein of potato with non-specific lipid acyl hydrolase activity [1].

Although its *in vivo* function is still subject to debate, a single variant in PNPLA3 (rs738409) was recently associated with liver fat content in individuals of various ethnicities [1]. This variant soon emerged as a key genetic determinant of advanced injury in various etiologies of liver disease, such as chronic viral hepatitis, alcoholic or non-alcoholic liver disease, and hepatocellular carcinoma (HCC).

In this review, we briefly summarize the main hypotheses on the cellular functions of PNPLA3 and the role of its sequence variation as a risk factor in the progression of major forms of liver disease.

### *Role of PNPLA3 in lipid metabolism*

In humans, the *PNPLA3* gene is localized on the long arm of chromosome 22 and encodes for a transmembrane polypeptide chain of 481 amino acids. Among the 9 patatin domain-containing proteins encoded by our genome, the structure of PNPLA3 is most closely related to that of the second member of the family (PNPLA2), which is the triacylglycerol hydrolase of the adipose tissue [2]. Early attempts to characterize its biochemical function showed that recombinant PNPLA3 protein expressed in Sf9 cells had predominantly acyl glycerol hydrolase activity [2]. The enzyme was active on the three major classes of glycerolipids (triacylglycerol, diacylglycerol, and monoacylglycerol), with a preference for oleic acid as the fatty acid moiety. The non-synonymous C>G polymorphism rs738409 leads to the substitution of an isoleucine with a methionine residue in codon 148. The longer side chain of methionine at this position impairs substrate access to the catalytic serine of the enzyme's active site, leading to a significant decrease of glycerolipid hydrolase activity of the protein [2]. These results were supported by an independent study employing a different expression system for the PNPLA3 [3]. The loss of function of the variant protein implied by these results may explain the higher liver fat content observed in carriers of the I148M form. However, *in vivo* studies failed to support the loss-of-function theory, as mice deficient in PNPLA3 did not develop fatty liver disease or metabolic syndrome [4,5].

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Another group of researchers described an anabolic function of PNPLA3, which was found to convert lysophosphatidic acid into phosphatidic acid and thus promote cellular lipid synthesis [6]. The lysophosphatidic acid acyl transferase (LPAAT) activity of the I148M variant of PNPLA3 was higher than that of the wild-type protein, both *in vitro* and *in vivo*. Conversely, adiponutrin-deficient mice showed decreased hepatic LPAAT activity, but normal triglyceride (TG) hydrolase function. This gain-of-function theory is further supported by the observation that chronic overexpression of PNPLA3 I148M in mouse liver results in development of hepatic steatosis [7]. In this model, the variant enzyme seems to exert three effects on liver TG metabolism: increased synthesis of fatty acids and TG, reduced TG hydrolysis, and relative depletion of TG long-chain polyunsaturated fatty acids.

PNPLA3 expression regulation suggests that the enzyme has a more important role in lipid anabolism than in catabolic processes [8]. PNPLA3 levels are highly controlled by nutritional stimuli [9]. This regulation is modulated *via* transcription factors such as liver X receptor (LXR) and sterol response element binding factor 1 (SREBF1) [10]. During fasting, the low levels of SREBF1 entrain a decrease in PNPLA3 transcription and an efficient degradation of the protein, resulting in very low levels of PNPLA3 in the liver. When glicemia increases, insulin induces SREBF1 which, in turn, upregulates PNPLA3 expression and increases fatty acid biosynthesis. Thus, PNPLA3 is induced when the liver synthesizes TG and includes them into lipid droplets [10]. Using an animal model, it was also shown that PNPLA3 regulation in the adipose tissue mirrors that in the liver [9].

A unifying hypothesis on PNPLA3 function was recently proposed [8]. Accordingly, adiponutrin may play dual roles, in both the biosynthesis and hydrolysis of lipids, depending on various factors related to the nutritional environment, such as sources of lipids, or to possible interactions with as yet unidentified cofactors.

*PNPLA3 and liver steatosis*

*PNPLA3* polymorphism rs738409 was first identified in a genome-wide association study as an independent factor associated with liver fat content [1]. Interestingly, it was not linked with major changes in biochemical markers of glucose or lipid metabolism. No association was observed between *PNPLA3* allele G and BMI, fasting serum glucose, insulin or homeostatic model assessment of insulin resistance (HOMA-IR). It was also not associated with lipid profile markers such as plasma triglycerides, total cholesterol, high-density-lipoprotein cholesterol or low-density-lipoprotein cholesterol.

Several subsequent studies assessed the association of PNPLA3 polymorphism with liver fat accumulation in its various forms of increasing severity: from simple steatosis to NAFLD and NASH. Data from the meta-analysis performed by Sookoian and Pirola [11] provides suggestive evidence that rs738409 is a strong modifier of NAFLD natural history. Among individuals without steatosis of secondary causes, including alcohol abuse, chronic hepatitis B or C, total parenteral nutrition, and use of steatosis-inducing drugs, carriers of GG homozygote genotype had a liver fat content with about 70% higher than CC homozygotes. GG homozygotes also had more than three-fold greater risk of higher necro-inflammatory scores and fibrosis when compared with CC genotype individuals. NASH was also more frequent in patients with GG genotype. rs738409 polymorphism seems to provide an additive genetic risk for NAFLD development. Interestingly, the meta-analysis revealed a negative association between the effect of PNPLA3 polymorphism on liver fat content and male gender. This implies that PNPLA3 I148M variant more effectively increases the risk of NAFLD in females than in males. It is possible that the modulation of lipogenic genes such as SREBP1c by estrogens be responsible for this sexual dimorphism [11].

A more recent meta-analysis by Xu et al. [12] did not find a significant association between the rs738409 polymorphism and simple steatosis, but showed the same link between *PNPLA3* and the risk of NAFLD and NASH.

*PNPLA3 and HCV infection*

Liver steatosis accompanies CHC in up to two thirds of cases [13] and is favored by a series of factors including alcohol consumption, overweight/ obesity, hyperglycemia, diabetes and viral genotype 3 [14]. The presence of steatosis contributes to histological progression in CHC and is independently associated with advanced fibrosis [13]. Liver steatosis is also an independent negative predictor for response to interferon-based antiviral therapy in CHC [15,16].

There is increasing evidence that cellular lipids, and particularly lipid droplets (LDs), play a key role in the production of infectious HCV particles [17]. The recruitment of HCV replication complexes induces the aposition of LDs and endoplasmic reticulum-derived membranes, generating a lipid membranous complex wherein virion assembly can proceed [18].

Impairment of HCV core protein binding to LDs sharply decreases the formation of viral replication complexes and the production of infectious virions [19]. It is still not clear whether the cytosolic LDs are recruited to the endoplasmic reticulum where virus assembly takes place or if they serve themselves as a platform for the initiation of assembly [20]. However, a fraction of HCV particles circulating *in vivo* in infected patients are associated with triglycerides and ApoB, and their formation seems to be linked to the process of VLDL assembly [20-22]. These less dense particles also exhibit higher infectivity [23].

PNPLA3 is a protein tightly associated with LDs and contributes to their formation [24,25]. The I148M form contributes to intracellular lipid accumulation and increases LDs size compared with the wild-type protein [26], a phenomenon compatible with the observation that transgenic mice overexpressing PNPLA3 I148M develp a NAFLD-like phenotype [24]. Triglyceride accumulation may promote HCV replication [27]. On the other hand, PNPLA3 influences VLDL secretion from hepatocytes, both in humans and *in vitro*, and the I148M motation results in a loss-of-function [25]. Thus, carriers of PNPLA3 allele G have a lower VLDL secretion than CC homozygotes for the same amount of liver fat and it was proposed that the PNPLA3 variant may reduce lipidation of nascent VLDL [25]. In this context, we might expect PNPLA3 I148M to negatively affect secretion of lipidated, more infectious, HCV particles.

It is not clear so far whether PNPLA3 or its variant I148M affect HCV life cycle. It is however well documented that PNPLA3 I148M influences the clinical course of chronic hepatitis C.

Besides the decrease of TG hydrolase and increased LPAAT activities, impairment of TG export from hepatocytes is another mechanism by which PNPLA3 I148M variant promotes intracellular fat accumulation [25]. *In vivo*, genotype rs738409 GG of PNPLA3 contributes as an independent risk factor for the development of liver steatosis and advanced injury in CHC patients of various ethnic origins. Thus, in Caucasian CHC patients from Belgium, Germany and France, adiponutrin genotype GG was associated with steatosis, fibrosis stage, and fibrosis progression [28]. In a Swiss cohort, the same genetic marker was linked with a high risk of steatosis in patients infected with HCV genotype non-3 [29]. In Italian CHC patients, rs738409 GG was a risk factor for steatosis, fibrosis stage, cirrhosis and poor response to antiviral treatment and HCC development [30]. PNPLA3 genotype was also associated with the presence of steatosis, severe necro-inflammation and advanced fibrosis in Japanese HCV carriers [31].

Further on, PNPLA3 genotype seems to interact with IL-28B genotype, a well-known predictor of response to antiviral therapy [32], in the development of CHC-related hepatic steatosis. In this regard, PNPLA3 allele G favored steatosis in patients carrying IL-28B genotypes CT or TT, but not CC [33]. Valenti et al. showed that PNPLA3 GG patients infected with HCV genotype non-3, also carrying IL-28B CC genotype, were protected against steatosis [34].

Despite the strong association of PNPLA3 I148M variant with processes that favor HCV-related disease progression, such as lipid biosynthesis, lipid droplet formation and liver steatosis, it has a negligible influence on viral clearance achievement following antiviral treatment in unselected CHC patients [35,36]. However, PNPLA3 GG may negatively affect sustained viral response rates in difficult-to-cure CHC patients infected with viral genotype 1 or 4 and presenting advanced pretreatment fibrosis [36].

PNPLA3 genotype GG is an important risk factor for CHC disease progression. A suggestive association is the increasing prevalence of this genotype among patients with increasing disease severity: 4% in CHC without steatosis, 17% in CHC with steatosis, and 24% in HCC (30). PNPLA3 allele G is linked with a higher risk of clinical deterioration, defined as the development of ascites, hepatic encephalopathy, variceal hemorrhage, HCC, or liver-related death in patients with CHC-related cirrhosis [37]. The association remains significant even in age, sex, and race-adjusted analysis.

*PNPLA3 and liver fibrosis*

The effect of rs738409 genotype on liver fibrosis severity was assessed in a recent meta-analysis summarizing 24 studies and 9915 patients [38]. The included studies were mostly performed in Europe and in the US. There was no significant heterogeneity among studies. Advanced fibrosis was significantly associated with PNPLA3 genotype, in the dominant as well as in the recessive genetic models. In the recessive model, which is the most commonly used, the GG carrier status was more strongly associated with advanced fibrosis than CG and GG genotypes. The effect of PNPLA3 genotype on fibrosis stage was similar among studies conducted in the US, Europe and elsewhere. GG genotype had also similar effect on fibrosis in patients with various etiologies of liver disease, including NAFLD, ALD and CHC [38]. The same effect of rs738409 was observed in Asian populations. In Japanese patients with CHC, the rs738409 GG genotype was associated with advanced fibrosis, as well as with the presence of steatosis and severe necroinflammatory activity [31].

The I148M variant of PNPLA3 was also associated with increased risk of cirrhosis in patients with underlying liver disease, genotype GG carriers having a more than three-fold risk of cirrhosis than CC individuals [39]. Another meta-analysis underlined a clear association of rs738409 G allele with alcoholic liver cirrhosis, in an additive genetic model for this variant [40].

Interestingly, PNPLA3 genotype seems to interact with age at infection in determining fibrosis progression in CHC patients [41]. Thus, PNPLA3 GG genotype increased fibrosis progression rates in individuals infected at older age. Additionally, PNPLA3 impact on fibrosis progression was more pronounced in patients at higher risk of altered hepatic lipid metabolism, such as overweight patients, with grade 2-3 steatosis and infected with HCV genotype 3 [41]. A stronger effect of PNPLA3 genotype on CHC severity in the presence of additional metabolic disturbances is also supported by the observation that GG genotype diabetic patients had an almost 9-fold risk

increase for the presence of advanced fibrosis compared with non-diabetics [42].

It is known that hepatic stellate cells (HSCs) play a major role in the development of liver fibrosis [43]. A novel link between PNPLA3 variant, HSCs and the genetic predisposition to chronic liver disease was recently proposed by Pirazzi et al. [44]. The authors showed that PNPLA3 is highly expressed in human HSCs. PNPLA3 expression in primary HSCs was regulated by retinol availability and insulin. Thus, PNPLA3 was downregulated when retinol was abundant, whereas retinol deficiency resulted in an increased expression of the protein. Moreover, PNPLA3 was able to hydrolyze retinyl palmitate and allow the extracellular release of retinol. The retinyl esterase activity of PNPLA3 was restricted to HSCs and not observable in hepatocytes. The I148M variant of the enzyme lacked the retinyl-esterase activity and promoted retinol retention within HSCs, in parralel with decreased circulating levels of retinol and retinol binding protein 4. This in turn may interact with chronic liver injury [44], since loss of retinyl esters-containing LDs was suggested to diminish hepatic fibrogenesis and carcinogenesis [45].

*PNPLA3 and risk of hepatocellular carcinoma*

HCC is the fifth most frequent form of cancer and the third cause of cancer-related death worldwide [46]. In Europe and the United States, the rise of HCC incidence was mainly related to CHC cases.

In a meta-analysis including nine independent studies, 2937 patients were assessed with respect to the risk of developing HCC related to their rs738409 genotype [38]. With the exception of one study conducted in Japan, all the others were performed in Europe, with Caucasian patients. Depending on the genetic model, there was a significant risk of HCC development associated with rs738409 alleles. In the recessive model, GG genotype carriers were significantly more likely to develop HCC than CG and CC carriers. *PNPLA3* was also independently associated with the risk of HCC in a subset of 6 studies including patients with liver cirrhosis. In the dominant model, CG and GG patients had higher risk of HCC development than CC patients. These results suggest that the role of PNPLA3 variant in liver pathology is not limited to development of steatosis and NAFLD, but it also promotes fibrosis progression towards advanced stages and HCC development. Interestingly, the association of *PNPLA3* genotype with HCC was not observed in the subgroup analysis including HCV-related cirrhosis patients [38]. Similar results were provided by a separate meta-analysis with individual patient data including 2503 participants with cirrhosis, which found a tight association between rs738409 and overall HCC [47]. The strength of association between allele G and HCC was high in ALD patients and less evident in CHC-related cirrhosis. In a French and Belgian cohort of cirrhotic patients, rs738409 genotypes did not influence the risk of HCC development or death in HCV-infected individuals, whereas genotype GG independently predicted the risk of HCC in ALD-related cirrhosis [48].

In a different cohort from UK, the risk of HCC development conferred by *PNPLA3* rs738409 GG genotype in NAFLD patients was 5 times higher than CC genotype in participants with the same underlying liver disease, and 12 times higher than CC genotype in the general population [49].

PNPLA3 genotype GG predicts HCC occurrence at an earlier stage in ALD and NAFLD and is a negative predictor for patient survival, being associated with more diffuse HCC at presentation [50].

It was suggested that, when hepatic microenvironment is altered by steatosis/ steatohepatitis, the liver may be predisposed to cancer even in the absence of cirrhosis. Several mechanisms have been proposed to explain the procarcinogenic effect of the I148M variant [51]: intensified lipogenesis and availability of fatty acids as energy source for rapidly proliferating cells, altered adipokine secretion influencing microinflammation and insulin resistance, low-level hepatic inflammation promoting release of tumor necrosis factor alpha and interleukin-6, lipotoxicity altering intracellular signaling, mitochondrial damage and oxiative stress due to lipid peroxidation.

## CONCLUSION

In conclusion, adiponutrin is a new key determinant of liver disease progression. Future studies are required to establish the cellular roles of PNPLA3 and the mechanisms driving its involvement in liver pathology. Hopefully, further understanding of the pathophysiological role of PNPLA3 and its variant in the development liver steatosis, fibrosis and carcinogenesis, will enable the discovery of new therapeutic targets. Also, genetic testing of PNPLA3 polymorphism may be included in diagnostic protocols meant to identify individuals at risk of aggressive forms of liver disease and to allow a better personalization of clinical monitoring and treatment of these patients.

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## ROLUL POLIMORFISMULUI PNPLA3 I148M IN BOLILE CRONICE HEPATICE

**REZUMAT**

PNPLA3 este o enzima exprimata preferential la nivelul ficatului, avand rol atat in anabolismul, cat si in catabolismul lipidelor. Un polimorfism punctiform la nivelul genei PNPLA3 (rs738409 C>G), care are drept consecinta substitutia isoleucinei cu metionina in pozitia 148 a lantului polipeptidic, a fost descris recent drept un determinant esential al continutului hepatic de lipide. Varianta I148M a fost asociata cu dezvoltarea intregului spectru de afectare hepatica asociata acumularii de lipide, de la steatoza simpla la steatohepatita. Studii ulterioare au evidentiat o asociere stransa dintre varianta PNPLA3 I148M si progresia fibrozei hepatice catre stadii avansate, dezvoltarea cirozei, evolutia clinica in hepatita cronica de tip C si carcinogeneza hepatica. Polimorfismul PNPLA3 rs738409 reprezinta un factor genetic predictiv important si o potentiala tinta terapeutica in patologia hepatica. In acest review, vom trece in revista principalele ipoteze actuale asupra functiilor celulare ale PNPLA3 si rolul variantei I148M ca factor de risc in progresia bolilor hepatice cronice.

**Cuvinte-cheie:** PNPLA3, afectiune hepatica, fibroza, steatoza, cancer hepatic, factor de risc genetic

# INCIDENCE AND ANTIBIOTIC SUSCEPTIBILITY OF MICROORGANISMS ISOLATED FROM FEMALE GENITAL TRACT FROM OUTPATIENTS

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**ABSTRACT**

The aim of the study was to determine the etiology of genital infections and antibiotic sensitivity of strains of *Mycoplasma hominis (M. hominis)* and *Ureaplasma urealyticum (U. urealyticum)* isolated from outpatients. **Matherials and Methods**: The study included 130 women with clinical diagnosis of genital infection, aged between 18 and 67 years. Data were collected from the medical records of patients who presented to laboratory Intermed Service Lab Cluj-Napoca during the period February- July 2015. We used A.F. GENITAL SYSTEM, a 24-well system containing dehydrated biochemical and antibiotic substrates for presumptive identification and susceptibility testing of bacteria from genital secretions. **Results:** The distribution percentage of the infection of the lower genital tract was: 61% vaginosis (79 patients), 9.48% candidosis (12 patients), candidosis + vaginosis 18.9% (25 patients). The distribution of Gram-positive bacteria isolated was: *E. faecalis*- 83%, *S. agalactiae*- 15% and *S. aureus* -2%. *E. coli* was the most frequently isolated Gram-negative bacteria, followed by *G. vaginalis* - 43.28%, *N. gonorrhoeae* - 4.47%, *Pseudomonas aeruginosa* 2.98% and *Proteus spp.* 1.5%. We diagnosed 37 patients (28.45%) with candidosis. *M. hominis* and *U. urealyticum* were isolated from 36,92% of patients. **Conclusions:** Age group 21-30 presented most genital infections. From the total number of genital infections, bacterial vaginosis had the highest prevalence. *E. faecalis* was the most frequently isolated Gram-positive bacteria and *E. coli* the most commonly isolated Gram-negative bacteria. *U. urealyticum* was the most commonly isolated bacteria from the infections with *Mycoplasma spp* and *U. urealyticum*. All *M. hominis* strains were sensitive to Tetracycline, Pefloxacin, Ofloxacin, Doxycycline, Minocycline and Clindamycin.

**Keywords:** female genital tract, outpatients, *Mycoplasma hominis, Ureaplasma urealyticum*

## INTRODUCTION

In recent years the incidence of local or systemic complications of asymptomatic female genital tract infections increased significantly [3,4,8,9]. The possibility of simultaneously contamination with two or more sexually transmitted microorganisms and the increased percent of chronicization requires an early diagnosis and a specific treatement [11].

The increased frequency of genital infections with obstetric and gynecologic complications and antibiotic resistance of isolated bacteria was the motivation of this study.

The aim of the study was to determine the etiology of genital infections and antibiotic sensitivity of strains of *Mycoplasma hominis (M. hominis)* and *Ureaplasma urealyticum (U. urealyticum)* isolated from outpatients.

The study had the following objectives: identification of bacterial agents, antibiotic susceptibility testing of *M. hominis* strains and *U. urealyticum* and the results interpretation by comparing them with data from the literature.

## MATERIALS AND METHODS

To achieve the objectives an observational, retrospective study on a representative sample was carried.

The study included 130 women with clinical diagnosis of genital infection, aged between 18 and 67 years. Data were collected from the medical records of patients who presented to laboratory Intermed Service Lab Cluj-Napoca during the period February- July 2015.

Received 15th of September 2015. Accepted 20th of October 2015. Address for correspondence: Laura M Simon, Microbiology Department, UMF ''Iuliu Haţieganu" Cluj-Napoca, 6th Pasteur Street, RO-400349, Cluj-Napoca, Romania; phone/fax: +40264597257, e-mail: lauramihaelasimon@yahoo.com

The method used was **A.F. GENITAL SYSTEM**, a 24-well system containing dehydrated biochemical and antibiotic substrates for presumptive identification and susceptibility testing of bacteria from genital secretions.

**Collection of pathological products.** The pathological products were vaginal or cervix secretions collected with a steril synthetic fibre swab. We used Stuart and Amies mediums incorporated into sterile tubes attached to sterile fiber swabs. The samples were sent for inoculation in the **A.F. GENITAL SYSTEM** immediately after they were taken.

**A.F. GENITAL SYSTEM** allows the detection, semi-quantitative count, presumptive identification and susceptibility test of *M. hominis* and *U. urealyticum*, the detection and presumptive identification of the microorganisms most frequently isolated from vaginal and urethral swabs and seminal fluid, such as: *Escherichia coli*, *Proteus spp./Providencia spp*., *Pseudomonas spp*., *Gardnerella vaginalis, Staphylococcus aureus (S. aureus), Enterococcus faecalis, Neisseria gonorrhoeae, Streptococcus agalactiae* (Group B) (*S. agalactiae)* and *Candida spp.*The tests were interpreted after color changing in different wells and microscopic examination.

The presence of *Candida* spp. was determined by microscope (40x) examination of a drop of culture liquid assessing the presence of chlamydospores and hyphae. The presence of *S. aureus was* confirmed by the coagulase test. The presence of *S. agalactiae* was confirmed with agglutination test STREPTO B latex KIT**.**

Every batch of **A.F. GENITAL SYSTEM** was subjected to quality control using the following microorganisms as referrence: *M. hominis* ATCC 23114, *U. urealyticum* ATCC 27618, *Gardnerella vaginalis* ATCC 14018, *S. aureus* ATCC 25923, *Streptococcus faecalis* ATCC 19433, *C. albicans* ATCC 10231, *Escherichia coli* ATCC 5922, *Proteus mirabilis* ATCC 25933, *Neisseria gonorrhoeae* ATCC 19424, *S. agalactiae* ATCC 13813, *Ps. aeruginosa* ATCC 27853.


**Fig. 1.** *M. hominis* negative, *U. urealyticum* negative, *Trichomonas vaginalis* negative, *Escherichia coli* negative, *Proteus spp.* negative, *Pseudomonas spp.* negative, *Gardnerella vaginalis* negative, *S. aureus* negative, *Enterococcus faecalis* negative, *Neisseria gonorrhoeae* negative, *S.agalactiae* negative and *Candida spp* ***pozitive.***


**Fig. 2**. *M. hominis* negative, *U. urealyticum* negative, *Trichomonas vaginalis* negative, *Escherichia coli* negative, *Proteus spp.* negative, *Pseudomonas spp.* negative, *Gardnerella vaginalis* ***pozitive***, *S. aureus* negative, *Enterococcus faecalis* negative, *Neisseria gonorrhoeae* negative, *S. agalactiae* and *Candida spp.* negative.

**Statistical analysis**

The data collected were placed in an Excel database which was then processed to obtain statistical results. The database was created as spreadsheets using Microsoft Office 2013, depending on variables pursued. Individual tables were created for each variable that was monitored. Based on the tables the current graphics were generated.

The following statistical softwares: Epi Info™, G*Power Win_3.1.9.2 and R for Windows 3.2.0 were used. The sample size and statistical parameters: Se, Sp, PPV, NPV, P were computed using the mentioned tools.

The size of representative sample was determined by the prevalence of genital tract infections in the general population.

The null hypothesis of the study: no association between genital tract infections and isolated bacteria.

Alternative hypothesis of the study: there is association between genital tract infections and isolated bacteria.

Factors that may invalidate the results were poor inoculum standardization, inadequate materials, **A.F. GENITAL SYSTEM** expired, improper temperatures and incubation times.

Data obtained by using statistical tools had the following percentage distributions: by patients group age, for isolated Gram-positive bacteria, for isolated Gram-negative bacteria, for *C. albicans* strains, for *M. hominis* and *U. urealyticum* strains, for *M. hominis* sensitivity to antibiotics, for *U. urealyticum* sensitivity to antibiotics, the most frequent bacteria associated with *U. urealyticum*, the most frequent bacteria associated with *M. hominis*, the most frequent bacteria associated with *E. faecalis.*

## RESULTS

Based on the data collected Table I was compiled:

**Table I.** Contingency Table

| Test result | Infection present | No infection | Total |
|---|---|---|---|
| Test + | 114 | 2 | 116 |
| Test - | 2 | 12 | 14 |
| Total | 116 | 14 | 130 |

### Diagnostic test performance

Hi-square test was applied and $p < 0.005$ was obtained, rejecting the null hypothesis and accepting the alternative hypothesis. The test is very highly significant since p-value < 0.001 (with 95% confidence interval).

The following statistical parameters were calculated:

1. Sensitivity (Se) = 0.983; is the proportion of patients correctly identified as positive subjects
2. The specificity (Sp) = 0.857; is the proportion of urged subjects correctly identified as negative
3. Prevalence (P) = 0.89; the number of cases of illness at a given time relative to total population.
4. Positive predictive value (PPV) = 0.983; It is the probability of a subject with a positive test to have the disease.
5. The negative predictive value (NPV) = 0.857; is the probability of a subject with a negative test to not have the disease.

Due to Se and Sp values the test is highly sensitive and highly specific. Values are close to 100% which implies a high diagnostic quality.

Age patients included in the study was the age group as shown in Figure 3.

From the 130 patients included in the study, 8% (11 patients) were aged between 18-20 years, 58% (75 patients) were aged between 21-30 years, 21% (27 patients) were aged between 31-40 years and 13% (17 patients) were aged over 40 years.

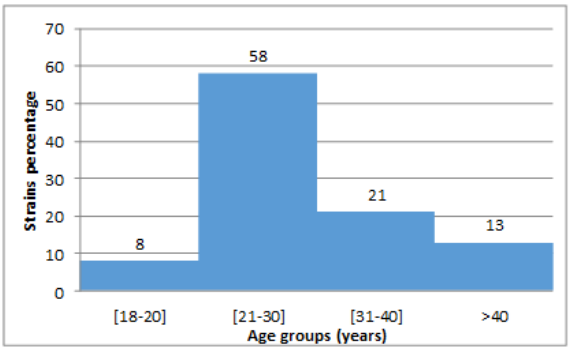


**Fig. 3.** Percentage distribution of patients by age

The distribution percentage of the infection of the lower genital tract as shown in Figure 4 was: 61% vaginosis (79 patients), 9.48% candidosis (12 patients), candidosis + vaginosis 18.9% (25 patients), and no-infection- 10.34% (14 patients).

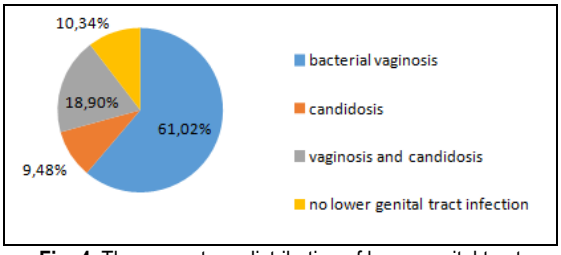


**Fig. 4.** The percentage distribution of lower genital tract infections

The distribution of Gram-positive bacteria isolated was: *E. faecalis*- 83%, *S. agalactiae*- 15% and *S. aureus* -2% (Figure 5).

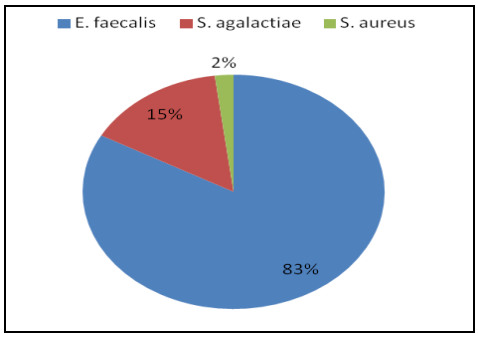


**Fig. 5.** The percentage distribution of isolated Gram-positive bacteria

*E. coli* (37 strains) was the most frequently isolated at 47.77% Gram-negative bacteria, *G. vaginalis* had a percentage of 43.28% (34 strains), *N. gonorrhoeae* had a share of 4.47% (8 strains), *Pseudomonas aeruginosa* -2.98% (7 strains) and *Proteus spp.* -1.5% (6 strains) (Figure 6).

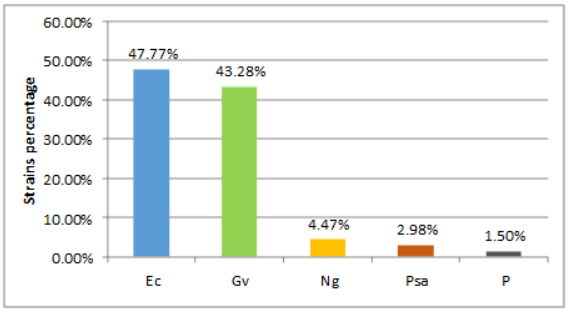


**Fig. 6.** The percentage distribution of Gram-negative bacteria (Legend: Ec=*E. coli*, Gv=*G. vaginalis*, Ng=*N. gonorrhoeae*, Psa=*P. aeruginosa*, P=*Proteus*)

We diagnosed 37 patients (28.45%) with candidosis (Figure 7).

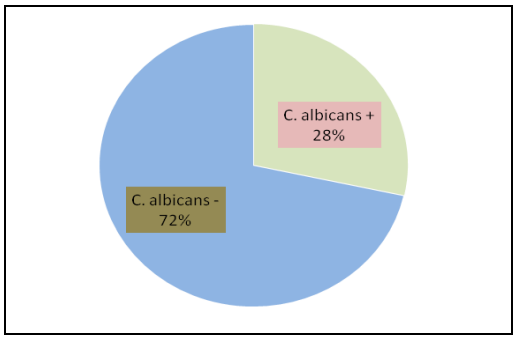


**Fig. 7.** Percentage distribution of strains of *C. albicans*

From 130 patients, *M. hominis* and *U. urealyticum* were isolated from 48 patients (36,92%), as shown in Figure 8. We isolated *U. urealyticum* from 42 patients (32.3%), *M. hominis* from 3 patients (2,3%). The combination of the two bacteria occurred at 3 patients (2,3%).

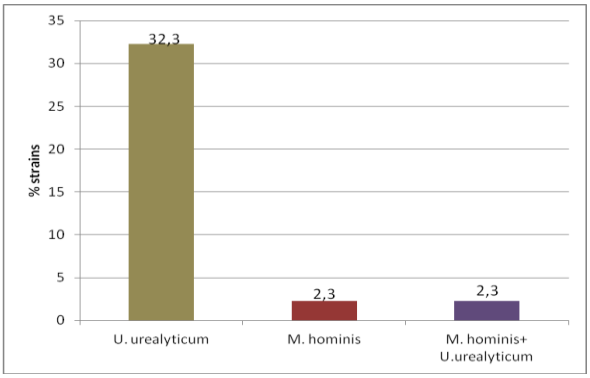


**Fig. 8.** Percentage distribution of *M. hominis* and *U. urealyticum* strains

All strains of *M. hominis* (3 strains) were sensitive to Tetracycline, Pefloxacin, Ofloxacin, Doxycycline, Minocycline, Clindamycin and 2 strains (66.66%) were susceptible to Erythromycin, Clarithromycin and Josamycin (Figure 9).

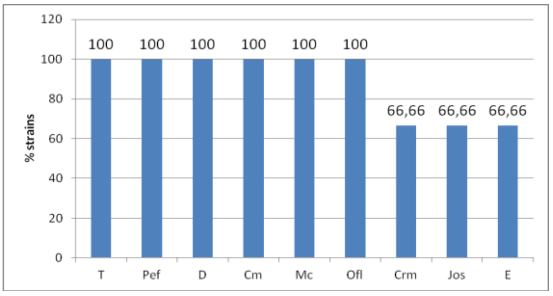


**Fig. 9.** Antibiotic susceptibility of *M. hominis* strains (Legend: T=Tetracycline, Pef=Pefloxacin, Ofl=Ofloxacin, D=Doxycycline, Mc=Minocycline, Cm=Clindamycin, E=Erythromycin, Crm=Clarithromycin, Jos= Josamycin)

A percentage of 73.68% (31 strains) of *U. Urealyticum* (42 strains) were sensitive to Tetracycline, Pefloxacin, Doxycycline, Clindamycin, 76.31% (32 strains) were susceptible to Ofloxacin, and Minocycline, 65.78% (28 strains) were sensitive to Clarithromycin and 55.26% (23 strains) were susceptible to Josamycin and Erythromycin (Figure 10).

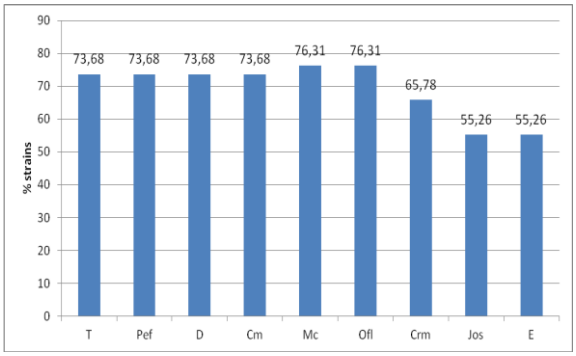


**Fig. 10.** Antibiotic susceptibility of *U. urealyticum* strains (Legend: T=Tetracycline, Pef=Pefloxacin, D=Doxycycline, Cm=Clindamycin, Mc=Minocycline, Ofl=Ofloxacin, Crm=Clarithromycin, Jos= Josamycin)

In the co-infection with *M. hominis* and *U. urealyticum*, 50% of strains were susceptible to Tetracycline, Pefloxacin, Doxycycline, Erythromycin, Minocycline, Josamycin, Clindamycin and all strains were susceptible to Ofloxacin and Clarithromycin (Figure 11).

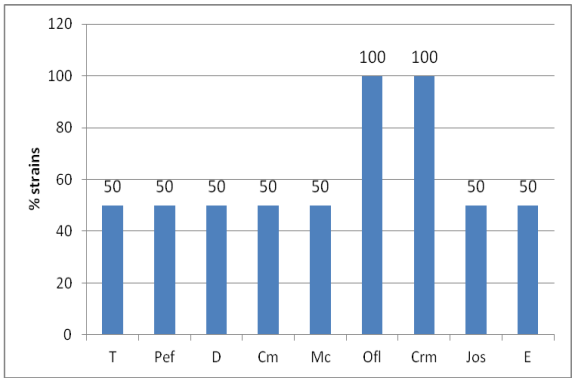


**Fig. 11.** Graphical representation of antibiotic susceptibility *of M. hominis* and *U. urealyticum* strains (Legend: T=Tetracycline, Pef=Pefloxacin, D=Doxycycline, E=Erythromycin, C=Clindamycin, Jos=Josamycin, Mc=Minocycline, Ofl=Ofloxacin, Crm=Clarithromycin)

In Figure 12 is shown the percentage association of *U. urealyticum* strains with other bacteria. 7 strains of *U. urealyticum* (15%) were associated with *G. vaginalis*, 24 strains (60.5%) have been associated with *E. faecalis*, 4 strains (6%) with *S. agalactiae,* and a percentage of 18.5% (9 strains) have been associated with *C. albicans*.

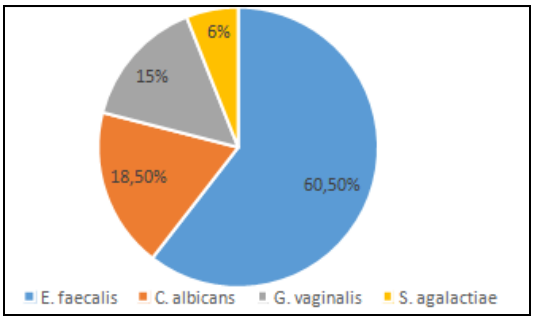


**Fig. 12.** Percentage distribution of *U. urealyticum* strains associated with other microorganisms

*M. hominis* strains have been associated in a proportion of 50% with *G. vaginalis*, 25% with *E. faecalis* and 25% were associated with *C. albicans* (Figure 13).

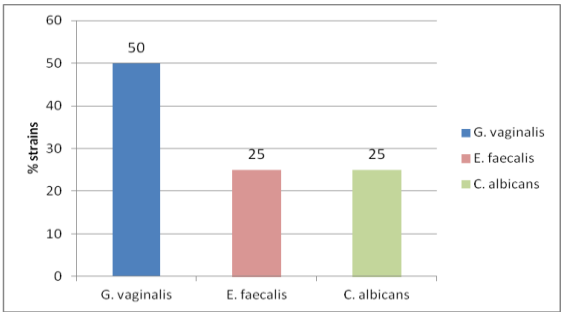


**Fig. 13.** Percentage distribution of *M. hominis* strains associated with other microorganisms

From 68 isolated strains of *E. faecalis,* 16 were associated with *G. vaginalis* (23.52%), 14 with *C. albicans* (20.58%), 23 strains with *E. coli* (33.82%), and 15 strains (22.05%) weren't associated with any bacteria (Figure 14).

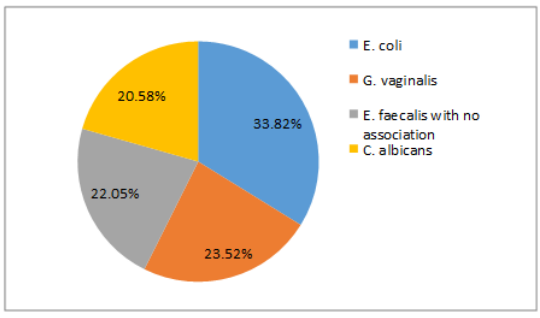


**Fig. 14.** Percentage distribution of *E. faecalis* strains associated with other microorganisms

## DISCUSSION

A study in Brazil on 100 sexually active women watching the prevalence and risk factors for bacterial vaginosis and other vulvovaginitis in sexually active women showed that the prevalence of bacterial vaginosis was 20%, the prevalence of candidosis was 22% and their coinfection was 2% [7].

In our study, out of 130 women aged between 18 and over 40 years, the prevalence of bacterial vaginosis was 61.02%, the prevalence of candidosis was 9.4% and the prevalence of bacterial vaginosis associated with candidosis was 18.90%.

A survey conducted in China highlighted the prevalence of genital tract infections with *M. hominis* and *U. urealyticum* for women in outpatient. From a total of 6051 women a percentage of 31% accounted for infections with *Ureaplasma* and 0.7% was the infection with *Mycoplasma* [13].

Atefeh M. et al conducted a study in 2013 in Iran to diagnose genital infections with *Mycoplasma* in infertile women. Out of 104 patients, 39 (37.5%) were diagnosed with *U. urealyticum*, 3 patients (2.9%) with *M. hominis* and the coinfection was 3.8% [1].

In a study conducted in Poland on a sample of 161 women (60 fertile and 101 infertile), the results revealed a number of 9 infertile women (9%) and 5 fertile females (8%) infected with *U. urealyticum*. The presence of *M. hominis* has been identified only in four infertile women in a proportion of 4%.

In our study on 130 women aged between 18 and over 40 years, the prevalence of *U. urealyticum* and *M. hominis* was 36,92%, also higher for *U. urealyticum.*

Another study that revealed *U. urealyticum* sensitivity, which was 94.6% for: Doxycycline, Tetracycline, Minocycline; 69.2% for: Azithromycin, Erythromycin, Clarithromycin, Roxithromycin. *M. hominis* was 100% sensitive to Doxycycline, Tetracycline, Minocycline and 95.6% to Azithromycin, Erythromycin, Clarithromycin, Roxithromycin [13].

In our study antibiotic sensitivity of *U. urealyticum* was: 73.68% to Tetracycline, Pefloxacin, Doxycycline, Clindamycin, 76.31% to Ofloxacin, and Minocycline, 65.78% to Clarithromycin and 55.26% to Josamycin and Erythromycin. All strains of *M. hominis* strains were sensitive to Tetracycline, Pefloxacin, Ofloxacin, Doxycycline, Minocycline, Clindamycin and 2 strains (66.66%) were susceptible to Erythromycin, Clarithromycin and Josamycin.

In previous studies in Hungary between 2008 - 2011 on a sample of 4466 patients aged 21 - 60 years showed the incidence and sensitivity of genital mycoplasmas. *U. urealyticum* incidence was 8.5% and 0.91% for *M. hominis*. *U. urealyticum* was sensitive to Tetracycline (95.9%), Doxycycline (97.32%) and Azithromycin (85.79%) and resistant to Erythromycin (81.23%), Clindamycin (75.06%), Ofloxacin (25.2%). *M. hominis* was sensitive to Clindamycin, Ofloxacin, Doxycycline (95%) and Tetracycline (82.9%) [10].

Studies available have shown the prevalence of sexually transmitted infections in 296 women aged

16-49. *N. gonorrhoeae* infection prevalence was 5.1% (15 women) [6].

Out of 130 cases examined, our results highlighted that *N. gonorrhoeae* was isolated in 4.47% of cases.

In Portland, out of 215 women surveyed, 147 (68%) showed no infection, 41 (19.1%) were infected with candidosis, and 27 (12.6%) were diagnosed with infectious vaginitis. 6 patients (22.8%) diagnosed with infectious vaginitis had *S. agalactiae* as etiologic agent [5].

Our results revealed the candidosis prevalence with 9.4% and *S. agalactiae* prevalence from isolated Gram-positive bacteria was 15%.

Earlier work identified that from 200 women with symptoms of lower genital infection, bacterial vaginosis prevalence was 51.5%, and *G. vaginalis* was isolated in 8.7% of cases of vaginosis [2].

Our results showed prevalence of bacterial vaginosis at 61.02% and 43.28% prevalence of G. vaginalis from isolated Gram-negative bacteria.

Another study showed the prevalence of microorganisms in the vaginal secretion of fertile and infertile women. 952 patients were included over a period of 2 years who have carried out tests on the vaginal discharge for: *G. vaginalis*, *T. vaginalis*, *Candida*, *S. agalactiae*, *M. hominis*, *U. urealyticum* and *C. trachomatis*. The most common organisms identified in fertile women was 12.1% *Candida*, 26.6% *G. vaginalis* and *S. agalactiae* at 9.2% [12].

The most common microorganisms isolated from our study were: 26.15% *G. vaginalis*, 28.45% *Candida* and *S. agalactiae* at 12.76%.

## CONCLUSIONS

- A.F GENITAL SYSTEM test has a high diagnostic quality and is highly sensitive and highly specific due to Se amd Sp being close to 100%;
- Age group 21-30 presented most genital infections;
- From the total number of genital infections, bacterial vaginosis had the highest prevalence;
- *Enterococcus faecalis* was the most frequently isolated Gram-positive bacteria and *Escherichia coli* the most commonly isolated Gram-negative bacteria;
- *U. urealyticum* was the most commonly isolated bacteria in the infections with *Mycoplasma spp* and *U. urealyticum;*
- All *M. hominis* strains were sensitive to Tetracycline, Pefloxacin, Ofloxacin, Doxycycline, Minocycline and Clindamycin;
- The incidence of infections wih *M. hominis* and *U. urealyticum* coincides with the incidence reported in the literature;
- The asymptomatic pathology determined by *Mycoplasmas* causes long-term health consequences, pose serious issues for individual and couple health;
- It is necessary to develop guidelines for diagnosis and treatment of non-specific genital infections guided by their incidence in our population, as well the current profile of sensitivity to antibiotics;
- This study, through the given limits, draws attention to some etiological, diagnostic, treatment and prevention aspects of non-specific genital infections in Romanian female population;
- Developing an effective prevention program in compliance with the rules of sexual health and screening actions are particularly important.

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## INCIDENTA SI SENSIBILITATEA LA ANTIBIOTICE A MICROORGANISMELOR IZOLATE DE LA NIVELUL TRACTULUI GENITAL FEMININ LA PACIENTE DIN AMBULATOR

**REZUMAT**
Scopul acestui studiu a fost de a determina etiologia infectiilor genitale si sensibilitate la antibiotice a tulpinilor de *Mycoplasma hominis (M. hominis)* si *Ureaplasma urealyticum (U. urealyticum)* izolate de la paciente din ambulator. Material si Metoda: Am luat in studiu 130 femei avind diagnostic clinic de infectii genitale, cu varsta cuprinsa intre 18 si 67 ani. Datele s-au colectat din buletinele de analiza ale pacientelor care s-au prezentat la Intermed Service Lab Cluj-Napoca în perioada februarie – iulie 2015. Am utilizat **A.F GENITAL SYSTEM**, care reprezintă un sistem de 24 de godeuri conținând substraturi biochimice deshidratate și antibiotice, cu scopul identificării prezumtive și testării sensibilității bacteriilor izolate din secrețiile genitale. Rezultate: Repartiția procentuală a infecțiilor de tract genital inferior a fost: 61% vaginoză (79 paciente), 9.48% candidoză (12 paciente), candidoză + vaginoză 18.9% (25 paciente). Repartiția procentuală a bacteriilor Gram-pozitive a fost: *E. faecalis* - 83%, *S. agalactiae* 15%, iar *S. aureus*- 2%. *E. coli* a fost izolată la 47.77% din paciente (37 tulpini), *G. vaginalis* la 43.28% (34 tulpini), *N. gonorrhoeae* la 4.47% (8 tulpini), *Pseudomonas aeruginosa* la 2.98% (7 tulpini) iar *Proteus spp.* la 1.5% (6 tulpini). 37 de paciente (28.45%) au fost diagnosticate cu candidoză. *M. hominis* și *U. urealyticum* au fost izolate la 36,92% din paciente. Concluzii: Grupa de vârstă 21-30 ani a prezentat cele mai multe infecții genitale. Din totalul infecțiilor genitale, vaginoza bacteriană a avut prevalența cea mai mare. *E. faecalis* a fost cel mai frecvent izolata bacterie Gram-pozitiva iar *E. coli* a fost cel mai frecvent izolata bacterie Gram-negativa. Dintre tulpinile de *M. hominis* și *U. urealyticum,* cea mai frecvent izolata a fost *U. urealyticum.* Toate tulpinile de *M. hominis* au fost sensibile la Tetraciclină, Pefloxacină, Ofloxacină, Doxiciclină, Minociclină, Clindamicină.
**Cuvinte cheie:** tractului genital feminin, ambulator, *Mycoplasma hominis, Ureaplasma urealyticum*

# ETIOLOGY, FREQUENCY AND ANTIMICROBIAL RESISTANCE OF *ENTEROBACTERIACEAE* INFECTIONS IN AN INTENSIVE CARE UNIT

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**ABSTRACT**

Infections produced by Gram-negative bacilli continue to be of key importance in the ICU, as they are considered important determinants of hospital mortality, prolonged hospitalization and increased health-care costs as compared to infections caused by nonresistant bacteria.

We investigated antimicrobial resistance and bacterial distribution of *Enterobacteriaceae* isolated from the ICU of the Timisoara Emergency Clinical County Hospital (TECCH), Romania. The purpose of this study is to provide up-to-date information on the frequency, microbial etiology, and susceptibilities of *Enterobacteriaceae* infections, with the goal of improving their management.

The study group was represented by patients who received antibiotic treatment in the ICU and from whom enterobacteria were isolated in the collected specimens. From 1 January 2012 to 31 December 2013 a total of 1,605 patients were included in our study group. Our study revealed that 43.08% of the infections in the ICU, are produced by *Enterobacteriaceae*, and the 3 most frequently isolated agents of this family are *Klebsiella pneumoniae, Proteus mirabilis* and *Escherichia coli*.

We recorded low percents of beta-lactam sensitive enterobacteria. *K. pneumoniae* and *P. mirabilis* strains were more frequently classified in the ESBL phenotype, while in *E. coli* strains HPAZA was the most frequently identified phenotype. *E. coli* strains were more sensitive to aminoglycosides as compared to *K. pneumoniae* and *P. mirabilis.* Although differences regarding the antimicrobial resistance patterns of *K. pneumoniae, P. mirabilis* and *E.coli* infections have been identified, high resistance to most commonly used antibiotics has been found.

## INTRODUCTION

Thanks to the progress in medical care and the improvement in patient treatment over the last decade, effective treatment is now possible for many Intensive Care Unit (ICU) patients who previously could have been lost [1,2]. Antibiotics have contributed greatly to improvements in health, but, in addition to this, their irrational use led to the emergence of antimicrobial resistant microorganisms [3-5]

Antimicrobial resistant microorganisms mainly develop in ICU's, and this seems due to massive antibiotic use [1,3]. It is alarming that a limited number of antimicrobials retain some potency against these increasingly resistant pathogens and novel therapies currently in the developmental pipeline appear to be years away from the wide scale clinical use [6-8].

Infections produced by Gram-negative bacilli (i.e. *Escherichia coli, Klebsiella pneumoniae* etc.) continue to be of key importance in the ICU, as they are considered important determinants of hospital mortality, prolonged hospitalization and increased health-care costs as compared to infections caused by nonresistant bacteria [6-9].

We investigated antimicrobial resistance and bacterial distribution of *Enterobacteriaceae* isolated from the ICU of the Timisoara Emergency Clinical County Hospital (TECCH), Romania.

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The purpose of this study is to provide up-to-date information on the frequency, microbial etiology, and susceptibilities of *Enterobacteriaceae* infections, with the goal of improving their management.

## MATERIALS AND METHODS

Data were collected in the ICU of the Timisoara Emergency Clinical County Hospital (TECCH), Romania. The study data were recorded for 24 months, between 01.01.2012 and 31.12.2013, for all ICU admitted patients who received antibiotic treatment.

For each patient we recorded: age, gender, admission diagnosis, underlying surgical pathology, presence of invasive procedures, infection sites, isolated germs, susceptibility pattern, type, dose and length of antibiotic therapy, days of ICU stay and ICU status after discharge. Microbiology results were obtained from the Microbiology Laboratory of the hospital.

Cultures were performed based on the clinical status of the patient. All patients with clinical signs of infection received empirical antimicrobial therapy. After culture results were available, antibiotic treatments were either maintained or adapted according to the results of standard sensitivity testing.

All isolated microbial strains were identified using the Vitek 2 Compact automated system (bioMérieux).

The anitimcrobial sensitivity of germs isolated in collected specimens was determined by measuring the MIC (minimal inhibitory concentration), with further classification into resistance phenotypes by use of the Vitek 2 Compact system, based on the CLSI standard (Clinical Laboratory and Standards Institute Inc.).

Copy strains - defined as an isolate with the same susceptibility pattern throughout a 1-month period in the same patient – were excluded.

Institutional review board approval was obtained before study initiation.

### Statistical analysis methodology

The statistical analysis of the database was performed using the SPSS version 10.0. Continuous numerical variables characterized by mean value and standard deviation were tested regarding the type of data distribution using the Kolmogorov-Smirnov test. Numerical variables with normal distribution were compared using the unpaired t test, those with non-Gaussian distribution were compared using the nonparametric Mann-Whitney test, and dichotomic variables characterized by percent values were compared using the chi-squared test with Fisher correction. All statistical tests were calculated with two ends, and the p value for statistical significance was set at values ≤ 0.05.

## RESULTS

The study group is represented by patients who received antibiotic treatment in the ICU and from whom enterobacteria were isolated in the collected specimens. From 1 January 2012 to 31 December 2013 a total of 1,605 patients were included in our study group.

The mean patient age was 60.23, 42.39% of patients were female, and 57.61% were male. The mean length of ICU stay was 17.32 days and the mean length of antibiotic therapy was 12.21 days.

The strains were cultured from bronchial aspirate (238 isolates, 41.90%), urine (111 isolates, 19.54%), blood (67 isolates, 11.79%), wound secretion (55 isolates, 9.68%), catheters (40 isolates, 7.04%), peritoneal fluid (19 isolates, 3.34%), pus (17 isolates, 2.99%), CSF (6 isolates, 1.05%), pleural fluid (5 isolates, 0.88%), nasal exudate (3 isolates, 0.53%), conjunctival secretion (2 isolates, 0.35%), sputum (2 isolates, 0.35%), faeces (1 isolate, 0.17%), urethral secretion (1 isolate, 0.17%) and vaginal secretion (1 isolate, 0.17%). Enterobacteria were isolated in 568 biological specimens.

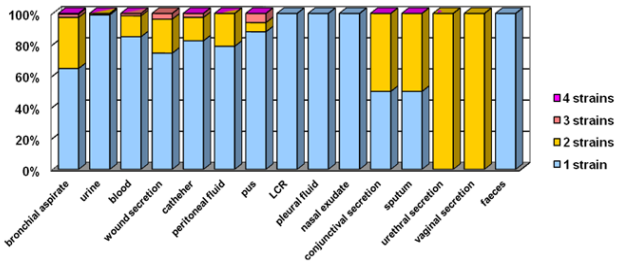


**Fig. 1.** Distribution of bacterial load depending on the type of biological specimen

As shown in Figure 1, the majority of infections were monomicrobial but in certain samples collected from urethral and vaginal infection sites (with a very low prevalence in the present study) two microorganisms were isolated in the same specimen.

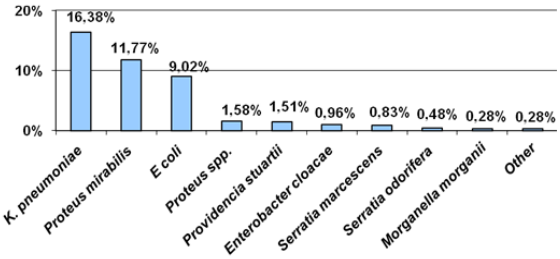


**Fig. 2**. Incidence of circulating *Enterobacteriaceae* species in the ICU during the period between 01.01.2012-31.12.2013

During the studied period, our study revealed that 43.08% of the infections in the intensive care unit, are produced by *Enterobacteriaceae*, and the 3 most frequently isolated agents of this family are *Klebsiella pneumoniae, Proteus mirabilis* and *Escherichia coli* (Figure 2).

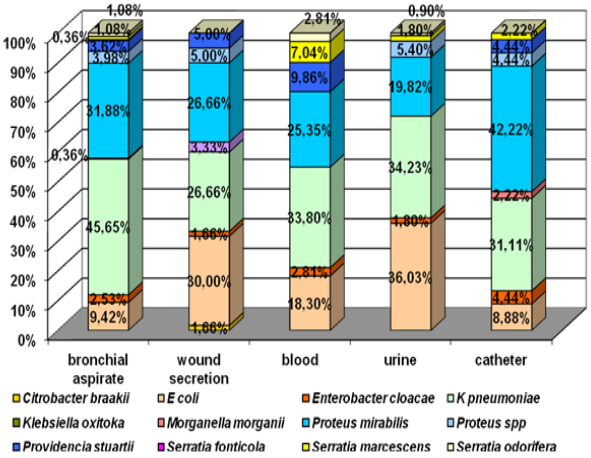


**Fig. 3.** Distribution of *Enterobacteriaceae* species in biological specimens

*Enterobacteriaceae* species isolated in our study are variably distributed in biological specimens as shown in Figure 3. Thus, the most frequently involved in lower respiratory tract infections are: *K. pneumoniae, P. mirabilis, E. coli, Proteus spp, P.* stuartii; in urinary tract infections the most frequently involved are: *E. coli, K. pneumoniae, P. mirabilis, Proteus spp., Enterobacter colacae, Serratia* marcescens; in blood stream infections *K. pneumoniae, P. mirabilis, E. coli, P. stuartii* were isolated in higher percents.

Infections were defined according to the International Sepsis Forum Consensus Conference on Definitions of Infections in the Intensive Care Unit (10). The organism associated with infection was defined by the isolation of the germ in a biological material in the presence of signs and symptoms of infection.

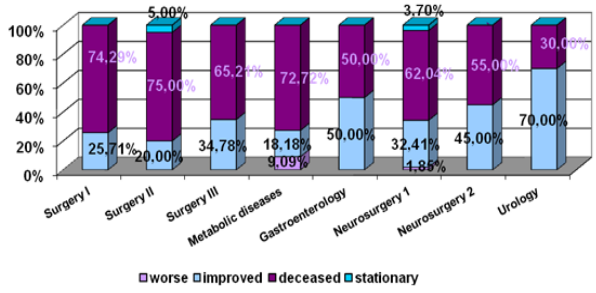


**Fig. 4.** Fatality rates in infectious with *Enterobacteriaceae* depending on originating hospital department

Moreover, ICU mortality of the study group was very high (61.70%), and distribution in hospital departments is shown in the above figure, as is the evolution of cases upon ICU discharge, also depending on the originating hospital department.

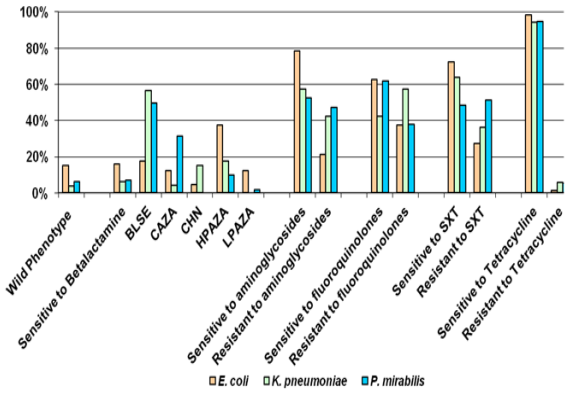


**Fig. 5.** Distribution of the most prevalent strains according to the resistance phenotype
Legend: ESBL - extended spectrum beta-lactamase, CAZA - cephalosporinase, CHN - high level cephalosporinase, LPAZA - low level penicilinase, HPAZA - high level penicilinase, SXT – sulfamethoxazole and trimethoprim

We recorded low percentage of beta-lactam sensitive enterobacteria. *K. pneumoniae* and *P. mirabilis* strains were more frequently classified in the ESBL phenotype, while in *E. coli* strains HPAZA was the most frequently identified phenotype. *E. coli* strains were more sensitive to aminoglycosides as compared to *K. pneumoniae* and *P. mirabilis.* Fluoroquinolone sensitivity was similar for *E. coli* and *P. mirabilis*, a lower percent being recorded in *K. pneumoniae* strains. Regarding trimethoprim-sulfamethoxazole, sensitive strains of *E.coli* and *K.pneumoniae* exceeded the number of resistant strains. Tetracycline sensitivity was maintained in most tested enterobacteria.

## DISCUSSION

Searching for the means to understand, control, and prevent the emergence and spread of infections and antimicrobial resistance has become a public health priority. The prevention, control, and treatment of ICU infections demand thorough knowledge of the infection incidence and infection site rates, the occurrence rates of organisms and their antimicrobial resistance profiles [11].

Since each hospital unit has a distinct bacteriological profile and antibiotic resistance pattern, knowledge of these differences is critical for planning effective therapy and reducing infection-related costs, morbidity, and mortality [11,12].

A large European single-day point prevalence study of nosocomial ICU infections, reported *Enterobacteriaceae* as most common (34.4%), with *Pseudomonas* second most common (28.7%) [12].

Resistance in Gram negative bacilli, including *Escherichia coli*, *Klebsiella* spp, is of great concern, because a number of reports have documented mechanisms whereby these microorganisms have become resistant to all available antibacterial agents used in therapy [13,14].

*Enteriobacteriaceae* are the most common Gram-negative isolates in laboratories. They can be isolated from numerous sites, the majority from: urine, blood, peritoneal cavity, respiratory tract but also from other sites like cerebrospinal fluid, synovial fluid, abscesses [15,16].

This ICU is also a surgical ICU, and in this type of department the use of antibiotics not only for therapy but also for prophylaxis contributes to an increase in total antibiotic consumption, but also to the selective pressure by antibiotics, and to the pharmaceutical budget [15,17].

The objective of the present prospective observational study was the identification and analysis of the demographic, clinical, and microbiological characteristics of the patients who receive antimicrobial treatment and from whom microorganisms of the *Enterobacteriaceae* family have been isolated during their hospitalization in ICU of our hospital. In particular, we evaluated the occurrence rates of the pathogens and their susceptibility to antibiotics.

The top three specimen types collected in the present study, similarly to several studies in the literature, [11,12] were sputum, urine and blood. It is emphasized that Gram negative bacilli are mainly responsible for pneumonia, urinary tract infection and septicaemia in the studied ICU.

The microbiological profile of infections included *K. pneumoniae*, *P. mirabilis, E. coli, Proteus* spp, *Providencia stuartii, Enterobacter cloacae* and *Serratia marcescens* as the most frequently isolated species; similar results have also been found in other studies [11,18]. *K. pneumoniae* was the most common, while *P. mirabilis* and *E. coli* were second and third most frequently identified pathogens in our study population, respectively.

Although differences regarding the antimicrobial resistance patterns of *K. pneumoniae, P. mirabilis* and *E.coli* infections have been identified, high resistance to most commonly used antibiotics has been found in several studies [11,16].

*E. coli* and *Klebsiella* can produce extended-spectrum beta-lactamases (ESBLs). ESBLs are widely prevalent in Latin America, with rates as high as 32.6% for *K. pneumoniae* and 11.8% for *E. coli* in the ICU [11,19]. The present study showed a prevalence of ESBLs-production ranging from 56.72%-49.70% in *Klebsiella pneumoniae* and *Proteus mirabilis*. The high prevalence of ESBLs-producing isolates described in this study was probably due to the large amount of third-generation cephalosporins consumed by ICU patients.

Significant differences with regard to infection rates, the rates of occurrence of organisms, infection sites incidence, and antimicrobial resistance profiles have been identified between different countries, between centres in the same country, and even between the departments of a hospital [11]. There is also a big difference in antimicrobial resistance rates between different European countries, as shown by the European Antimicrobial Surveillance System (EARRS) [5].

In conclusion, infections are a very important problem in our ICU, associated with high incidence, mortality, and multi-drug resistance of the responsible microorganisms. Eradication requires implementation of rigorous infection control measures, prudent antibiotic use, and effective antimicrobial therapy. Recognition of clinical and microbiological characteristics of these patients is essential for prevention and treatment purposes.

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## ETIOLOGIA, FRECVENTA SI REZISTENTA LA ANTIBIOTICE A ENTEROBACTERIILOR DIN INFECTII ALE PACIENTILOR INTR-O SECTIE DE TERAPIE INTENSIVA

**REZUMAT**

Infecţiile produse de bacilii Gram-negativi sunt frecvente în secţiile de terapie intensivă (TI), fiind considerate importanti factori de risc în cresterea mortalitatii, spitalizarea prelungită, dar şi în creşterea costurilor, în comparaţie cu infecţiile produse de bacterii non-rezistente.

In cadrul acestui studiu am investigat rezistenţa microbiană şi distribuţia bacteriilor aparţinând familiei *Enterobacteriaceae,* în secţia de TI a Spitalului Judeţean din Timişoara (SCJUT), România. Scopul acestui studiu a fost determinarea frecvenţei, etiologiei microbiene şi a sensibilităţii la antibiotice a enterobacteriilor identificate, in vederea îmbunătăţirii managementului acestor infecţii.

Grupul de studiu a fost reprezentat de pacienţii care au primit tratament antibiotic pe durata spitalizării în secţia de TI şi cărora le-au fost izolate enterobacterii. Au fost incluşi în studiu 1,605 pacineţi internaţi pe perioada 1 Ianuarie 2012 - 31 Decembrie 2013.

S-a observant că 43,08% dintre infecţii au fost produse de bacterii aparţinând familiei *Enterobacteriaceae*, iar cele mai frecvent izolate bacterii aparţinând acestei familii au fost: *Klebsiella pneumoniae, Proteus mirabilis* şi *Escherichia coli.*

S-au identificat procente scăzute ale sensibilităţii enterobacteriilor la beta-lactamine. Tulpinile de *K. pneumoniae* şi *P. mirabilis* au apartinut cel mai frecvent fenotipului BLSE, iar cele de *E. coli* fenotipului HPAZA. Tulpinile de *E. coli* au fost mai sensibile la aminoglicozide decât cele de *K. pneumoniae* şi *P. mirabilis.* Deşi există diferenţe între fenotipurile de rezistenţa ale celor mai izolate trei tipuri de enterobacterii (*K. pneumoniae, P. mirabilis* şi *E.coli* ) in general se observă un grad mare de rezistenţă la antibioticele cel mai frecvent utilizate.

# BINGE DRINKING REALITY IN RELATION TO ASPECTS OF SEXUAL BEHAVIOUR AMONG STUDENTS

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**ABSTRACT**
The study aims was to investigate some aspects of sexual behavior associated with binge drinking among students from Timis County, Romania. We applied a transversal population study to the representative sample of 2076 students, 62.49% girls and 37.51% boys with a mean age of 21.09 years with SD 1.48. The boys practice binge drinking significantly more often than girls, the difference is small to medium: prevalence of 22.1% for boys and 8.7% girls, 1-2 times in the last month. Girls forced into sexual relation practiced binge drinking significantly more often compared to those who were not forced. The frequency of practicing binge drinking is significantly higher in students who have sex with partners of one night. The frequency of practicing binge drinking is significantly higher in students who consumed alcohol intercourse average size of the gap is small to medium in boys and girls.
**Keywords:** students, binge drinking, sexual behavior

## INTRODUCTION

Alcohol is one of the most commonly used substances [1]. The quantities of alcohol consumed and the health problems due to the alcohol abuse are specific to each age group. Characteristic for the young adults is the increased alcohol consumption in one occasion - binge drinking, intoxication, drunk driving and assault [2]. Compared with adults, young people consume less alcohol, but per occasion, quantities consumed by young adults exceed those consumed by adults [3].

Alcohol plays an important role in sexual risk behavior, including unwanted, unintended and unprotected sexual activity, also sex with multiple partners. All of these behaviors increase the chance of unwanted pregnancies and contacting sexually transmitted diseases including HIV infection [4]. In pregnant women, alcohol consumption leads to the termination of the evolving pregnancy, miscarriage, physical and / or mental fetal malformations [2].

In adolescents and young people, alcohol consumption increases the risk of inadequate physical and sexual assault [5], and lifetime alcohol abuse.

We proposed a study on manifestations of sexual behavior (sexual assault, sex with one night partners, alcohol associated intercourse) associated with binge drinking among students.

## MATERIAL AND METHOD

The representative sample of students in the study totaled 2076 students from higher education institutions from Timiș county, 62.49% (1296) girls and 37.51% (778) boys. The average age was 21.09 years with SD 1.48, ages ranging from 18-25 years old.

The method used was the transversal population study based on the use of CORT Questionnaire 2004 on health risk behaviors in adolescents and young people. The questionnaire was validated by the Ethics Committee of the University of Medicine and Pharmacy "Victor Babeș" Timişoara.

The study was conducted with the written approval of the higher education establishments from Timiş.

Inclusion of young people in the study carried out only after their expressed consent of each participant in the study, with respect for all the individual rights.

The processing and interpretation of data was done by using modern statistical methods and advanced medical PASW 18 software (SPSS 18) 2010. The value of statistical significance was set at $p < 0.05$, except in cases where the Bonferroni correction was applied, the acceptable threshold level was stated in the text. For ordinal data comparisons we used Mann-Whitney and Kruskal test-Wallis. Chi-square test was used for ordinal data / nominal.

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## RESULTS

### 1. Binge drinking

By practicing binge-drinking or increased consumption of more than 5 servings of alcohol on one occasion, we have: for boys group 11.6% (89) have practiced binge drinking more than 3 times in the last month, 22.1% (170) have practiced 1-2 times, and 66.3% (510) did not practice binge drinking in the last month; 2.5% for the group of girls (32) have practiced binge drinking more than 3 times in the last month, 8.7% (112) have practiced 1-2 times, and 88.8% (1145) have not practiced binge drinking last month. The frequency of practicing binge drinking is significantly higher in boys than girls, U = 381 799, z = -12.63, p <0.001, r = 0.27, the size difference is considered small to medium according to the Cohen's criteria (Figure 1).

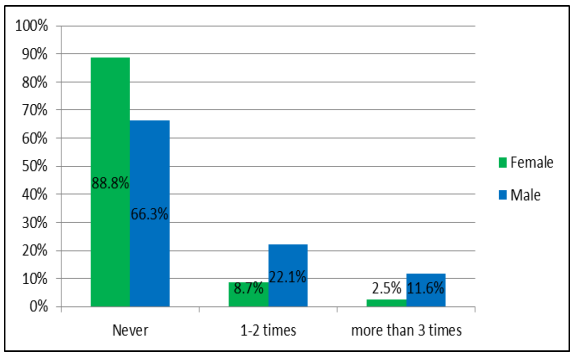


**Fig. 1.** Percentage distribution of students according to frequency of binge drinking practice last month, by gender

### 2. The situation when they had forced intercourse

A percentage of 3.0% (23) of the boys and 4.8% (62) of girls admit that they were obliged to have sexual intercourse against their will. The prevalence of forced sexual relations is higher among girls compared to boys, U = 484409.5, z = -2.01, p = 0.044, r = 0.044, the size difference is very small.

The prevalence of binge drinking in boys who were forced to have sex against their will was 40.9% (9) and those who did not report this incident was 33.6% (249). We could not find a relationship between the frequency of binge drinking and the claim that they had been forced to have sexual relations, p = 0.324 (Figure 2).

The prevalence of binge drinking among girls who were forced to have sex against their will was 24.2% (15) and those who did not report this incident was 10.6% (129). The girls who were forced to have sex against their will, practiced more often binge drinking, compared with those who were not forced, U = 32 718, z = -3.23, p = 0.001, r = 0.09, the difference is small in size (Figure 3).

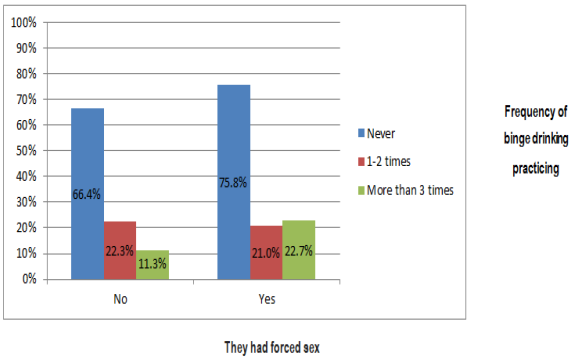


**Fig. 2.** Percentage distribution of students according to whether a situation when they had forced sex and frequency of binge drinking practicing during the past month among boys

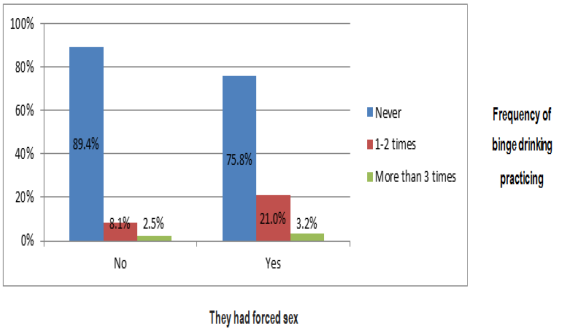


**Fig. 3.** Percentage distribution of students according to whether a situation when they had forced sex and frequency of binge drinking practicing during the past month among girls

### 3. Sex with one night partner

35.1% (264) of boys and 7.9% (101) of girls practiced sex with one night partners. Compared to girls, boys practiced 6.3 times more common, $\chi 2$ (1) = 237.03, p <0.001.

In the group of boys, 49.0% (128) of those who have sex with a one night partner, practiced binge drinking and 26.3% (128) of those who did not practiced sex with one night partners practiced binge drinking. The frequency of practicing binge drinking is significantly higher in boys who have sex with one night partners, U = 48 961, z = -5.22, p <0.001, r = 0.15, the size difference is small (Figure 4).

In the group of girls, 26.7% (27) of those who have sex with a one night partner, practiced binge drinking, and 9.8% (115) of those who did not practiced sex with one night partners practiced binge drinking . The frequency of practicing binge drinking is significantly higher in girls who have sex with one night partners, U = 48 511, z = -6.32, p <0.001, r = 0.23, the size difference is small (Figure 5).

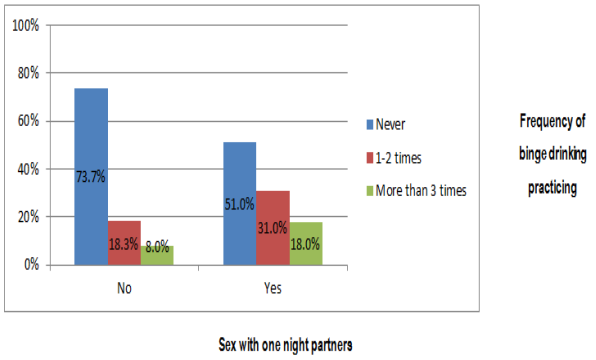


**Fig. 4.** Percentage distribution of students according practicing sex with one night partners and frequency of binge drinking practicing during the past month among boys

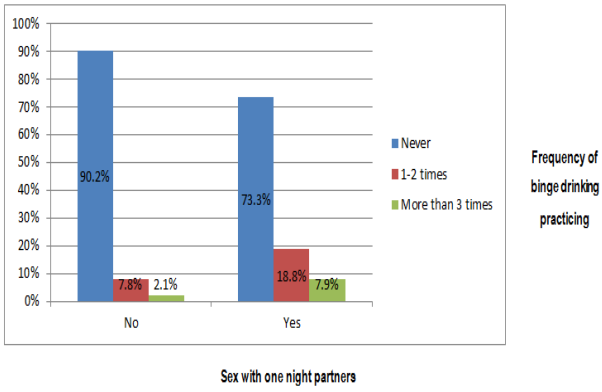


**Fig. 5.** Percentage distribution of students according practicing sex with one night partners and frequency of binge drinking practicing during the past month among girls

### 4. The use of alcohol associated with sex

A percentage of 29.1% (219) of boys and 15.7% (200) of the girls use alcohol during sex, boys 2.21 times more often than girls, $\chi 2$ (1) = 52.20, p <0.001.

In the group of boys, 62.6% (136) of those who practice sex associated with alcohol consumption practice binge drinking, and 22.6% (120) of those who do not practice associating alcohol and sex, practice binge drinking. The frequency of practicing binge drinking is significantly higher in boys who consumed alcohol during intercourse, U = 33,293, z = -10.79, p <0.001, r = 0.39, the size difference is medium (Figure 6).

In the group of girls, 28.5% (57) of those who practice sex associated with alcohol consumption practice binge drinking, and 7.9% (85) of those who do not practice associating alcohol and sex, practice binge drinking. The frequency of practicing binge drinking is significantly higher in girls who consumed alcohol during intercourse, U = 84923.5, z = -8.51, p <0.001, r = 0.23, the difference is small to medium size (Figure 7).

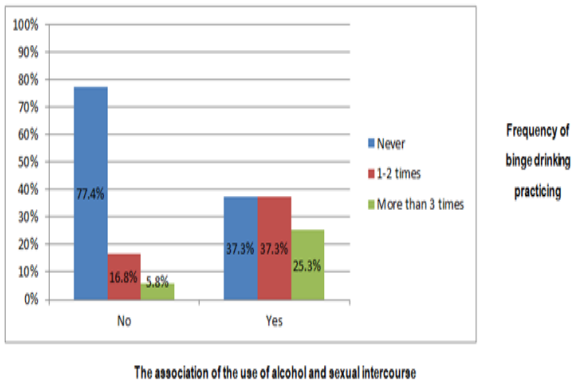


**Fig. 6.** Percentage distribution of students according to the association of the use of alcohol and sexual intercourse and frequency of binge drinking practicing during the last month among boys

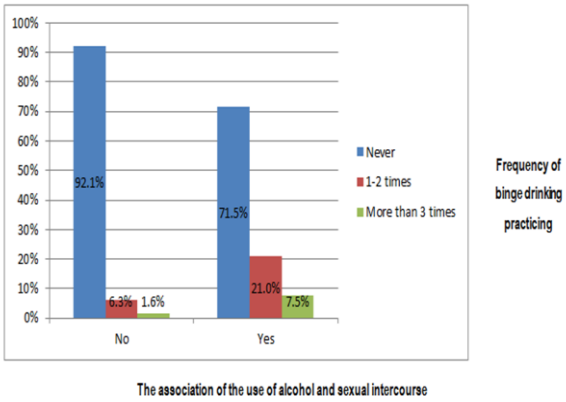


**Fig. 7.** Percentage distribution of students according to the association of the use of alcohol and sexual intercourse and frequency of binge drinking practicing during the last month among girls

## DISCUSSION

In a study by Boyd and colleagues [6], it was shown that the students witch are practicing binge drinking were associated with sexual risk behaviors and sexual assault.

Given the sensitive nature of the subject, coercion or sexual activity is hard to investigate. Studies have shown the different ways of evaluating the forced sexual activity. In some cases compulsion was defining in any sexual activity that was not consensual. In other cases it was heavily defined as rape or with men that trade money and/or gifts for sex [7-10].

Other authors [11] found the association between promiscuous unprotected sexual experiences and drinking, especially binge drinking practice and the early onset of alcohol consumption.

In multiple studies, young people reported early onset of sexual activity, multiple sexual partners, one night sexual partners, the use of psychoactive substances and sexual abuse during childhood. A relatively small percentage said that they used condom during the last sexual intercourse. Unprotected sex was related to no behavioral intentions to use condoms, pregnancy, a casual partner and people who practiced anal sex [12,13].

Alcohol consumption occurs in association with sexual behavior in a social and cultural variety. Where young people use alcohol before they engage in sexual relations occur behaviors risk such as unprotected sex [14,15]. Young people are less likely to adopt safe procedures for the sexual behavior when are drunk. The perception that alcohol has an uninhibited effect propels some people to consume alcohol and then to engage in behaviors that normally would not attend. Alcohol should be recognized as a risk factor in the transmission of HIV and other sexually transmitted infections. The synergy between sexual behavior and alcohol consumption multiplies enormously the negative potential consequences of the two separate behaviors [16,17].

## CONCLUSIONS

The prevalence of binge drinking practicing 1-2 times in the last month is 22.1% for boys and 8.7% girls, and more than 3 times in the last month is 11.6% for boys and 2.5% girls. The boys practice binge drinking significantly more often than girls, the difference is small to medium size.

A percentage of 3.0% of boys and 4.8% of girls admit that they were obliged to have sexual intercourse against their will. The prevalence of forced sexual relations is higher among girls compared to boys, the size difference is very small. Girls forced into sexual activity practiced binge drinking significantly more often compared to those who were not forced.

35.1% of boys and 7.9% of girls practiced sex with a one night partner, boys practicing 6.3 times more frequent than girls. In the group of boys, 49.0% of those who have sex with one night partners practice binge drinking. In the group of girls, 26.7% of those who have sex with one night partners practice binge drinking. The frequency of practicing binge drinking is significantly higher in students who have sex with one night partners, the difference size is small.

A percentage of 29.1% of boys and 15.7% of girls practice the use of alcohol during sex, boys 2.21 times more frequently than girls. The prevalence of binge drinking between students who practice sexual activity associated with alcohol is 62.6% for boys and 28.5% girls. The frequency of practicing binge drinking is significantly higher in students who consumed alcohol during intercourse, the difference size is medium for boys and small for girls.

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## ABUZUL DE ALCOOL ÎN RELAȚIE CU ASPECTELE COMPORTAMENTULUI SEXUAL ÎN RÂNDUL STUDENȚILOR

**REZUMAT**

Acest studiu își propune investigarea unor aspecte ale comportamentului sexual asociate cu abuzul de alcool în rândul studenților din județul Timiș, Romania. Am aplicat un studiu populațional transversal unui lot reprezentativ de 2076 studenți, 62,49% fete și 37,51% băieți, cu vârsta medie de 21,09 ani și SD 1,48. Persoanele de sex masculin prezintă episoade de abuz de alcool semnificativ mai frecvent decât cele de sex feminin, diferența fiind mică spre medie: prevalența 22,1% pentru băieți și 8,7% pentru fete, de 1-2 ori în ultima lună. Persoanele de sex feminin forțate să aibă relații sexuale abuzează de alcool mai frecvent decât cele care nu au fost forțate. Frecvența episoadelor de abuz de alcool este semnificativ mai crescută la studenții care au relații sexuale ocazionale. Frecvența episoadelor de abuz de alcool este mai mare în rândul studenților care consumă alcool în timpul contactului sexual, diferența dintre băieți și fete fiind mică spre medie.

**Cuvinte cheie:** studenți, abuzul de alcool, comportament sexual

# FURIN-CLEAVABLE INSULIN EXPRESSED IN HUMAN MESENCHYMAL STEM CELLS AS DIABETES THERAPY

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**ABSTRACT**

Despite recent progress in the chemically-induced transdifferentiation of human and rat MSC into insulin-producing cells, the efficiency and clinical aplicability of factor-based conversions is still questionable. In contrast, genetically-modified hMSCs, capable of long-term, controlled secretion of biologically-active mature insulin, represent a promising therapeutical solution for the treatment of diabetes. In the present study, we explored the posibility of generating plasmid vectors carrying mutant construct human proinsulin construct, where the PC1/3 and PC2 cleavage sites had been altered by site-directed mutagenesis to form furin-cleavable sites and which can be successfully transcribed correctly and at very high levels, following the in vitro non-viral transfection into human mesenchymal stem cells.

**Keywords:** furin-cleavable insulin; mesenchymal stem cells

## INTRODUCTION

Currently recognized as a major public health concern of both highly industrialized and developing countries, diabetes, a metabolic disorder affecting more than 220 million people worldwide, warrants a definitive cure. Exogenous insulin supply, the current treatment, is not fully capable of achieving tight control of glucose regulation [1], while pancreatic islet transplantation is not a viable solution due to the shortage of donors [2]. As Type 1 diabetes (T1D) is caused by the selective autoimmune destruction of the highly specialized insulin-secreting β-cells, it follows logically that the disease is a good candidate for gene therapy to correct insulin defficiency.

The possibility of ectopic insulin expression by gene transfer in a non-β-cell has been explored since 1983 [3], but further experiments have shown that simple replacement of insulin expression is not likely to be useful as a therapy for T1D unless it contains a system to regulate insulin levels in response to minute variations of blood glucose. Considering the complex machinery of stimulus-secretion coupling in β-cells, a successful gene therapy for diabetes should deliver in the target cells, separately or in combination, at least two key elements: a regulatory system responsive to glucose concentrations, and appropriate processing of proinsulin into mature, biologically active insulin [4]. Correct proinsulin processing in non-β cells has been overcome by expressing a modified single-chain insulin [5], lacking C-peptide, which has a higher biological activity than proinsulin, but still only about 30% of that of the normally-occurring insulin, and does not require any enzymatic cleaving. A different approach focused on engineering a mutated version of the proinsulin gene, in which the aminoacid cleavage sites recognized by β-cell-specific proteases PC1/3 and PC2 have been replaced with the tetrabasic sites recognized by the more ubiquitary endoprotease furin [6].

The introduction of furin consensus sequences at the B-chain/C- peptide and C-peptide/A-chain junctions increased the processing of proinsulin to mature, biologically active insulin [7-10]; however, the production of furin-cleavable proinsulin was shown to be sensitive to the aminoacid substitutions used [11].

Ideally, both the cause and the consequence of T1D should be addressed [12] in a combined therapy pairing β-cell replacement by genetic engineering of insulin secreting cells with protection of the newly transplanted β-cells from the recurrence of islet-specific autoimmune attack. Mesenchymal stem cells (MSCs), which generate minimal immune reactivity and have anti-inflammatory [13] and immunomodulatory [14-16] effects, have become favourites in recently initiated or on-going clinical trials involving the use of stem cells for the treatment of diabetes mellitus, matching the more comprehensive interest in the potential of MSCs to alleviate abnormal immune responses in graft-versus-host-disease [17],

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solid organ transplantation [18] and Crohn's disease, among others. The precise therapeutic mechanism through which MSCs would be able to treat T1D, either by release of regulatory cytokines such as TGF-β or IL-10 or by cell-cycle inhibition of lymphocyte proliferation [19], has yet to be conclusively determined.

In the present study, we explored the posibility of generating plasmid vectors carrying mutant construct human proinsulin construct, where the PC1/3 and PC2 cleavage sites had been altered by site-directed mutagenesis to form furin-cleavable sites and which can be successfully transcribed correctly and at very high levels, following the in vitro non-viral transfection into human mesenchymal stem cells.

## MATERIALS AND METHODS

### Isolation and culture of hMSC

Human MSCs were isolated by adherence to plastic, from bone marrow aspirates obtained by sternal punction from healthy donors. Bone marrow samples were obtained after informed consent elaborated under an approved protocol, according to the World Medical Association Declaration of Helsinki. Prior to the transfection experiments, all hMSC samples were maintained in culture and expanded for 5-7 passages in alpha-minimum essential medium (α-MEM; Gibco BRL, Life Technologies, Carlsbad, CA, USA), supplemented with 10% fetal calf serum (FCS; PromoCell, Heidelberg, Germany) and 2% penicillin/streptomycin mixture (Pen/Strep, 10,000 IU/ml; PromoCell), by incubation at 37°C in 5% CO2 atmosphere. Medium replacement was performed every third day and, upon reaching 80% to 90% confluence, the cells were subcultured using 0.25% Trypsin-EDTA solution (Sigma, St. Louis, MO, USA) followed by centrifugation (10 minutes, 300xg) and were replated in T75 culture flasks at a density of 10,000 cells/cm2 to ensure optimal proliferation.

### Expression plasmids

Total RNA was isolated from human pancreas using the Absolutely RNA Miniprep Kit (Agilent Genomics, La Jolla, CA, USA) and its concentration was determined on the ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). Proinsulin coding sequence (CDS) was cloned by RT-PCR (OneStep RT-PCR Kit; Qiagen, Valencia, CA, USA) and GatewayTM BP recombination into the pDONR/Zeo vector (Life Technologies) using primers designed to introduce an attB1 and a Kozak sequence at the 5' end and an attB2 and stop-codon at the 3' end: forward 5'-GGGGACAAGTTTGTACAAAAAA GCAGGCTTCACCATGGCCCTGTGGATGCGC-3', reverse 5'-GGGGACCACT TTGTACAAGAAAGCTGGGTCCTAGTTGCAGTAGTTCT CCAGCTGGTAGAG. The proinsulin CDS was further subcloned by GatewayTM LR recombination into the destination vectors pcDNA-DEST53 and pcDNA-DEST47 (Life Technologies), which contain a neomycin selection marker and a N-terminal or C-terminal, respectively, Cycle3 GFP gene. The expression clones, DEST53-hIns and DEST47-hIns, were transformed into supercompetent OneShot OmniMAX 2 T1 E.coli and isolated with the PureLink HQ Mini Plasmid Purification Kit (both from Life Technologies).

### Mutant proinsulin construction

We analysed six furin cleavage sites with the neural prediction network ProP 1.0 [20] and chose to reconstruct the sites resulting in the highest probability of cleavage after the R56 and R89 aminoacids of proinsulin. The furin-cleavable consensus sequences were generated using QuikChange II Site-directed Mutagenesis Kit (Agilent Genomics) and the plasmid expression clone DEST-53-hIns as substrate. The mutagenesis sense primers were as follows: for the B-chain/C-peptide junction 5'-GAACGAGGCTTCTTCTACACACCCAGGAGCA AGCGGGAGGCAGAGG-3', and for the C-peptide/A-chain 5'-TGGAGGGG TCCCGGCAGAAGCGTGG. Mutagenesis was performed in two steps, following the manufacturer's instructions. First, a single-mutant insulin was generated, in a plasmid vector, by PCR amplification using the first set of mutagenesis primers. The DpnI-treated plasmid was transformed into supercompetent bacteria. The single-mutant insulin plasmid was isolated and subjected to another PCR amplification, using the second set of primers. The resulting double-mutant proinsulin 2M in the expression vector DEST53-2M was isolated after transformation into supercompetent OneShot OmniMAX 2 T1 bacteria and was back-cloned into an entry Gateway vector, which, subsequently served for generating the expression vector DEST47-2M, containing a double-mutant furin-cleavable proinsulin.

### DNA sequencing of plasmid vectors

The transgene presence and correct orientation in the DEST53-hIns and DEST47-hIns expression clones, as well as in the plasmids carrying the single- and double-mutant insulin constructs, was confirmed by capillary electrophoresis DNA sequencing with the ABI 3130 Genetic Analyzer using the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA). The primers were purchased from Life Technologies. Prior to sequencing, the PCR products were purified by ethanol/EDTA precipitation and denatured with HiDi Formamide (Applied Biosystems). Raw data was processed with SeqAnalysis 5.3 software (Applied Biosystems), while

contig assembly was perfomed with the DNA Workbench program (CLC Bio, Aarhus, Denmark).

**Lipofection of hMSC**

Prior to the transfection, hMSCs were permitted to reach 80-90% confluence, and the complete culture medium was changed to medium without antibiotics 30 minutes before lipofection. The expression plasmids DEST53-hIns and DEST53-2M were purified at transfection grade from competent E.coli OneShot OmniMAX 2 T1 using the S.N.A.P. MidiPrep Kit and diluted in serum-free OptiMEM. Lipofectamine 2000 was mixed and diluted at the appropriate amount in serum-free OptiMEM. The diluted DNA was mixed with Lipofectamine 2000 in a ratio of 1:2.5, and the liposomal-DNA complexes formed after a 20- minute incubation at room temperature were added to the hMSC. We used as a positive control for assessing transfection rate the pmaxGFP plasmid (Amaxa Lonza). The transfection medium was changed to complete growth medium after 16-20 hours and the transgene expression was evaluated at different time points. All transfection reagents were purchased from Invitrogen.

**qRT-PCR assay**

We isolated total RNA from hMSC by means of the GenElute Mammalian Total RNA Miniprep Kit (Sigma) and determined its concentration with the ND-1000 spectrophotometer. Sample purity, as indicated by the 260/280 absorbance ratio, was 1.9-2.02. We used 1 ug total RNA for every 20 ul reverse transcription reaction performed with the AccuScript High Fidelity 1st Strand cDNA Synthesis Kit (Stratagene Agilent Technologies). cDNA samples were analyzed by quantitative real-time PCR, using the LightCycler 480 SYBR Green I Master (Roche, Florence, SC, USA). HPRT1 was chosen as a suitable reference gene. The primers for Gen-Ins, 2M-Ins and N-Ins were designed using the open-access program Primer3. The 2M-Ins primers were chosen to anneal only to the mutant insulin, the N-Ins only to the wild-type insulin, while Gen-Ins can amplify both variants. Furin primer sequences were chosen from qPrimerDepot and HPRT1 primer sequences from Harvard Primer Bank databases. We performed a relative basic quantitation based on the ΔΔCt method with the LightCycler480 Software (Roche).

**Statistical analysis**

All statistical tests and values were calculated with GraphPad Prism 4 (GraphPad Software, San Diego, CA, USA). Data were expressed as mean ± s.e.m. Groups were compared using one-way ANOVA for normally-distributed data or the Mann-Whitney U test for nonparametric values. $P<0.05$ was considered to be significant.

## RESULTS

**Furin expression in mesenchymal stem cells**

Human mesenchymal stem cells ubicuitary express the proconvertase enzyme furin, which can successfully replace the pancreatic endoproteases in processing proinsulin to the active, mature hormone. We analyzed six different batches of MSCs isolated in our laboratory and compared the expression of furin in MSCs with that of human embryonic stem cell (hESC) and other cell lines available. We also performed a metaprofile analysis on a public microarray database, Genevestigator V3 [21], to confirm that furin is constantly expressed in MSCs, allowing the mutant insulin construct to be cleaved in target cells.

**Construction of insulin plasmids**

The wild-type insulin plasmid DEST53-hIns was obtained by subcloning the 333 bp human preproinsulin CDS, including the stop-codon, into the pcDNA-DEST53 destination vector. As described in the Methods section, we isolated 1740 ng/uL total undegraded RNA from 39 mg human pancreas. By PCR amplification of human insulin CDS with attB-containing primers, we obtained a 397 bp product, corresponding to the insulin CDS and the primer-generated Gateway recombination segments. This product was further subcloned, by recombination reactions into destination vectors, capable of inducing transgene expression in eukaryotic cells. The enzymatic recombination reactions, followed by amplifi- cation of the vectors by transforming supercompetent bacteria, were highly efficient and resulted in numerous plasmid-carrying colonies on selective agar plates. We chose 5 colonies for plasmid isolation with the PureLink HQ MiniPrep kit and downstream sequencing. Finally, pending analysis of sequencing data, one of the plasmids was isolated with a transfection-grade midiprep kit. Sequencing results documented a synonimous mutation C306G, with the same aminoacid, isoleucine, being transcribed at position 102. Otherwise, the reading frame for GFP was the same to that of insulin and we concluded that the proteins can be expressed simultaneously following transfection of the DEST53-hIns vector.

To ensure proinsulin processing in nonendocrine cells, we engineered a double mutant proinsulin (2M), in which the PC1/3 and PC2 cleavage sites were altered by site-directed mutagenesis to form consensus tetrabasic cleavage sites, containing the motif R-X-[K/R]-R, recognized by the subtilisin/kexin-like enzyme furin expressed in hMSC (Fig. 1). According to the ProP 1.0 neural prediction network, replacing proinsulin aminoacids 53-55 with Arg-Ser-Lys residues and L89 with Arg results in a 0.728 probability of cleavage of the mutant proinsulin after R56 and 0.751 after R89, respectively.

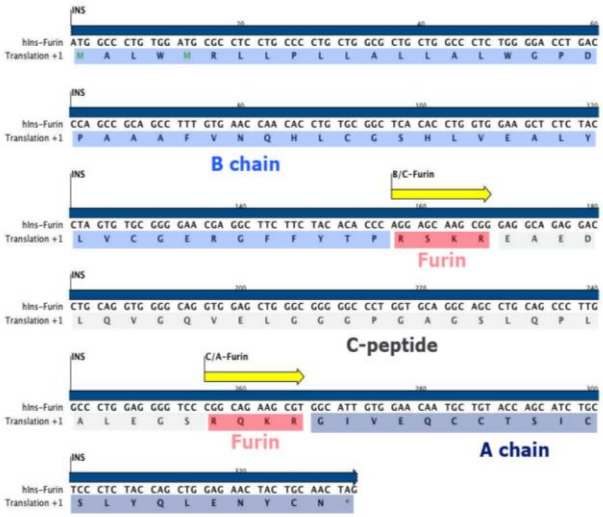


**Fig. 1 Mutant insulin cleavage sites.** The dibasic sequences Arg55-Arg56, where the B chain is cleaved from the C-peptide, and Lys88-Arg89, where the C-peptide is separated from the A-chain from human insulin were replaced by consensus amino acid sequences recognized by furin (shown in red).

We also analyzed several other tetrabasic motifs both for furin and a more general proconvertase cleavage probability, as well as the energy cost of mismatches when constructing the cleavage sites by in vitro mutagenesis (QuikChange Primer Design Program, Agilent Genomics). DNA sequencing confirmed the successful mutagenesis of insulin clevage sites.

### Non-viral gene transfer in hMSC

We achieved a 40-50% rate of transfection with 90-100% hMSC cell viability following the lipofection of the control plasmid pmaxGFP, at a ratio of DNA: Lipofectamine 2000 of 1: 2.5, as documented by fluorescence microscopy 24 hours from transfection (Fig. 2).

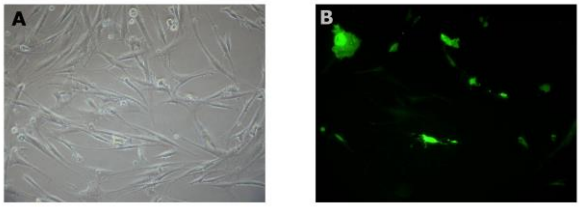


**Fig. 2 Rate of transfection of hMSC with pmaxGFP.** The transfection rate is about 50%, 24 hours from lipofection, while cell viability is 100%. Optic and fluorescence microscopy, 100x.

### Insulin transcription following lipofection of hMSC

We transfected by lipofection hMSC at passages 5-7 with the expression vectors DEST53-hIns/2M and DEST47-hIns/2M to assess the potential for insulin expression, in non- neuroendocrine cells. qPCR analysis of relative insulin mRNA expression showed a 105 times more intense transcription of the transgene in the transfected cells comparative to that in the INS-1 transgenic insulinoma cell line, which was used as a positive calibrator. The presented data was derived from five experiments, performed in duplicates, at different time points and we verified insulin expression with several primer pairs, designed to bind to different complementary regions within the preproinsulin sequence. The insulin mRNA detected in the transfected hMSC was transcribed entirely from our plasmid vectors as the endogenous insulin primers designed to bind targets outside the preproinsulin CDS cloned in the DEST47/53 plasmids detected only negligeable quantities, similar in the controls and transfected cells (Fig. 3).

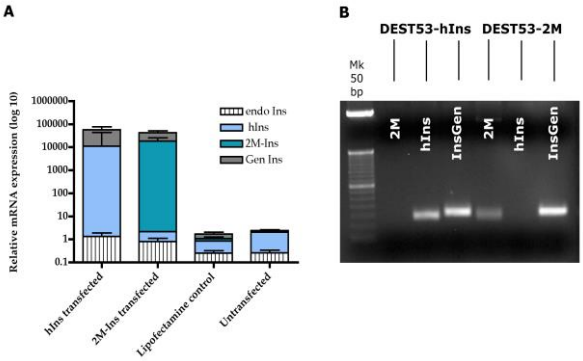


**Fig. 3 A Relative mRNA expression of different proinsulin variants, detected by real-time qPCR.** Endogenous insulin is present at almost undetectable levels and does not account for insulin expression in the transfected cells. mRNA level of expresson for mutant 2M-insulin is similar to that of the wild-type insulin. B We designed and tested on the plasmid vectors spe- cific primer pairs that allow us to differentiate between the 2M-insulin and the native insulin. Gen-Ins primers amplify both the normal and the mutan transgenes, while the 2M and the hIns primers amplify only the particular sequences of the engineered and the normal insulin.

Interestingly, the level of expression of mutant proinsulin mRNA was similar to that of the native proinsulin, when transfected into the same batch of MSCs, using the same plasmid backbone. This is an important observation because previous studies reported reduction of more than 70% of furin-cleavable insulin expression [9,11,22].

There was no significant difference in mRNA expression when hMSCs were transfected with either the GFP-insulin fusion protein construct or with the proinsulin alone (Fig. 4A). On the other hand, the level of insulin transcription after lipofection varied significantly ($p<0.0003$) between individual batches of hMSC (Fig. 4B). The MSCs were isolated from patients of different clinical backgrounds and age groups. The transfection was performed in identical experimental settings (DNA quantity,

DNA:Lipofectamine 2000 ratio) for all samples, and mRNA expression was assayed 24 hours post-transfection.

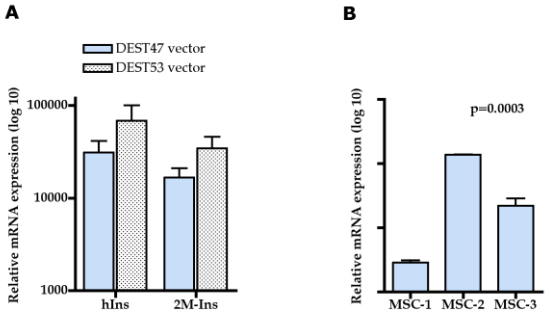


**Fig. 4. A. Relative mRNA expression of native and mutant proinsulin,** respectively, determined by real-time qPCR. Following lipofection of either the DEST53 or DEST47 vectors, which differ in their capacity to generate GFP-fusion constructs, the expression of the two in- suliant variants does not vary significantly ( p >0.05, N=4 for each vector). **B**. **Proinsulin mRNA levels** after lipofection of hMSC samples derived from different patients were heterogenous.

## DISCUSSION

Despite progress in the chemically-induced transdifferentiation of human and rat MSC [23-25] into insulin-producing cells or the even more momentous conversion of embryonic stem cells into insulin-secreting cells reported by Douglas Melton and his team [26], genetically-modified hMSCs, capable of long-term, controlled secretion of biologically-active mature insulin, still represent a promising therapeutical solution for the treatment of diabetes mellitus [27].

The current strategies for gene therapy in diabetes with the objective of correcting the underlying metabolic disfunction and restoring normal glucose levels involve the transfer of genes to express different variants of insulin [28,29], transcription factors closely related to pancreatic β-cell ontogeny [30,31] or proteins that control glucose metabolism [32]. In this experimental study, we reported the cloning of human preproinsulin CDS into a GatewayTM non-viral expression vector in view of a straighforward and efficient approach to obtaining a clinically-relevant number of insulin-producing cells from hMSC.

As hMSC do not express the pancreatic-specific endopeptidases PC1/3 and PC2, which convert proinsulin into the mature, active hormone, we modified the proinsulin proconvertase recognition sequences by in situ mutagenesis. The tetrabasic furin-cleavable motifs RSKR and RQKR replaced the wild-type proinsulin cleavage sites in the mutant proinsulin 2M, to ensure the correct processing of proinsulin, a key requirement of any long-term therapeutic solution for diabetes. To the best of our knowledege, the 2M proinsulin we constructed, with Arg-Ser-Lys-Arg : Glu-Ala (B:C cleavage site) and Arg-Gln-Lys-Arg : Gly- Ile (C:A cleavage site), has not been previously reported in the scientific literature, although similar furin-recognition sites have also been reconstructed. The motifs were chosen due to the high probability to be cleaved by the ubiquitary enzyme furin, following a bioinformatics analysis with the ProP 1.0 prediction server [20]. They are also very likely to be cleaved by other furin-like proconvertases, as predicted by the same program, which can ensure the posibility of expression in various cell types and tissues. The presence of furin was documented in the hMSC used in our experiments, and we confirmed that it is constantly expressed at least at medium levels, sufficient for an efficient processing, by screening a free functional genomics microarray database (Genevestigator V3, [21]). We then proceeded to cloning the 2M proinsulin in the DEST53-2M and DEST-47-2M non-viral plasmid vectors.

Transgene expression from the plasmid vectors DEST53-hIns/2M and DEST47-hIns/ 2M was controlled by the CMV constitutive promoter. The CMV promoter is frequently employed in gene transfer experiments due to its high efficiency in most cells [33] and we encountered few literature mentions of transcriptional inactivation due to promoter sequence methylation [34]. The next step would be to replace the CMV promoter with an endogenous, glucose-sensitive promoter, such as the EGFR1 (early growth response-1) element identified by Chen et al. [28], induced by rising glucose levels, in the absence of any protein synthesis, which also activates several genes invoved in glucose homeostasis [35]. The DEST53 plasmid vector controls the expression of a fusion GFP-insulin construct, with the GFP located at the N terminus of the protein. As we cloned the insulin CDS with a stop-codon, this would prevent the fusion of insulin with GFP at the C terminus, thus generating only the transgenic insulin. The insulin mRNA expression directed from either vector did not differ significantly. We have yet to discern whether the N-terminal GFP will interfere with protein folding or signal protein cleavage.

The advantages of using a non-viral transfection method by lipofection include the relative ease and safety of the procedure, with high levels of transgene expression in less than 24 hours from transfection in 40-50% of the cells, and very high cell viability. As the expression plasmids also contain an eukaryotic antibiotic resistance gene, we can also select only the transfected cells.

Several research groups testing a series of genetically engineered human and rat insulins reported a decrease by almost 90% of furin-cleavable proinsulin expression following transfection [9,22,36,37]. Hay et al. attributed this observation to an instability of proinsulin structure caused by the basic amino acids (arginine and

lysine) present in the furin consensus sites [11]. Surprinsingly, the lipofection of DEST53-2M expression plasmid into hMSC resulted in a level of insulin transcription similar to the wild-type insulin.

In conclusion, our data demonstrate that human mesenchymal stem cells transfected with wild-type and a mutant furin-cleavable human proinsulin were capable of expressing insulin and the mutant proinsulin was transcribed at a level comparable to wild-type insulin. Subsequently, we will further analyze the processing, secretion kinetics and physiological effect of the newly engineered proinsulin.

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## OPȚIUNI TERAPEUTICE ÎN DIABET: INSULINA CLIVABILĂ DE FURINA ȘI EXPRIMATĂ ÎN CELULE STEM MEZENCHIMALE

**REZUMAT**

În pofida unor progrese recente în domeniul transdiferențierii chimice a celulelor mezenchimale umane sau de șobolan în celule secretoare de insulină, eficiența și aplicabilitatea clinică a acestor metode este chestionabilă. În contrast, celule mezenchimale umane modificate genetic, capabile de secreție pe termen lung, controlabilă, a insulinei mature, activă biologic, ar putea reprezenta o soluție terapeutică promițătoare pentru tratarea diabetului. În acest studiu, am explorat posibilitatea de a obține vectori genetici de tip plasmid pe care să conțină un construct de proinsulină umană, în care situsurile de clivare ale enzimelor PC1/3 și PC2 au fost modificate genetic prin mutageneză în situsuri de clivare penmtru furină. Am urmarit ca această transgenă pentru insulină sa poată fi transcrisa corect și la un nivel înalt de expresie prin transferul non-viral în celule stem mezenchimale umane.

**Cuvinte-cheie:** insulină, furina, celule stem mezenchimale

# THE INVOLVEMENT OF *ENTEROCOCCUS SPP.* STRAINS IN THE AETIOLOGY OF INFECTIONS IN HOSPITALIZED PATIENTS

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**ABSTRACT**

The present study was motivated by the low rate of morbidity due to enterococcal infections reported at a national level and associated with a high risk of such infections in hospitals. The aims of the present study are to: i) Specify the aetiology of infections diagnosed in a Cluj university hospital from 2010 to 2012; ii) Investigate the associations between enterococci and other microorganisms involved in the aetiology of infections in the above-mentioned hospital; iii) Study the distribution by various medical services of infections caused by *Enterococcus* spp.

**Material and method.** During the period January 2010 – December 2012, of the 878 bacterial strains isolated from different pathological products collected from hospitalized patients, 137 were identified by the Vitek 2 Compact automatic system as being species of the genus *Enterococcus*.

**Results.** The species most frequently isolated in hospitalized patients was *E. faecalis* (64.23%), followed by *E. faecium (*27.73%). 8.04 % were other Enterococcus species encountered only in the hospital environment: *E. durans* (2.19%), *E. casseliflavus* (2.19%), *E. gallinarium* (2.19%), and *E. avium* (1.45%). *E.faecalis* was most frequently isolated from urine samples (39.77%), followed by CVC (14.8%), pus from fistula/drainage tube, and wound secretions and puncture fluids (9.1%). *E. faecium* was most frequently isolated from pus from fistula/drainage tube (34.21%), followed by wound secretions (23.7%), puncture fluids (18.42%), and respiratory secretions (13.15%).

**Conclusions.** The specification of the aetiology of enterococcal infections in hospitalized patients, the distribution of these infections by medical service, and the incidence of species and bacterial associations have all proved the importance of knowledge on enterococci.

**Key words:** Enterococci, infections, aetiology, hospital, patients.

## INTRODUCTION

Enterococci, although they are part of the intestinal flora commensals, may become nosocomial pathogens.

In the past three decades, a constant growth in the number of infections caused by species of the genus *Enterococcus* has been reported, a phenomenon probably caused by a growth in the population that shows risk factors (chronic inflammations, surgery, intestinal neoplasia), but also by the emergence of enterococci strains with multiple resistance to antibiotics, especially Vancomycin [1].

In hospitals, enterococci infections are one of the most important therapeutic challenges, due to their intrinsic resistance, on the one hand, and the constant growth of acquired resistance, on the other hand.

In this era of increased resistance of microorganisms to antibiotics, it is mandatory to give due consideration to the judicious use of antibiotics, which is certainly also helpful in stopping the emergence of resistant and virulent enterococci strains.

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## MATERIAL AND METHOD

The present study was conducted in a prospective manner, in that the *Enterococcus* spp. strains involved in the aetiology of infections in hospitalized patients were observed, by ward, during the period January 2010 – December 2012.

The hospitalized patients were from:

- the Intensive Care ward (54 beds)
- 2 General Surgery wards (130 beds)
- the Orthopaedics ward (60 beds)
- the Gastroenterology ward (50 beds)
- the Neonatal ward (40 beds)
- the Obstetrics and Gynaecology wards (85 beds)

In the hospital, data from the Clinical Laboratory's Microbiology Compartment was used. In order to collect data from the hospital, we used the standard surveillance data sheet, presented in the annex of Health Ministry Order no. 10/2005.

For each year studied (2010, 2011, and 2012), data was collected both about the enterococci strains that proved to be a unique etiological agent and the enterococci strains isolated in association with other microorganisms.

The samples were normally collected from patients by specialized hospital staff, on account of the fact that the observance of sample-collecting and transportation rules for pathological products intended for bacteriological analysis influences the different stages and the accuracy of the diagnosis.

Urine, blood, and cephalorachidian fluid samples were collected, as well as samples from purulent collections, bronchial aspirate, sputum, and central venous catheters. The collected samples were sown on Columbia gelose supplemented with sheep blood (5%), and then incubated at 37°C for 24 hrs.

In order to isolate the enterococci, we also used the Bile Esculin Azide Agar (Sanimed) medium, CPS (bioMérieux), Trypticase Soy Agar with 5% sheep blood (bioMérieux), and the Uriselect (BioRad) chromogenic medium [2].

The identification of bacteria belonging to the genus *Enterococcus* was done based on culture and biochemical characters.

The observance of the culture characters involved the presence of the following types of colony:

- on gelose-blood, type S, small, white-grey, non-haemolytic colonies - *E. faecalis*, beta-haemolytic colonies - *E durans,* or alpha-haemolytic colonies*;*
- on the Bile Esculin Azide Agar medium, the enterococci develop white-grey colonies with a black halo;
- by wiping with a wad the 24 hour culture obtained on the Trypticase Soy Agar medium and 5% sheep blood, one can notice the medium's yellow colouring- *E. casseliflavus, E. mundtii, E. sulfureus*;
- on the chromogenic CPS medium, the enterococci colonies are green and small.

In the hospital, the isolated enterococci cultures were identified via the Vitek 2 Compact automatic system (bioMérieux). The Vitek 2 cards for the identification of Gram-positive cocci are standardized products that contain miniature biochemical tests.

The *Enterococcus* species identified by VITEK 2 GP: *E.faecalis, E.faecium, E.avium, E. casseliflavus, E.cecorum, E.columbae, E.durans, E.gallinarum, E.hirae, E. raffinosus, E. Saccharolyticus.*

## RESULTS

During the study, 878 bacterial strains isolated from different pathological products from hospitalized patients were processed in the hospital laboratory.

**Table I.** The distribution of the strains isolated during the study, by pathological product collected from hospitalized patients

| Pathological product | 2012 | 2011 | 2010 | Total | |
|---|---|---|---|---|---|
| | | | | No. | % |
| Urine | 49 | 44 | 42 | 135 | 15.37 |
| Puncture fluids | 11 | 16 | 14 | 41 | 4.66 |
| Respiratory secretions (bronchial aspirate/tracheal secretion) | 102 | 96 | 96 | 294 | 33.53 |
| Pus from fistula/drainage tube | 16 | 17 | 23 | 56 | 6.37 |
| Wound secretions | 46 | 39 | 54 | 139 | 15.83 |
| Central venous catheter | 16 | 19 | 23 | 58 | 6.60 |
| Blood | 36 | 35 | 33 | 104 | 11.84 |
| Other products | 15 | 19 | 17 | 51 | 5.80 |
| **Total** | **291** | **285** | **302** | **878** | **100** |

The isolated bacterial strains came from: respiratory secretions, 294 (33.53%), wound secretions, 139 (15.83%), urine, 135 (15.37%), blood, 104 (11.84%), central venous catheter, 58 (6.60%), pus from fistula/drainage tube, 56 (6.37%), puncture fluid, 41 (4.66%), and other products, 51 (5.80%).

Of the 878 bacterial strains isolated, 137 strains (15.6%) belonged to the genus *Enterococcus*; their origin from different pathological products is shown in Table II.

**Table II.** The distribution of *Enterococcus* strains isolated in the hospital, from various pathological products

| Pathological product | 2012 | 2011 | 2010 | Total | | P |
|---|---|---|---|---|---|---|
| | No. of strains | No. of strains | No. of strains | No. | % | |
| Urine | 14 | 13 | 13 | 40 | 29.19 | 0.993 |
| Puncture fluids | 7 | 6 | 7 | 20 | 14.59 | 0.954 |
| Respiratory secretions (bronchial aspirate/ tracheal secretion) | 4 | 5 | 4 | 13 | 9.48 | 0.900 |
| Pus from fistula/ drainage tube | 8 | 9 | 8 | 25 | 18.24 | 0.929 |
| Wound secretions | 6 | 5 | 6 | 17 | 12.48 | 0.946 |
| Central vascular catheter | 5 | 4 | 4 | 13 | 9.48 | 0.952 |
| Blood | 1 | 1 | 1 | 3 | 2.18 | 0.999 |
| Bile | 1 | 1 | 1 | 3 | 2.18 | 0.999 |
| Cervical secretion | 1 | 1 | 1 | 3 | 2.18 | 0.999 |
| **Total** | **47** | **45** | **45** | **137** | **100** | / |

It can be noticed that during the three-year study period, no statistically-significant differences were recorded in the incidence of enterococci strains isolated in hospital.

The main types of enterococcal infections diagnosed in the hospital over the three-year study period are shown in Table III.

**Table III.** The distribution of infections caused by enterococci

| Infections caused | Number | % |
|---|---|---|
| Urinary tract infections | 40 | 29.19 |
| Respiratory infections | 13 | 9.48 |
| Post-operative infections | 42 | 30.72 |
| Bacteraemias/septicaemias | 3 | 2.18 |
| Gynaecological infections | 3 | 2.18 |
| Peritonitis | 20 | 14.59 |
| Cholecystitis | 3 | 2.18 |
| Infections caused by the application of the catheter | 13 | 9.48 |
| **Total** | **137** | **100** |

The most frequent infections are post-operative infections (30.72%), followed by urinary tract infections (29.19%), peritonitis (14.59%), infections caused by the application of the central venous catheter (9.48%), and respiratory infections (9.48%).

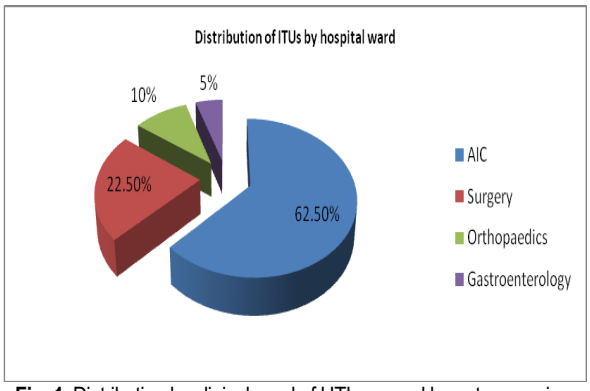


**Fig. 1.** Distribution by clinical ward of UTIs caused by enterococci

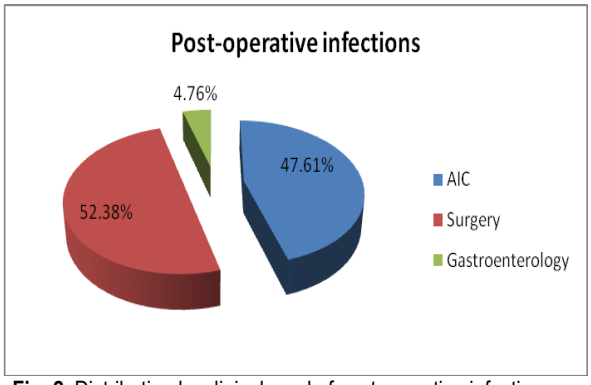


**Fig. 2.** Distribution by clinical ward of post-operative infections caused by enterococci

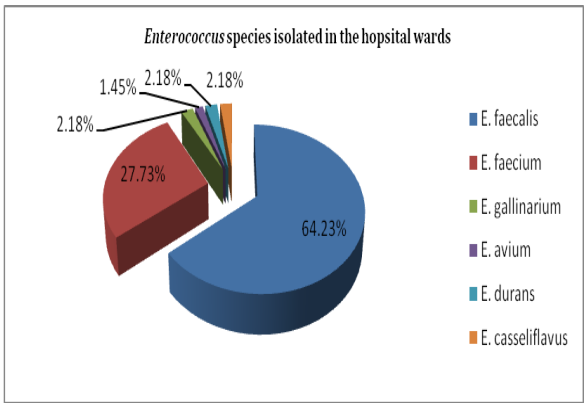


**Fig. 3.** Distribution by species of the enterococci strains isolated in the hospital wards

As Figure 3 indicates, the species most frequently isolated was *E. faecalis* (64.23%), followed by *E. faecium* (27.73%).

Other enterococci species encountered in the hospital environment isolated in a percentage of 8.04% were: *E. durans* (2.19%), *E. casseliflavus* (2.19%), *E. gallinarium* (2.19%), *and E. avium* (1.47%).

**Table IV.** Distribution of *E. faecalis* strains isolated in the hospital

| Pathological product | 2012 | 2011 | 2010 | Total | |
|---|---|---|---|---|---|
| | No. of strains | No. of strains | No. of strains | No. | % |
| Urine | 11 | 12 | 12 | 35 | 39.77 |
| Puncture fluids | 3 | 2 | 3 | 8 | 9.1 |
| Respiratory secretions (bronchialaspirate/ tracheal secretion) | 2 | 2 | 2 | 6 | 6.81 |
| Pus from fistula/drainage tube | 3 | 2 | 4 | 9 | 10.22 |
| Wound secretions | 2 | 2 | 4 | 8 | 9.1 |
| Central venous catheter | 5 | 4 | 4 | 13 | 14.8 |
| Blood | 1 | 1 | 1 | 3 | 3.40 |
| Bile | 1 | 1 | 1 | 3 | 3.40 |
| Cervical secretion | 1 | 1 | 1 | 3 | 3.40 |
| **Total** | **29** | **27** | **32** | **88** | **100** |

*E.faecalis* was most frequently isolated from urine samples (39.77%), followed by CVC (14.8%), pus from fistula/drainage tube (10.22%), and wound secretions and puncture fluids (9.1%).

**Table V**. Distribution of the *E. faecium* strains isolated in the hospital

| Pathological product | 2012 | 2011 | 2010 | Total | |
|---|---|---|---|---|---|
| | No. of strains | No. of strains | No. of strains | No. of strains | % |
| Urine | 2 | 1 | 1 | 4 | **10.52** |
| Puncture fluids | 3 | 2 | 2 | 7 | **18.42** |
| Respiratory secretions (bronchial aspirate/tracheal secretion) | 1 | 2 | 2 | 5 | **13.15** |
| Pus from fistula/drainage tube | 4 | 5 | 4 | 13 | **34.21** |
| Wound secretions | 4 | 3 | 2 | 9 | **23.7** |
| **Total** | **13** | **13** | **12** | **38** | **100** |

*E. faecium* was most frequently isolated from pus from fistula/drainage tube (34.21%), followed by wound secretions (23.7%), puncture fluids (18.42%), and respiratory secretions (13.15%).

**Table VI.** Comparative presentation of the *E. faecalis* and *E. faecium* strains isolated in hospital from diverse pathological products

| Pathological product | E. faecalis (n=88) | E. faecium (n=38) | p |
|---|---|---|---|
| Urine | 35 | 4 | **0.001** |
| Puncture fluids | 8 | 7 | 0.146 |
| Respiratory secretions (bronchial aspirate/tracheal secretion) | 6 | 5 | 0.304 |
| Pus from fistula/drainage tube | 9 | 13 | **0.001** |
| Wound secretions | 8 | 9 | **0.027** |
| Central venous catheter | 13 | 0 | **0.009** |
| Blood | 3 | 0 | 0.553 |
| Bile | 3 | 0 | 0.553 |
| Cervical secretion | 3 | 0 | 0.553 |

Table VI indicates a higher statistically-significant incidence of *E. faecium* in pus and wound secretions, while *E faecalis* is, in a statistically-significant manner, more present in urine and on central venous catheters.

In the numerous pathological products collected from the hospitalized patients, the enterococci were isolated alongside other bacterial strains. In the following graph, we show the microbial associations involved in the aetiology of the infections comprised in the present study.

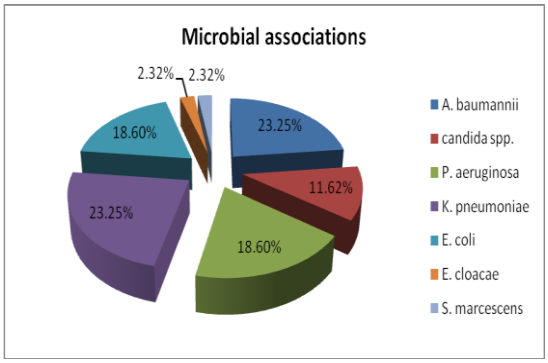


**Fig. 4**. Distribution of the microbial associations of the isolated *Enterococcus* spp. strains

As Figure 4 indicates, the enterococci were more frequently isolated in association with *Acinetobacter baumannii, Klebsiella pneumoniae, Pseudomonas aeruginosa,* and *E. coli*.

## DISCUSSION

The aim of the present study was to evaluate the involvement of the *Enterococcus* spp. strains in the aetiology of certain infections diagnosed in hospitalized patients and in outpatients.

According to the results presented above, we isolated and identified a total number of 137 *Enterococcus* spp. strains involved in the aetiology of certain infections in hospitalized patients, which represent 15.6% of the total germs isolated during the period in question.

By comparing the isolation of enterococci from various pathological products, in the hospital we noted a higher extremely statistically-significant prevalence in pus, wound secretions ($p<0,001$), and puncture fluids ($p<0,001$). The most frequently isolated enterococcus species were *E. faecalis* and *E. faecium,* which is consistent with the data provided by the specialty literature [3].

On the whole, *E. faecalis* was represented by 88 strains *(64.23%). E. faecium* totalled 38 strains *(27.73%).*

By comparing the distribution by pathological product, we noted that the *E. faecalis* strains were better represented as compared to the *E. faecium* strains in urine ($p<0.001$), pus ($p<0.001$), wound secretions

(p=0.027), and central venous catheter secretions (p=0.009).

Other Enterococcus species included in this study were *E. gallinarium, E. durans*, and *E. casseliflavus,* with 3 strains each (1.36%), and *E. avium,* with 2 strains (0.91%).

The *Enterococcus* species are part of the normal intestinal flora, enterococci being considered as low virulence germs. However, affecting the local intestinal defence mechanisms, usually by surgery, neoplasms, or chronic intestinal inflammations, causes invasive enterococcal infections, such as septicaemia, peritonitis, cholecystitis, and endocarditis. Due to the high incidence of the risk factors mentioned above, in recent years we have witnessed a constant rise in enterococcal infections [4].

In this study, in the case of hospitalized patients, the enterococci were more frequently involved in the aetiology of post-operative infections (30.72%), followed by urinary tract infections (29.19%), and peritonitis (14.59%).

Post-operative infections, irrespective of their localization, were more frequently mixed, being caused by associations of enterococci with Gram-negative bacilli, especially *Acinetobacter baumannii* and *Klebsiella pneumoniae*.

We did not notice any statistically-significant differences between the Anaesthesia and Intensive Care, and the Surgery wards, which are frequently faced with such infections (p=0,006), whose number is, nonetheless, larger in the surgery ward.

Although enterococci are frequently isolated from patients with intra-abdominal infections, their role in these infections is controversial.

In six studies conducted on patients with mixed abdominal infections, caused by enterococci and Gram-negative bacteria, antibiotics were administered that had no effect on the enterococci; it was observed that, in this case, the abdominal infections had a favourable evolution. Similar studies, conducted on outpatients who had mixed intra-abdominal infections, demonstrated a favourable evolution after the surgical treatment, in the absence of enterococcal antibiotherapy. Also, it was observed that the enterococci do not cause intra-abdominal infections when they are injected intraperitoneally into laboratory animals, except if they are associated with other, more virulent, microorganisms [5].

On the other hand, other researchers have demonstrated that the presence of enterococci can cause an increased occurrence rate of post-surgery infections and increased mortality. A study conducted on 330 patients indicated the isolation of enterococci in intra-abdominal collections as the cause for treatment failure [5,6]. Enterococci can also be involved in skin infections, soft tissue infections, or wound infections. Usually, these infections are mixed, the role of the enterococci in their aetiology being not fully known. Most frequently, enterococci are isolated from sore and ulcer infections of the diabetic foot [5]. In this study, the 17 *Enterococcus* strands come from wound infections.

The 2 species, *E. faecalis* and *E. faecium,* were almost identically represented, by 8 and 9 strands, respectively, which were associated with *P. aeruginosa* (5 cases), and *Acinetobacter baumannii* (4 cases).

In the case of hospitalized patients, surgical wound infections were included in the category of post-operative infections, and they were more frequently diagnosed in the Anaesthesia and Intensive Care, and Surgery wards.

Urinary tract infections ranked second in the case of hospitalized patients.

During the three-year period that the study spanned, we did not observe any statistically-significant differences, from one year to the next, in the incidence of UTI in the hospital.

As far as aetiology is concerned, *E. faecalis* was more present, and in a much more statistically-significant manner, as compared to the other Enterococcus species (p<0,001).

In the case of the hospitalized patients diagnosed with UTI, the enterococci were relatively frequently associated with enterobacteria, especially with wide spectrum beta-lactamase-secreting *E.coli* strains.

Although enterococcal UTI more frequently affect the urinary bladder, the ground on which they occur is completely different. Thus, in the general population, women without anomalies of the urinary tract or urological interventions have UTI more often. Enterococci, like other Gran-positive cocci, are more frequently associated with nosocomial urinary infections in patients with obstructive uropathy or after endourologic procedures [7-11].

It is considered that approximately 10% of hospitalized patients are catheterized, and of these, 20-25% develop UTI, the incidence of the infection being directly proportional to the length of catheterization. After a unique bladder catheterization, UTI is present in 1% of outpatients and 10% in hospitalized patients, it being more frequent in the elderly, in immunodepressed patients, and in those having urological diseases. Urinary infections are significantly more frequent in patients with long catheterization. In the first two weeks of catheterization, the relation between the onset of UTIs and catheterization is linear, 50% of patients developing UTI after 15 days of catheterization, and almost all patients after one month. According to Ferrar, all patients develop bacteriuria 4 days after catheterization, under the conditions of an open urinary drainage system, while the use of a closed drainage system is associated with bacteriuria 10 days later [12-16].

The data cumulated from 463 hospitals in the USA indicates enterococci as the third cause for UTIs associated with catheterization, the most isolated being the *E. faecium* and *E. faecalis* species [17].

In the specialty literature, UTIs caused by enterococci are associated with the antibiotic treatment in

previous cases, and often it's a case of recurring UTIs, diagnosed more frequently in the case of men over 50 years of age, with a urological pathology. Though relatively rarely, the enterococci can nevertheless cause pyelonephritis or renal abscess, accompanied by bacteraemia [18].

Ranking fourth in order of frequency, in the hospital, were respiratory infections and central venous catheter-related infections.

Respiratory infections are not frequently caused by enterococci, but ortotracheal intubation, assisted breathing, prolonged dorsal decubitus, and antibiotherapy, especially with cephalosporins, are favouring factors in the case of hospitalized patients. In our study we have diagnosed such infections in a number of 13 patients coming from the hospital's Anaesthesia and Intensive Care, and Surgery wards, which participated in our study. The majority of respiratory infections were mixed, 3 being caused by *E. faecalis* and *Acinetobacter baumannii*, 3 by *E. faecium* associated with *Acinetobacter baumannii*, and 3 were caused by *E. faecalis* in association with *Pseudomonas aeruginosa*, *Klebsiella pneumoniae* or *Acinetobacter baumannii*. By comparing the distribution of the *E. faecalis* and *E. faecium* strains involved in the aetiology of respiratory infections, we did not obtain any statistically-significant differences (p=0,304) during our study.

Of the enterococci, only *E. faecalis* was responsible for central venous catheter infections. As far as the distribution by ward of CVC infections is concerned, they were more present, in a statistically-significant manner, in the Anaesthesia and Intensive Care ward than they were in the Gastroenterology ward (p=0,030); this difference was no longer present when we compared the Anaesthesia and Intensive Care ward with the Surgery ward (p=0,620).

The bacteraemias caused by enterococci, although frequently reported in the specialty literature, were extremely rare in this study, in that only 3 such infections were identified: 2 in the Anaesthesia and Intensive Care ward and one in the Surgery ward. *E. faecalis* was isolated in all 3 cases of bacteraemia.

Both the bacteraemias and CVC infections caused by enterococci frequently recognize as their source of infection the gastrointestinal or genitourinary tracts of inpatients or outpatients. In hospital, other sources of infection can be the vascular or urinary catheters and the wounds, including burns. Approximately half the cases of enterococcal bacteraemias occur in immunocompromised patients and in those who are administered antibiotics [5].

If, in the case of outpatients, enterococcal infections are less severe, the infections diagnosed in hospital, due to the ground on which they occur, raise the length of hospitalization and the cost of treatment, and sometimes worsen the state of the patients. Under these conditions, it is necessary to strictly observe certain procedures for fighting and controlling infections.

The general control of infections, which also include enterococcal infections, involves isolating the patients who are likely to transmit the germs to other patients or medical staff, and the use of a "physical barrier" [19].

## CONCLUSIONS

The specification of the aetiology of enterococcal infections in hospitalized patients, the distribution of these infections by different medical services, the incidence of species and of bacterial associations have all demonstrated the importance of knowledge on enterococci.

The specification of the frequency and aetiology of the different types of enterococcal infections (urinary infections, post-operative infections, respiratory infections, bacteraemias/septicaemias, gynaecological infections, peritonitis, cholecystitis, and CVC infections) is genuinely helpful in the prevention and control of these infections, especially of those caused by VRE strains.

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## IMPLICAREA TULPINILOR DE *ENTEROCOCCUS SPP.* ÎN ETIOLOGIA INFECŢIILOR LA PACIENŢI SPITALIZAŢI

**REZUMAT**

**Obiective.** Prezentul studiu a fost motivat de morbiditatea scăzută prin infecții enterococice raportată la nivel național, asociată riscului crescut al unor astfel de infecții în spitale. Cercetarea de față și-a propus: i) Precizarea etiologiei infecțiilor diagnosticate în cadrul unui spital universitar clujean în perioada 2010-2012; ii) Investigarea asociațiilor dintre enterococi și alte microrganisme implicate în etiologia infecțiilor în spitalul menționat; iii) Studierea repartiției infecțiilor determinate de *Enterococcus* spp. pe diferite servicii medicale.

**Material şi metodă.** In perioada ianuarie 2010- decembrie 2012 din cele 878 de tulpini bacteriene izolate din diferite produse patologice provenite de la pacienti spitalizaţi, 137au fost identificate cu sistemul automat Vitek2 Compact ca fiind specii din genul *Enterococcus*.

**Rezultate.** Specia cel mai frecvent izolată la pacienţii spitalizaţi a fost *E. faecalis* (64.23%) urmată de *E. faecium (*27.73%). Alte specii de enterococi întâlnite doar în mediul spitalicesc s-au izolat în procent de 8.04 %: *E. durans* (2.19%), *E. casseliflavus* (2.19%), *E. gallinarium* (2.19%), *E. avium* (1.47%). *E.faecalis* a fost cel mai frecvent izolat din prelevatele de urină (39.77%), urmat de CVC (14.8%), puroi fistulă/tub de dren (10.22%), secreţiile de plagă şi lichidele de puncţie (9.1%). *E. faecium* a fost cel mai frecvent izolat din puroi fistulă/tub de dren (34.21%), urmat de secreţiile de plagă (23.7%), lichidele de puncţie (18.42%) şi secreţiile respiratorii (13.15%).

**Concluzii.** Precizarea etiologiei infecţiilor enterococice la pacienţii spitalizaţi, repartiţia acestor infecţii pe diferite servicii medicale, incidenţa speciilor şi a asocierilor bacteriene a demonstrat importanţa cunoaşterii enterococilor.

**Cuvinte cheie:** Enterococi, infecţii, etiologie, spital, pacienţi.

# THE ROLE OF BMI AND CO-MORBIDITIES IN HOSPITALIZATION LENGTH STAY IN SURGICAL REPAIR OF INCISIONAL HERNIA


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**ABSTRACT**

Introduction. BMI as risck factor and co-morbidities evaluated pre-operative can predict the hospitalization lenght stay in incizional hernia repair. We conducted a 18 months retrospective observational study on incizional hernia repair in our Clinics after registering to the EuraHS platform in order to evaluate the patients demographic characteristics (age, sex, BMI), co-morbidities, and surgical outcomes (early and late complications, total length of hospital stay).

Materials and methods. We have established the target group of 126 consecutive patients with incizional hernia repair. We collected data about demographics, comorbidities, and surgical outcomes (length of hospital stay, incidence of early and late complications). Data are presented as mean and range. Correlation coefficient r (Pearson), 95% confidence interval ($p<0.05$) with have been assessed for each analyzed variable.

Results. The age average of the group was 62.7 y. Sex ratio showed females preponderance (76%) when compared with males (24%). The midline localization with all the possibilities as describes on the Eura HS site has been seen in most of the cases (78.6%), followed by right iliac fossa localization (9.5%). None of the patients had a 4 SOC score. Score SOC 3 was the most encountered. Out of 126 cases, 54 presented BMI < 29.99; 72 had BMI > 29.99 and 7 more than 40. We have seen that BMI and SOC score are very strong statistically correlated with correlation coefficient r (Pearson) = 0.36, and $p = 0.0001$. The length of total hospital stay has registered values between 2 and 38 days. The BMI and total hospital stay are strongly correlated: correlation coeficient r (Pearson) = 0.27, and $p = 0.002$. Between SOC score and total hospital stay, there have been no statistical correlation ($r = 0.14$, and $p = 0.12$ (> 0.05). Intraoperative we reported 2 cases of iatrogen bowel lesions. In terms of surgical complications we had 9 cases (7.14%) of acute or early post-operative complications (4 cases of hemorrhage, 3 cases of bowel obstruction and 2 cases of hematoma) and 5 (3.96%) late post-operative complications (3 hernia recurrence and 2 parietal abcesses) as per EuraHS classification, in patients with BMI >30.

Conclusions. BMI has a good correlation with SOC score and total hospital stay. Appropiate definitions and classifications as provided by EuraHS platform could enable the evaluation of patients prior and after abdominal wall surgery in a unitary manner and thus studies and meta-analysis of this pathology could be improved.



**Key words:** SOC score, BMI, hospital stay, surgical outcomes


## INTRODUCTION

Incisional hernia is a common complication of abdominal surgery. In a clinical review, Sanders [1] summarized that the reported incidence after a midline laparoscopy ranges from 3% to 20% and it is double if the index operation is complicated by wound infection [2]. About 50% of incisional hernias are detected within one year of surgery, but they can occur several years afterwards, with a subsequent risk of 2% a year [2, 3]. Millions of abdominal incisions are performed each year worldwide, so incisional hernias are a major problem, both in terms of morbidity and socioeconomic cost [1].

Every incizional hernia repair is a challenge for the surgeon, with different factors related to the patient and hernia type that potentially could influence the postoperative outcomes.

Despite considerable improvements in prosthetics used for hernia surgery, the incidence of incision hernias and the recurrence rates after repair remain high. Arguably, no other benign disease has seen so little improvement in terms of surgical outcome [1]. For predicting the development of early (less than six months after surgery) incisional hernia models have been published recently [4]. Van Ramshorst and colleagues [5] identified the major independent risk factors as age, sex,


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chronic pulmonary disease, ascites, jaundice, anemia, and emergency surgery, type of surgery, postoperative coughing, and wound infection.

Consequently the length of hospital stay is a valuable indicator of patient care needs before and after surgery. The main objective of this study is to identify the role of BMI and co-morbidities variables that significantly predict the length of hospital stay. Another objective is to implement definitions and classifications proposed by the international online platform for registration and outcome measurement EuraHS (European Registry for Abdominal Wall HerniaS) in defining certain variables in order to collect data in a standardized manner [6].

## MATERIALS AND METHODS

Electronic medical records of all consecutive patients for whom an alloplastic substitution in an open repair procedure for abdominal wall defects was performed from July 2012 until December 2013 (18 months) at our Second Surgery Clinic of Timisoara Emergency County Hospital were retrospectively reviewed. For each patient a set of data have been recorded in order to evaluate their status pre-, intra-, and postoperative.

We have used definitions and classifications posted on the international online platform for registration and outcome measurement EuraHS (European Registry for Abdominal Wall HerniaS) for hernia class, SOC score for co-morbidities (Table I), early and late postoperative complications (Table II).

**Table I.** SOC score evaluation [6]

| **Severity of co-morbidity score SOC score** | |
|---|---|
| **SOC score** | **Definition** |
| 0 | No co-morbidities |
| 1 | Asymptomatic, no medical consultation needed in last 12 months |
| 2 | Stable disease, intermittent therapy and medical consultation needed ≤4x/year |
| 3 | Stable disease, continuous therapy with regular medical consultation >4x/year |
| 4 | Progressive disease, with changing or intensified therapy and frequent medical consultation >12x/year |

**Table II**. EuraHS classification of surgical complications [6]

| | |
|---|---|
| Intra-operative complications | Are complications occurring during the time of the patients' arrival in the operating room and the patient leaving the operating room |
| "Acute" or "early" post-operative complications | Are complications occurring during the hospitalisation or within 30 days postoperatively |
| Late post-operative complications | Are complications related to the hernia repair occurring after discharge and more than 30 days postoperatively |

We have used histograms to report the number of observations/frequency of data of certain figures or belonging to a certain interval. Statistic parameters have been calculated as mean values, and standard deviation, using chi-square analysis. Correlation coefficient *r* (Pearson), 95% confidence interval ($p<0.05$) with have been assessed for each analyzed variable.

## RESULTS

During the above mentioned period, a total number of 134 abdominal wall hernia repairs have been performed. Because of lack of information about BMI and other data, 8 patients have been excluded from the present study. Where excluded the patients for whom a laparoscopic approach has been performed.

During pre-operative evaluation a set of variables have been used: age, sex, BMI, hernia class and SOC score as defined by EuraHS. For postoperative outcomes we have used EuraHS classification of surgical complications.

The age average of the group was 62.7 y. Sex ratio showed females preponderance (76%) when compared with males (24%).

Considering class hernia distribution the midline localization with all the possibilities as describes on the EuraHS site has been seen in most of the cases (78.6%), followed by right iliac fossa localization (9.5%).

While evaluating co-morbidities we have noticed that none of the patients had a 4 SOC score. Score SOC 3 was the most encountered as it is showed in Figure 1.

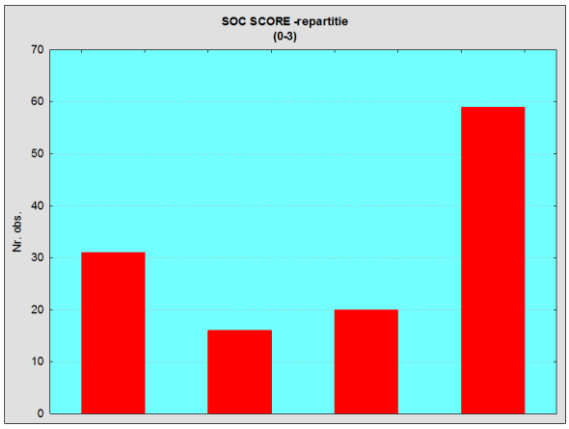


**Fig. 1.** SOC score based repartition

Since weight is a risk factor for hernia formation we have calculated the BMI for each patient. Out of 126 cases, 54 presented BMI < 29.99; 72 had BMI > 29.99 and 7 more than 40. The total repartition had a normal theoretical Gaussian distribution as it is illustrated in Figure 2.

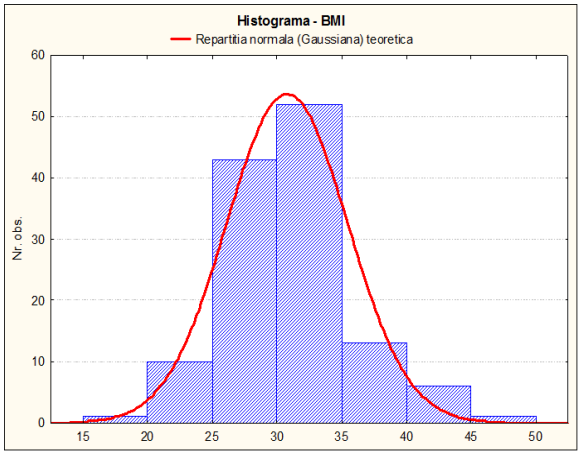


**Fig. 2.** BMI distribution

We have analyzed the BMI and SOC score behavior. We have concluded that the two variables are very strong statistically correlated with correlation coefficient *r* (Pearson) = 0.36, and *p* = 0.0001 (Figure 3).

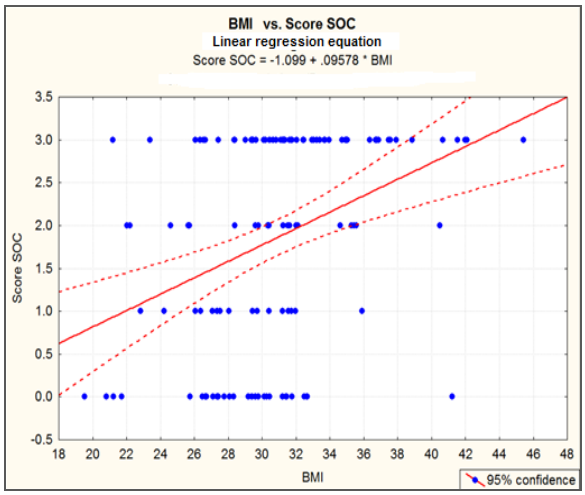


**Fig. 3.** Correlation between BMI and SOC score

The length of total hospital stay has registered values between 2 and 38 days. The correlation between BMI and the length of hospital stay is presented in the graph bellow (Figure 4). The BMI and total hospital stay are strongly correlated and the statistic calculated parameters are: correlation coeficient r (Pearson) = 0.27, and p = 0.002.

Between SOC score and total hospital stay, there have been no statistical correlation (r = 0.14, and p = 0.12 (> 0.05). While intra-operative evaluation we noticed that no complications have occurred.

In terms of surgical complications we had 9 cases (7.14%) of acute or early post-operative complications (4 cases of hemorrhage and 3 cases of bowel obstruction and 2 cases of hematoma) and 5 cases (3.96%) of late post-operative complications (3 hernia recurrence and 2 parietal abcesses) as per EuraHS classification.

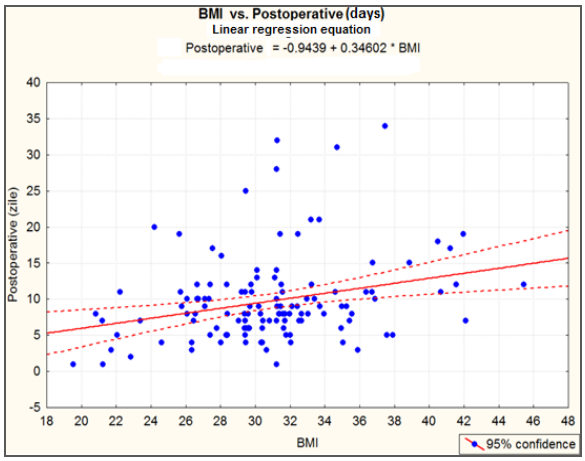


**Fig. 4.** Correlation between BMI and the length of hospital stay

## DISCUSSION

Surgical hernia repairs is one of the most common operative procedures and also the most commun elective procedures performed by general surgeons since it occurs in about 11% of laparotomies [7].

Preoperative appreciation of patient characteristics may help choosing the type of procedure repair and predict for surgical outcomes. Several investigators have attempted to identify risk factors for postoperative wound morbidity in patients undergoing abdominal wall reconstruction [8, 9, 10, 11]. A lot of important risk factors for predicting surgical site occurrence (SSO) were identified. Patients with obesity, a smoking history, diabetes, and chronic obstructive pulmonary disease had a16% risk of developing a complication as SSO [10]. Obesity has long been considered a risk factor for the development of primary and incisional ventral hernias [12].

We conducted a 18 months retrospective observational study on incizional hernia repair in our Clinics after registering to the EuraHS platform in order to evaluate the patients demographic characteristics (age, sex, BMI), comorbidities, and some surgical outcomes (early and late complications, total length of hospital stay). The group consisted in 126 patients with the age average of 62.7 y. Sex ratio showed females preponderance (76%) when compared with males (24%). More than two third of patient had a midline localization of abdominal wall defect (78.6%), followed by right iliac fossa localization (9.5%). Since we could notice that 72 patients from the group had BMI > 29.99 and 7 more than 40 we have looked for the statistical correlation of BMI with SOC score and total hospital stay. In both cases we observed a strong statistic correlation in terms of correlation ceoficient (r, Pearson) with p<0.05. In terms of surgical outcomes we had 9 cases (7.14%) of acute or early post-operative complications (4 cases of hemorrhage and 3 cases of bowel obstruction and 2

cases of hematoma) and 5 cases (3.96%) of late post-operative complications (3 hernia recurrence and 2 parietal abcesses) as per EuraHS classification, in patients with BMI >30.

This study has several limitations. The complex nature of our referral practice has enabled us to have a larger experience with comorbid patients undergoing ventral hernia repair reporting to EuraHS platform and longer follow-up, in order to evaluate the surgical outcomes on a larger scale.

## CONCLUSIONS

In our study, we could notice that BMI as risk factor showed a strong statistical correlation with SOC score and with total hospital stay.

In terms of surgical complications as defined by EuraHS we had 7.14% of cases with acute or early post-operative complications and 3.96% of cases with late post-operative complications, in patients with BMI >30.

Appropriate definitions and classifications as provided by EuraHS platform could enable the evaluation of patients prior and after abdominal wall surgery in a unitary manner and thus studies and meta-analysis of this pathology could be improved.

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## ROLUL IMC ȘI AL CO-MORBIDITĂȚILOR ASUPRA DURATEI DE SPITALIZARE ÎN CAZUL REPARĂRII CHIRURGICALE A EVENTRAȚIILOR POSTOPERATORII


**REZUMAT**

Introducere. Evaluarea preoperatorie a IMC ca factor de risc si a co-morbiditatilor pot previziona durata de spitalizare in cazul repararii eventratiei peretelui abdominal. Am efectuat un studiu retrospectiv, despre repararea eventratiilor postoperatorii in Clinica noastra pe o durata de 18 luni, dupa inregistrarea la platforma online EuraHS cu scopul de a evalua caracteristicile demografice ale pacientilor (varsta, sex, IMC), comorbiditatile si rezultatele postoperatorii (complicatii precoce si tardive si durata totala de spitalizare) .

Material si metoda. A fost selectionat retrospectiv un grup de 126 pacienti operati pentru eventratie. S-au colectat date despre caracteristicile demografice ale pacientilor (varsta, sex, IMC), comorbiditatile si rezultatele postoperatorii (complicatii precoce si tardive, durata totala de spitalizare). Datele sunt prezentate ca valori medii si procentuale. Coeficientul de corelatie (Pearson), si limita de confidenta 95% ($p<0.05$) a fost apreciata pentru fiecare variabila.

Rezultate. Varsta medie a grupului a fost de 62.7 ani cu preponderenta sexului feminin (76%) comparativ cu sexul masculin (24%). Localizarea eventratiei pe linia mediana, cu toate posibilitatile descrise de EuraHS a fost intalnita in majoritatea cazurilor (78.6%), urmata de localizarea in fosa iliaca dreapta (9.5%). Niciunul dintre pacienti a prezentat scorul SOC 4. Majoritatea au avut scorul SOC calculat in valoare de 3. Dintre cei 126 pacienti, 54 au prezentat BMI < 29.99; 72 au avut BMI > 29.99 si 7 mai mult de 40. S-a observat o puternica corelatie statistica intre BMI si scorul SOC cu coeficient de corelatie r (Pearson) = 0.36, si p = 0.0001. durata de spitalizare a avut valori cuprinse intre 2 si 38 zile. Deasemenea intre BMI si durata de spitalizare s-a observat o puternica corelatie statistica cu coeficient de corelatie r (Pearson) = 0.27 si p = 0.002. Intre scor SOC si durata de spitalizare nu s-a observat nicio corelatie(r = 0.14 si p = 0.12 (> 0.05). In timpul actului operator s-au semnalat 2 cazuri de leziuni intestinale iatrogene. In ceea ce priveste rata complicatiilor postoperatorii s-au semnalat 9 cazuri (7.14%) de complicatii precoce (4 cazuri de hemoragie, 3 cazuri de ocluzie si 2 cazuri de hematom) si 5 (3.96%)complicatii tardive (3 cazuri de recidiva si 2 cazuri de abces parietal), conform clasificarii EuraHS, la pacienti cu IMC >30.
Concluzii. IMC are o corelatie puternica cu durata de spitalizare. Definitii si clasificari adecvate, asa cum sunt disponibile online prin inregistrare la platforma EuraHS ofera o evaluare pre si postoperatorie a pacientilor intr-o maniera unitara si astfel creste calitatea studiilor si a meta-analizelor acestei patologii.
Key words: SOC score, BMI, hospital stay, surgical outcomes
**Cuvinte cheie:** scor SOC, IMC, durata de spitalizare, rezultate chirurgicale

# GABAERGIC POTENTIATION OF REDUCED BOWEL MOVEMENTS INDUCED WITH ZINC SULPHATE BY BACLOFEN


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**ABSTRACT**

This study evaluated the influence of orally administered zinc sulphate on bowel movement of rats by the faecal output and gastrointestinal transit time in the rats. The effects of zinc sulphate on feeding as well as water intake were also determined. The dose of zinc sulphate that yielded the maximal effect was administered alongside baclofen to investigate the role of GABA on bowel propulsion. All the three doses of zinc sulphate (1 $mgkg^{-1}$, 5 $mgkg^{-1}$ and 10 $mgkg^{-1}$) produced significant reduction ($p < 0.05$) in total faecal pellets produced in eight hours of study (12.00 ± 0.93, 9.17 ± 0.31 and 3.17 ± 0.17) respectively, compared with the control group (15.67 ± 1.38). Also, the three doses of zinc sulphate produced dose-dependent significant reduction ($p < 0.05$) in the faecal mass (1.78 ± 0.17, 2.50 ± 0.22 and 1.07 ± 0.10) respectively when compared with control group (3.39 ± 0.25).The total transit time of zinc sulphate (10 $mgkg^{-1}$) treated group (593.8 ± 2.84 min) increased significantly ($p < 0.0001$, $t = 26.98$) compared with the control group (251.2 ± 5.48 minutes). Zinc sulphate (5 $mgkg^{-1}$ and 10 $mgkg^{-1}$) significantly ($p < 0.05$) reduced food intake (10.83 ± 0.54 and 12.50 ± 0.51) respectively while (1 $mgkg^{-1}$) produced increase ($p < 0.05$) in food intake (22.50 ± 0.56) when compared with the control group (18.33 ± 0.67). Administration of zinc sulphate and zinc + baclofen significantly ($p < 0.001$, $f = 63.9$) reduced faecal pellet output (4.80 ± 0.74 and 1.60 ± 0.25) respectively when compared with control (10.2 ± 0.74). The study concluded that zinc sulphate reduced bowel movement in rats stimulating γ aminobutyric acid ($GABA_B$) receptors.



**Keywords:** Zinc sulphate, Bowel, $GABA_B$, Baclofen and Transit Time


## INTRODUCTION

Bowel movements (Motility) in the gastrointestinal tract is an involuntary and self-generated movement of the entire gut which accounts for the propulsion, mixing, and reservoir functions necessary for the orderly processing of ingested food and the elimination of waste products. Propulsion is the coordinated motion of ingested foods, liquids, gut secretions, and sloughed cells from the mucosa through the gastrointestinal tract. It propels the food from the stomach into the small intestine and along the small intestine, with appropriate timing for efficient digestion and absorption. Peristaltic waves propel undigested material into the large intestine and eliminate waste through defecation [1].

Trituration, the crushing and grinding of ingested food by the stomach, decreases particle size, increasing the surface area for action by digestive enzymes in the small intestine. Mixing movements mix pancreatic, biliary, and intestinal secretions with nutrients in the small intestine and bring products of digestion into contact with the absorptive surfaces of the mucosa. Reservoir functions are performed by the stomach and colon. The body of the stomach stores ingested food and exerts steady mechanical forces that are important determinants of gastric emptying. The colon holds material during the time required for the absorption of water and stores the residual material until defecation is convenient. Each of the specialized organs along the digestive tract exhibits a variety of motility patterns. These patterns differ depending on factors such as time after a meal, awake or sleeping state, and the presence of disease. Motor patterns that accomplish propulsion in the esophagus, small and large intestines are derived from a basic peristaltic reflex circuit in the ENS [1,2].

The musculature of the gastrointestinal tract is for the most part smooth muscle. Electrical slow waves and action potentials are the principal sources of electrical activity in the gastrointestinal musculature. Gut smooth muscles have properties of a functional electrical syncytium. A


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hierarchy of neural integrative centers in the central nervous system (CNS) and peripheral nervous system (PNS) regulates and controls the functioning of the gut. The gastrointestinal tract is innervated by the sympathetic, parasympathetic, and enteric divisions of the autonomic nervous system (ANS). Vagus nerves carry afferent sensory information to the brain and parasympathetic autonomic efferent signals to the digestive tract. Splanchnic nerves convey sensory information to the spinal cord and sympathetic autonomic efferent signals to the digestive tract. The enteric nervous system (ENS) functions as a mini-brain within the gut [1-5].

Existence of GABA neurons in enteric ganglia of many species has been described, including human and rodents. In the rat and human intestine, enteric GABAergic neurons have at least three distinguishable components viz; neurochemical populations and two morphological cell types (Krantis, 2000). Enteric GABAergic fibers are present in great quantity, branching extensively all around the ganglionated and non-ganglionated enteric nerve interconnection of the gut wall [6]. In addition to GABAergic nerve fibers, the mucosa of the rodent and human bowel have GABA in endocrine-like cells in the gastric antrum over the whole distance to the distal colon within them [6].

There is paucity of evidence on the role of GABA receptors in the alteration of gastrointestinal motility by zinc salts, hence this study.

## MATERIALS AND METHODS

**Animal Care and Management**

Twenty-four adult male Wistar rats weighing 120 – 180 g were obtained from the Animal House, College of Health Sciences, Obafemi Awolowo University, Ile-Ife. The rats were kept in plastic rat cages for two weeks to acclimatize in the Animal Holding, Department of Anatomy and Cell Biology, where they were given access to feed (Caps Feed, Osogbo) and water *ad-libitum*. Later, the animals were housed in modified cages in which food and water consumption as well as faecal pellet output can be assessed.

**Chemicals and Drugs**

Zinc sulphate was obtained from May and Baker Laboratory Chemicals Limited, Dagenham, England. Grade-S.L.R; CAS 7446-20-0 and Baclofen tablets was purchased from MA Halder TEVA UK. Limited 83915 T. For the experiments the both drugs were dissolved in distilled water for oral administration.

**Experimental Design**

The rats were randomly divided into four groups consisting six rats each. Group 1 which served as the control, was given distilled water while groups 2, 3 and 4 received 1, 5 and 10 mg/kg/day oral administration of zinc sulphate respectively.

**Test on Gastrointestinal Motility (Charcoal Meal) In Rats**

Gastrointestinal transit time in the rats was determined using the method of [7]. The rats were deprived of food for three hours but had free access to water. The dose of zinc sulphate (i.e. 10 $mgkg^{-1}$) that produced the most significant change in faecal pellet output was administered to rats of groups 2, 3 and 4. After 90 min, 0.3ml of an aqueous suspension of 5% charcoal in 10% water was administered to each animal orally. Sixty minutes later (time 150 min), the rats were given free access to food. They were observed at 20 min intervals until the first appearance of charcoal in their faecal pellet. Charcoal was observed on the faeces under normal light, using a hand lens to identify the black spots. The transit time was based on time for charcoal to be eliminated from the gastrointestinal tract [7].

**Test on $GABA_B$ Receptor:** $GABA_B$ receptor agonist (baclofen) was administered orally at 1 mg/kg 15 minutes before administration of zinc sulphate.

**Test to determine Faecal Pellet Output and Weight**

Collection of Faecal pellets followed the method of [8] with slight modifications. Faecal pellets were counted at two-hour intervals for eight hours following zinc salt administrations. The faecal droppings were collected on the absorbent paper placed beneath the rat perforated cages. The weight of the air-dried pellets were determined after 24-hours.

**Determination of food and water intake:** Food and water intake was determined during the experiment as described by [9].

***Statistical Analysis*:** Statistical analyses were carried out using Graphpad Prism 5.0 version software. Data are presented as mean ± SEM. Analysis of Variance (ANOVA) was performed followed by Newman Keuls' post-hoc test. A value of $p < 0.05$ was considered statistically significant.

## RESULTS

Pre-treatment with zinc sulphate (5 $mgkg^{-1}$ and 10 $mgkg^{-1}$) significantly reduced ($p < 0.05$) food intake (10.83 ± 0.54 and 12.50 ± 0.51) respectively while (1 $mgkg^{-1}$) produced increase ($p < 0.05$) in food intake (22.50 ± 0.56) when compared with the control group (18.33 ± 0.67) ($p < 0.05$) (Figure 1).

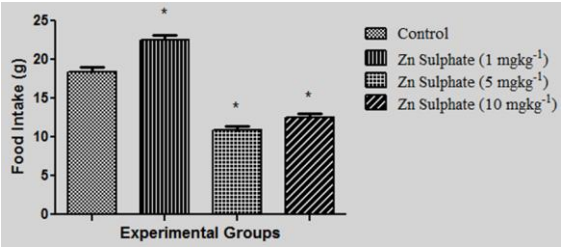


**Fig. 1. Effect of Zinc Sulphate Administration on Feeding:** Pre-treatment with zinc sulphate (5 mgkg$^{-1}$ and 10 mgkg$^{-1}$) significantly reduced ($p < 0.05$) food intake (10.83 ± 0.54 and 12.50 ± 0.51) respectively while (1 mgkg$^{-1}$) produced increase ($p < 0.05$) in food intake (22.50 ± 0.56) when compared with the control group (18.33 ± 0.67) ($p < 0.05$).

Pre-treatment with zinc sulphate (1 mgkg$^{-1}$, 5 mgkg$^{-1}$ and 10 mgkg$^{-1}$) produced a dose-dependent reduction ($p < 0.05$) in water intake (17.50 ± 0.66, 17.89 ± 0.61 and 16.74 ± 1.11) respectively compared with the control group (21.42 ± 0.93) (Figure 2).

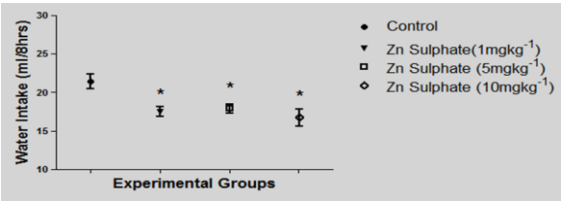


**Fig. 2. Effect of Zinc Sulphate Administration on Water Intake:** Pre-treatment with zinc sulphate (1 mgkg$^{-1}$, 5 mgkg$^{-1}$ and 10 mgkg$^{-1}$) produced a dose-dependent reduction ($p < 0.05$) in water intake (17.50 ± 0.66, 17.89 ± 0.61 and 16.74 ± 1.11) respectively compared with the control group (21.42 ± 0.93).

The three doses of zinc sulphate (1 mgkg$^{-1}$, 5 mgkg$^{-1}$ and 10 mgkg$^{-1}$) produced a significant dose dependent reduction ($p < 0.05$) in the number of total faecal pellets (12.00 ± 0.93, 9.17 ± 0.31 and 3.17 ± 0.17) respectively when compared with control group (15.67 ± 1.38) (Figure 3).

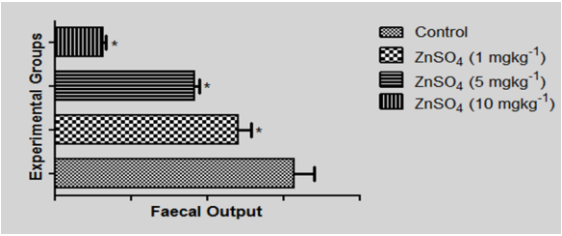


**Fig. 3. Effect of Zinc Sulphate Administration on Number of Faecal Pellets.** The three doses of zinc sulphate (1 mgkg$^{-1}$, 5 mgkg$^{-1}$ and 10 mgkg$^{-1}$) produced a significant dose dependent reduction ($p < 0.05$) in the number of total faecal pellets (12.00 ± 0.93, 9.17 ± 0.31 and 3.17 ± 0.17) respectively when compared with control group (15.67 ± 1.38).

All the three doses of zinc sulphate (1 mgkg$^{-1}$, 5 mgkg$^{-1}$ and 10 mgkg$^{-1}$) produced a significant reduction ($p < 0.05$) in the total faecal weight (1.78 ± 0.17, 2.50 ± 0.22 and 1.07 ± 0.10) respectively when compared with control group (3.39 ± 0.25). Of the three doses of zinc sulphate, 10 mgkg$^{-1}$ produced the highest reduction in faecal weight in 8 hours (1.07 ± 0.10) while 5 mgkg$^{-1}$ produced the least in 8 hours (2.50 ± 0.22) (Figure 4).

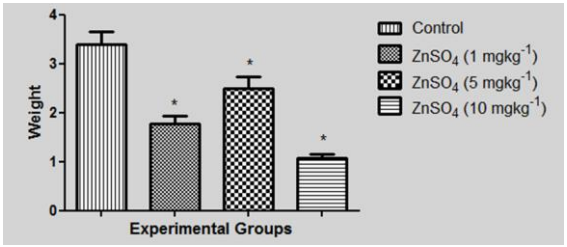


**Fig. 4. Effect of Zinc Sulphate Administration on the Weight of Faecal Pellets, * = significantly different from control ($p < 0.0001$).**

The total transit time of zinc sulphate (10 mgkg$^{-1}$) treated group (593.8 ± 2.84 min) increased significantly ($p < 0.0001$, $t = 26.98$) compared with the control group (251.2 ± 5.48 min) (Figure 5).

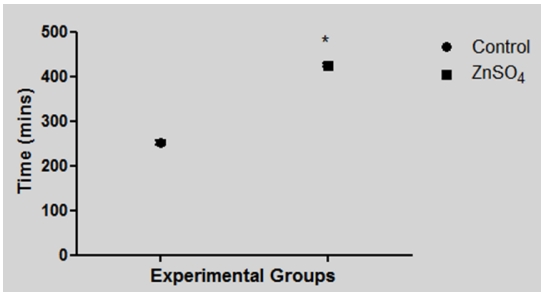


**Fig. 5. G.I Transit Time Induced by Zinc Sulphate, * = significantly different from control ($p < 0.0001$).** The total transit time of zinc sulphate (10 mgkg$^{-1}$) treated group (593.8 ± 2.84 min) increased significantly ($p < 0.0001$, $t = 26.98$) compared with the control group (251.2 ± 5.48 min).

Zinc sulphate and zinc + baclofen significantly ($p < 0.001$, $f = 63.9$) reduced faecal pellet output (4.80 ± 0.74 and 1.60 ± 0.25) respectively when compared with control (10.2 ± 0.74) (Figure 6).

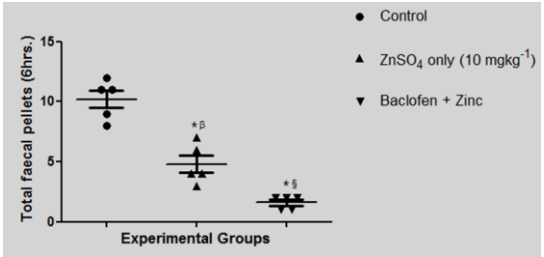


**Fig. 6. Baclofen Potentiation of Zinc Induced Reduction in Frequency of Defecation. * = significantly different from control; § = significantly different from $ZnSO_4$ only; β = significantly different from baclofen only ($p < 0.0001$, $f = 63.9$).**

Zinc sulphate and zinc + baclofen significantly ($p < 0.001$, $f = 63.9$) reduced faecal pellet output ($4.80 \pm 0.74$ and $1.60 \pm 0.25$) respectively when compared with control ($10.2 \pm 0.74$).

## DISCUSSIONS

**Zinc Sulphate Induced Alterations in Food Intake.**

In a report by [10], the messenger RNA (mRNA) expression of neuropeptide Y (NPY) and orexin in the hypothalamus significantly increased after three hours of oral administration of zinc which was not observed in the group given zinc sulphate intraperitoneally and the increased food consumption was inhibited by vagotomy. The route of administration used in this current study is the same as that of [10]. Also, the dose reported by the authors to have stimulated food intake (19 mmol/kg (3.0 mg/kg) of zinc sulphate (ZnSO4) in saline) also corresponds to the low dose of ($ZnSO_4$ 1 mg/kg) that increased food consumption in this current study. The only difference being the solvent. In this study, distilled water was used while Ohinata *et al.,* used saline. The mRNA expressions of hypothalamic peptides, such as orexin and NPY, increased after oral administration of zinc causing increased food intake.

Ghrelin is a potent orexigenic factor that is synthesized and released mainly from the oxyntic cells of the stomach, it is also released from duodenum, ileum, caecum and colon [11,12]. It is possible that at a low dose of zinc sulphate, ghrelin is released from the oxyntic cells which then increased food intake. This is because peripheral and centrally administered ghrelin have been shown to increase food intake in rodents [13,14] through the arcuate nucleus of the hypothalamus by increasing *c-fos* gene expression in the ARC Neuro-peptide Y (NPY)-synthesizing neurons [15].

Further, the anorexigenic effects of the zinc sulphate tested can be attributed to the peptide-YY secreted majorly from the L-cells of the distal gastrointestinal tract, particularly the ileum, colon and rectum [16,17]. This peptide PYY exists in two major forms: $PYY_{1–36}$ and $PYY_{3–36}$, the latter is the peripherally active anorectic signaling peptide. The observed anorectic effect of zinc salt at high doses therefore may be by directly releasing peptide PYY from the distal part of the gut. Peripheral administration of $PYY_{3–36}$ to rodents has been shown to inhibit food intake in rodents [18,19].

Another weight of evidence explaining the effect of zinc on appetite is by indirect release of Pancreatic Poly-peptide (PP-Peptide). It is synthesized from the cells at the periphery of the islets of the endocrine pancreas, and to a lesser extent in the exocrine pancreas, colon and rectum. Studies have identified $Zn^{2+}$ as a G Protein-Coupled Receptor 39 activating factor (GPR39) [20-22] and GPR39 is expressed in the endocrine pancreas [23]. Moreover, in the endocrine pancreas $Zn^{2+}$ functions as a chemical messenger co-stored and released [23] suggesting a correlation between Pancreatic Poly-peptide (PP-peptide) and $Zn^{2+}$. Peripheral treatment with PP-Peptide was shown to reduce food intake [24,25] just as higher dose of zinc in this study, also normal-weight human volunteers given an infusion of PP-Peptide demonstrated decreased appetite, and a 25% reduction in food intake over the following 24 h [26].

Although circulating PP is unable to cross the blood–brain barrier, it may exert its anorectic effect on the ARC via the area postrema [27]. This effect may occur via the $Y_5$ receptor as there is no response in $Y_5$ receptor-knockout mice, although the anorectic effect is not reduced by $Y_5$ receptor antisense oligonucleotides [28]. Following the peripheral administration of PP, the expression of hypothalamic NPY and orexin mRNA is significantly reduced [25]. PP may also exert some anorectic action via the vagal pathway to the brainstem, as vagotomy seems to reduce its efficacy [25].

**Zinc Sulphate Induced Effect on Water Intake, Faecal Pellet Output and Transit Time.**

The effect of zinc sulphate on water intake in this study is in agreement with a similar finding on the effect of zinc acetate on water intake. The probable mechanisms responsible for the observation were also extensively discussed in the report [9]. Zinc sulphate from previous studies have been shown to have an inhibitory effect on faecal pellet output. The observation from this experiment is also related with the findings of [9]. The detailed possible mechanisms by which zinc causes the reduction were as well discussed.

Zinc sulphate significantly increased the total transit time in the rats used for this experiment. The increased total transit time points to a reduction in propulsion velocity through the entire bowel of the experimental animals. Other details on the effect of zinc salts on gastrointestinal transit time has also been discussed in a previous report [9].

**GABAergic Potentiation of Reduced Bowel Movements Induced by Baclofen**

Baclofen significantly reduced the frequency of defecation as observed in this experiment. It exerted an additive effect when given together with zinc sulphate producing further reduction in faecal pellet output.

Gamma Amino-butyric Acid (GABA) exerts both stimulatory and inhibitory influence over enteric neuronal system, this is contingent upon the GABA receptor subtypes excited: stimulation is via $GABA_A$ receptors and inhibition is via $GABA_B$ receptors. GABA is implicated in the neural and endocrine/paracrine regulation of diverse functions within the gut. GABA can either stimulate (via $GABA_A$ receptors) or inhibit (via $GABA_B$ receptors) intrinsic cholinergic motor neurons that are responsible

for the ascending excitation limb of the peristaltic reflex. Thus perturbation of enteric GABA transmission could potentially contribute to the cholinergic hyper-contraction of the gut [6].

Within the mucosa, GABAergic nerve fibers branch extensively within the small nerve plexus located beneath the base of the crypts, which serve as the interface for the innervation of the mucosa [6]. This allows baclofen to exert its inhibitory influence on bowel motility as shown by the result of the current study.

In addition to the neural localization, GABA is synthesized, stored, and secreted by mucosal endocrine-like cells throughout the rat antrum and intestine [29,30]. The mucosa, therefore, may well be under the influence of GABA released from local neurons [31] as well as GABA secreted from endocrine cells. In the rodent gastric antrum, GABAergic endocrine-like cells resolve into subpopulations of cells colocalizing either gastrin (G cells) or somatostain (D cells). Baclofen may therefore modulate G cells' secretion by exerting inhibitory influence on the secretion of gastrin known to stimulate gastric motility on one hand while stimulating the secretion of somatostatin (D cells) from the gastric antrum. Baclofen may likewise stimulate the secretion of Gastric Inhibitory Peptide (GIP) by the K cells found in the antrum of the stomach to further inhibit gastric motility leading to reduction in faecal output.

In the rat, GABAergic neurons account for >5–8% of the total number of myenteric neurons in the large intestine, baclofen through its action on $GABA_B$ receptors is believed to trigger the release of somatostatin from D cells found in the pancreas and the small intestine to inhibit gastric motility. In the rodent intestine, GABAergic endocrine-like cells display a morphology similar to D-type endocrine cells [30]. GABA released from these cells into the local circulation or interstitial space may act as a classic gastrointestinal hormone or a local paracrine or autocrine factor. Baclofen may further stimulate the secretion of Gastric Inhibitory Peptide (GIP) from the K cells which are found in the jejunum and duodenum to also inhibit gastric motility.

Micro-dialysis sampling of myenteric plexus neurotransmitter release established a substantial inhibitory effect of the $GABA_B$ receptor agonist (baclofen) on dog's intestinal acetylcholine (ACh) release. This was sensitive to $GABA_B$ receptor antagonism [32]. It has been discovered that baclofen, a $GABA_B$ receptor agonist inhibit dog's intestinal Ach release. GABA antagonist was reported to reverse this inhibitory effect of baclofen on ACh release [32]. It is evident from various findings that $GABA_B$ receptors exert an inhibitory effect on release of ACh, without any significant effect on inhibitory neurotransmitter release or synthesis [33], this may partially explain the results as obtained in rats from this study. Further, in rat intestine, a neural circuit comprising GABA-, somatostatin-, and enkephalin-containing interneurons was suggested to be crucial for the regulation of descending relaxation [6].

Furthermore, GABA receptors was reported to modulate release of 5-Hydroxytryptamine (5-HT) from endocrine cells by Nakajima and colleagues [34]. $GABA_B$ receptors have also been shown to regulate the release of enterochromaffin cell-derived serotonin from guinea-pig small intestine, the action of baclofen from the result of this study can simply be thought to be by way of stimulating the release of 5-HT which binds to $5HT_3$ receptor while inhibiting $5HT_4$. The consequence of which is the slowing down of gastrokinetic activities, reduction in the rate of gastric emptying due to reduction in peristalsis of jejunum and duodenum.

In addition to GABA receptors modulating 5-HT release from enterochromaffin cells, it has also been reported to modulate histamine release from mast cells, gastric mucous secretion [35], prostaglandins released from interstitial cells [36], and mucosal electrolyte transport [31,37].

GABAergic myenteric interneurons in the human and rat colon fall into three neurochemically distinct, non-overlapping subpopulations: 1) neurons with somatostatin, which account for ~40% of the GABA population; 2) neurons with enkephalin, which account for ~10 % of the GABA population; and 3) neurons with NADPH diaphorase-related nitric oxide synthase (NOS) activity, which account for ~20% of the GABA population and neither somatostatin- nor enkephalin-positive cells show nitric oxide synthase (NOS) activity [5]. The reduction in bowel movement in this study may be a consequence of direct excitation of NOS activity by baclofen at enteric synapses. This is because, within the submucosa and mucosa, GABAergic cells were seen to co-localize either somatostatin or NOS, but not both [6].

Myenteric GABA neurons co-localizing enkephalin may represent inhibitory innervation of the muscularis, which modulates cholinergic mediated contractions [38,39], whereby the release of acetylcholine and other transmitters is attenuated [40]. This is one of the possible mechanisms by which baclofen reduced bowel motility as observed in the current study.

GABA and its receptor-mediated effects on bowel motility seem to be dependent on an intact ENS as isolated rat smooth muscle cells are unresponsive to addition of GABA. Moreover, independent of innervation by the central nervous system, peripheral $GABA_B$ receptor activation induces TTX- and atropine-sensitive gastric contractility *in vitro* [41], suggesting that baclofen locally increases gastric tone through activation of intrinsic cholinergic neurons.

The propulsive velocity of a dilated balloon along rabbit colonic preparations was significantly reduced by $GABA_B$ receptor activation with baclofen in a study by Tonini and colleagues [42]. This is in concert with the findings from our current study. Further, baclofen had a

minor inhibitory effect on colonic longitudinal muscle tone but a more significant inhibitory effect on TTX- and hyoscine-sensitive electrically stimulated responses, suggesting that the inhibitory effects of the $GABA_B$ agonist on colonic activity in the rabbit is dependent on cholinergic neurotransmission [42].

## CONCLUSION

It is evident that baclofen acting through $GABA_B$ receptors reduces bowel movements through many concerted mechanisms, modulating the activities of many endogenous neurotransmitters within the gut. We conclude that zinc sulphate induced reduction of propulsive movement within the gut is mediated by $GABA_B$ receptor stimulation. This observation opens new opportunities for understanding bowel function in health and disease.

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# BIODISTRIBUTION OF $FE_3O_4$/CITRIC ACID NANOPARTICLES IN A MURINE MODEL


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**ABSTRACT**

Iron oxide magnetic nanoparticles of about 20÷150 nm were synthesized by chemical co-precipitation of $Fe^{2+}$ and $Fe^{3+}$ in $NH_4OH$ solution and were coated with citric acid. The goal of this *in vivo* study was to analyze the biodistribution of MNPs in different organs by means of electron paramagnetic resonance (EPR) and histological analysis with Prussian blue after intravenous administration to Sprague Dawley rats. Their distribution was studied at 24 hours after intravenous administration through the jugular vein at a dose of 15 μmol of Fe/kg. The results showed larger deposits of nanoparticles in spleen and liver and not so significant ones in kidney, pancreas and lung.

We have studied these aspects with our novel iron oxide formulation, which can be used in the research of therapeutic areas and magnetic resonance imaging (MRI) and justify further assessment.



**Keywords:** MNP, biodistribution, murine model


## INTRODUCTION

Owing to the evolving field of bioapplications, magnetic iron oxide nanoparticles have found different directions such as magnetic separation and biodetection of cells, proteins, viruses etc., clinical diagnosis and therapy (magnetic resonance imaging and magnetic fluid hyperthermia), targeted drug delivery as well as cancer treatment [1]. When testing and designing innovative nanoparticles it should be taken into account important parameters like their pharmacokinetics properties and biodistribution characteristics. The first step in nanoparticles retention and cellular internalization is their passage from the bloodstream to the tissue of interest, followed by binding of molecular target so that it may reach an adequate concentration of nanoparticles in the desired tissue. However, a large percentage of systemically injected nanoparticles have a narrow therapeutic index because they are quickly eliminated from the bloodstream by the reticuloendothelial system and the mononuclear phagocyte system, of liver, spleen and bone marrow [2,3].

Also serum half-life and magnetic nanoparticles biodistribution may be influenced by coating materials, nanoparticle core and linker together with synthesis and purification procedures [4].

While naked magnetic nanoparticles have a hydrophobic nature they are not stable in normal physiological conditions and tend to form aggregates [5-7]. In water based magnetic fluids, the magnetic phase (mainly iron oxides) requires different coating materials that give them stability and compatibility with biological fluids [8-11].

Coating materials used to sterically stabilize the iron oxide nanoparticles often contain carboxyl functional groups. These functional groups are popular because they form stable covalent linkage between coating agents and iron oxide surface.

To stabilize the suspension of magnetic nanoparticles we used citric acid, which is adsorbed on the surface of freshly prepared iron oxide nanoparticles. The terminal carboxylic functional group of citric acid, not only that makes the nanoparticles water dispersible, but also provides a starting point for surface functionalization.


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Because a suspension of magnetic nanoparticles in an artificial physiological environment might not fully simulate different types of immune system cells or biological system, we focused our attention on evaluating the pharmacokinetics of nanoparticles in a *in vivo* situation, using a murine model.

The aim of this research is to investigate the biodistribution of biocompatible magnetite nanoparticles covered with citric acid in Sprague Dawley rats, after intravenous administration.

## MATERIALS AND METHODS

### 1. Magnetic nanoparticles

Citric acid-coated superparamagnetic iron oxide nanoparticles (MNPs) were obtained at the Institute of Macromolecular Chemistry "Petru Poni" (Iasi, Romania) (Figure 1).

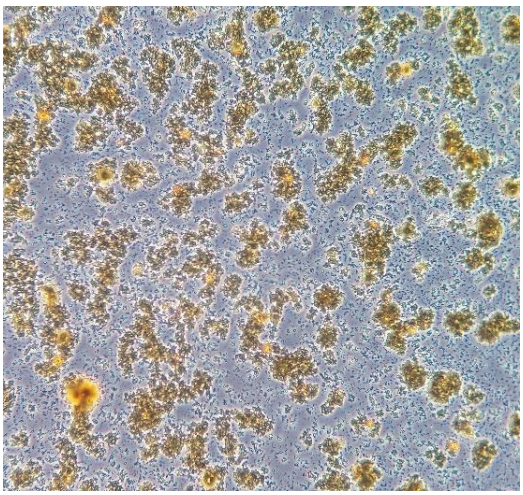

**Fig. 1.** MNPs seen at 20x with Olympus SZ-4045ESD Microscope

The coated magnetic nanoparticles were obtained *in situ* by chemical co-precipitation of $Fe^{3+}$ and $Fe^{2+}$ solutions with $NH_4OH$ solution, at pH 9–10, followed by dispersing in a solution of de-ionized water with citric acid. Briefly, 5.40 g of $FeCl_3$ x $6H_2O$ (1M) and 1.98 g of $FeCl_2$ x $4H_2O$ (2M), were dissolved in 25 mL HCl (2M) , and the resulted solutions were added to 90 mL of de-ionized water containing 3.25 g citric acid, under argon atmosphere and vigorously stirred. Further, 20 mL of $NH_4OH$ solution (5M) was added over the previous mixture and stirred for 30 min until the colour shift from orange to black indicating the formation of an iron oxide nanoparticles suspension. The suspension was heated to 80°C and stored for 2 hours followed by centrifugation for 5 minutes at 900 × g. The supernatant was eliminated and the coated nanoparticles were washed several times with water and then finally dispersed in 100 ml de-ionized water and sonicated for 3–5 min. The hydrodynamic diameters are between 20–150 nm.

### 2. Procedures

Our studies have been conducted using a group of ten Sprague–Dawley male rats of 20 weeks old and with an average weight of 400g. The animals have been raised in standard-sized cages for rats in sterile laboratory environment. All the experiments were approved by Ethics Committee of the University of Medicine and Pharmacy of Craiova, Romania, and were conducted in accordance with the European Legislation regarding animal rights, Directive 2010/63/EU.

The experimental models were anesthetized with an intraperitoneal injection using a mixture of 100 mg/kg ketamine hydrochloride (Richter, Germany) and 10 mg/kg, xylazine hydrochloride (Interchemie, Netherlands). A suspension of citric-acid coated magnetic nanoparticles (14 mg Fe/mL) was prepared in glycolyzed solution and injected slowly through the jugular vein at a dose of 15 µmol of Fe/kg. Control experiments were carried out on two rats without injecting them.

At 30 minutes after injection, blood sample were collected from rats tail vein, to be used as reference concentration in determining nanoparticle accumulation in organs. Animals were allowed to recover after the procedure and were monitored to be euthanized 24 hours after MNPs administration, with 120mg/kg pentobarbital sodium (Produlab Pharma B.V., Netherlands), intraperitoneally administered. Rats were then perfused transcardially with saline for 20 min, and various organs were collected (liver, spleen, pancreas, kidney, brain and muscle), washed thoroughly with saline, and were frozen at -80 °C [12]. These tissues samples were used for determination of magnetic nanoparticles content. Half of the samples were analyzed using electron paramagnetic resonance (EPR) following a lyophilization process [13].

The other half of the organ samples were kept overnight in a neutral buffered formalin 10% and ordinarily processed in paraffin. Sections of 3-4 µm thickness of the right lobe of the liver and spleen sections were sectioned axially and sections of the lung, heart and kidney were cut in the coronal plane. All sections were stained with Prussian Blue and counterstained with hematoxylin and eosin followed by examination under light microscopy [14,15].

## RESULTS

Magnetic nanoparticles are not delivered only to the liver, but they are also transferred to other organs of the rat [16]. Considering the concentration of nanoparticles in the sample at 30 minute after MNPs administration, as

the reference concentration, $C_{REF}$, the magnetic nanoparticles had the following organ concentrations, measured with EPR: Liver - (120 +/- 24) $C_{REF}$; Spleen - (706 +/- 141) $C_{REF}$; Kidney: (3.3 +/- 1.5) $C_{REF}$; Pancreas: (0.7 +/- 1.5) $C_{REF}$. In brain and muscle the MNP concentrations were approximately zero.

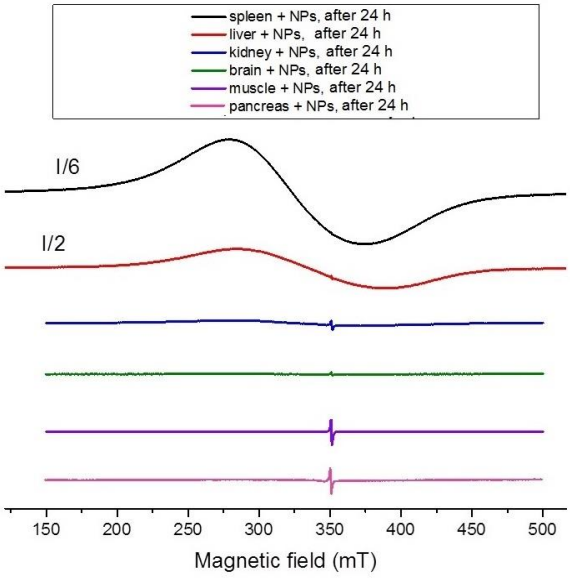


**Fig. 2.** EPR spectra at room temperature of different lyophilized rat organs 24 h after MNPs administration

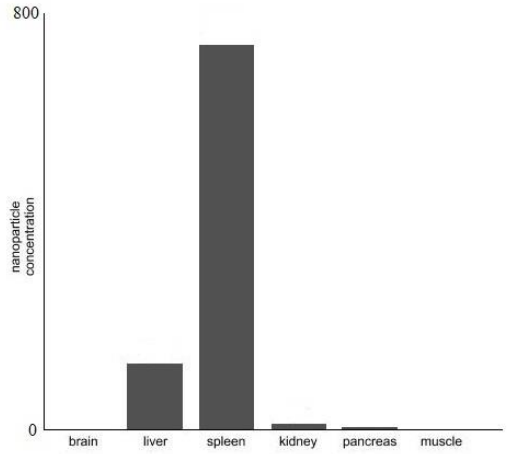


**Fig. 3**. MNP concentrations in organs 24 h after intravenous administration

Iron nanoparticles content in the spleen and liver is higher than in other organs studied (Figure 3), which is consistent with previous studies [17].

Histopathological assessment confirmed all the deposits using Prussian Blue staining (Figures 4-6). As expected, in spleen were found nanoparticle deposits in and out of the macrophages, especially in the red pulp. In the liver some deposits were noted in the periphery of the capillary sinusoids and also in Kupfer cells and Ito cells. Smaller deposits were found in the portal and centrilobular spaces. As for the kidneys, MNPs were primarily located within the proximal convoluted tubules and to a lesser extent in the distal convoluted tubules, more precisely in the epithelial cells which cover the collector tubes.

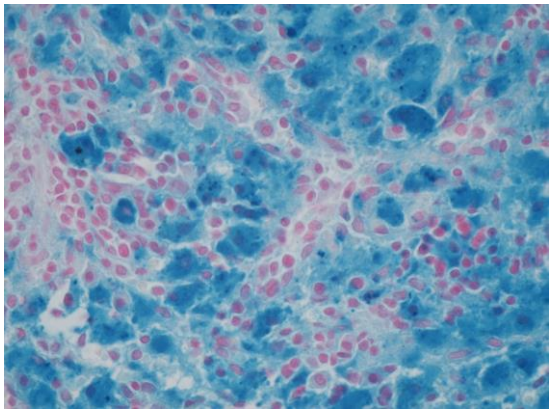

**Fig. 4.** MNP deposits within the spleen. Prussian Blue staining 40x

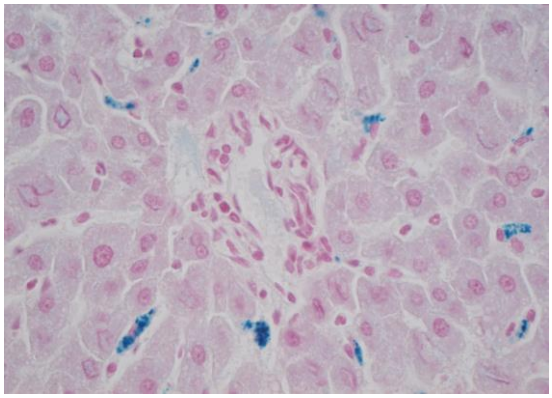

**Fig. 5.** Histological study of rats' livers sacrificed 24 hours after the intravenous administration of MNPs. Prussian Blue staining 40x

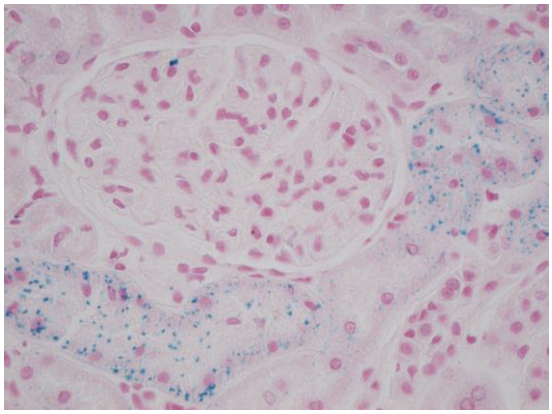

**Fig. 6.** MNP deposits within the kidney. Prussian Blue staining 40x

## DISCUSSION

This study had a few limitations because the number of rats was too small for an accurate analysis of the nanoparticles distribution and second, because due to technical challenges it could not determine the absolute concentration of MNPs but only a relative one.

Briley-Sæbø et al. reported that for nanoparticles with a diameter less than 40 nm, both the biodistribution and blood half-life of iron oxide particles are influenced by the material of the coating rather than the average particle size [18]. Our nanoparticles are coated with citric acid which contributes to the increase of the hydrodynamic diameter.

Gu *et al.* evaluated the biodistribution and degradation of PEGylated iron oxide nanocrystals in mice. The iron accumulated in the organs 24 h after intravenous injection (5 mgFe/kg) of Feridex and PEG phospholipid-coated IO nanocrystals, (5÷30 nm) confirm the results obtained in this study [19].

A more ample study was conducted by Jain el al. [20] in which they studied the distribution of iron oxide nanoparticles coated with oleic acid and pluronic in rats, after intravenous administration. From the analysis with inductively coupled plasma-mass spectrometry (ICP-MS), it was determined that about 55% of the injected nanoparticles were stored in the liver at 6 h but then declined to 20% after 24 h and raised at about 50% at 3 weeks after nanoparticle injection. An explanation of rising levels of iron in the liver at 3 weeks could be due to the intraportal transport of free iron released from biodegraded MNPs or or due to the formation of smaller particles as a result of degradation in the spleen.

Furthermore, Gamarra et al. [21] observed that dextran coated magnetite nanoparticles (80-150 nm) reach maximum concentration in liver at 95 min after the intravenous administration with the half-life of the nanoparticles in the blood of 11.6 ± 0.6 min measured by EPR.

This study highlighted that the type of iron oxide nanoparticles we used in intravenous administration in rats have been stored in the major tissues of the organism.

## CONCLUSIONS

The results suggest that MNPs, as characterized in our study, can be potentially used as contrast agent in MRI imaging, cancer therapy and drug delivery, distributing especially in spleen and liver.

A future objective of this research is to enhance the thermal ablation procedures in liver tumors through the use of computer-controlled radio-frequency ablation (RFA) needle combined with targeted intratumoral (MNP) delivery and induction of tumor hyperthermia as a novel method for ablating tumors. Although this technology holds interesting potential for the ablation of cancer, significant further research is needed.

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## BIODISTRIBUȚIA NANOPARTICULELOR DE $FE_3O_4$/ACID CITRIC ÎNTR-UN MODEL MURIN

**REZUMAT**

Nanoparticulele magnetice (MNP) de oxid de fier de aproximativ 20÷150 nm au fost sintetizate prin metoda chimică de coprecipitare a $Fe^{2+}$ și $Fe^{3+}$ în soluție de $NH_4OH$, după care acestea au fost acoperite cu acid citric.

Scopul acestui studiu *in vivo* a fost de a analiza biodistribuția MNP la nivelul diferitelor organe, după administrarea intravenoasă a MNP la șobolanii Sprague Dawley. Analiza a fost efectuată prin metoda rezonanței paramagnetice a electronilor (EPR), urmată de analiza histologică în colorația albastru de Prusia. Studiul distribuției MNP a fost efectuat la 24 de ore de la administrarea intravenoasă la nivelul venei jugulare, a unei doze de 15 µmol Fe/kg. Rezultatele au arătat o mai mare aglomerare a nanoparticulelor la nivelul splinei și ficatului, fără ca MNP să fie distribuite la nivelul rinichilor, pancreasului și plămânilor. Aceste aspecte au fost studiate utilizând o nouă formulare a oxidului de fier, care ar putea fi folosită în cercetări privind posibilitățile terapeutice ale acesteia, precum și în domeniul rezonanței magnetice nucleare (RMN), în studii viitoare.

**Cuvinte cheie:** MNP, biodistribuție, model murin

# BODY COMPOSITION ASSESSMENT IN PATIENTS WITH CHRONIC OBSTRUCTIVE PULMONARY DISEASE

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**ABSTRACT**

Chronic obstructive pulmonary disease (COPD) is a preventable and treatable disease with significant extra-pulmonary effects, which can contribute to serious complications in certain patients. The systemic component results in weight loss and muscle weakness, both of which contribute to progression of the disease and deterioration in quality of life. Malnutrition is one of the most investigated extra-pulmonary features in COPD patients. Loss of body weight and depletion of fat free mass (FFM) are common and severe risk factors for mortality in COPD.

Surrogate measures such as the well known Body Mass Index (BMI) give no indication of body composition, muscle mass or nutritional state. Different methods are used for nutritional assessments beyond BMI, such as bioelectrical impedance analysis (BIA). BIA is simple, inexpensive, quick and non-invasive technique for assessing body composition and its changes over time. Given the usefulness of BIA in determining body compartments and being a quick and simple method, its use could become generalized and constitute another instrument of measurement in COPD patients.

**Key words:** COPD, body composition, malnutrition, cachexia.

## INTRODUCTION

Chronic obstructive pulmonary disease (COPD) is a chronic inflammatory disorder of the lung with many extra-pulmonary effects, caused mainly by tobacco smoking, and is characterized by progressive, persistent airflow obstruction. There is a global increase in the number of patients suffering from COPD and the World Health Organization (WHO) estimates that COPD will become the third leading cause of death in the world until 2020. This progressive increase in the number of patients with COPD, especially in acute stages, will create a considerable medico-economic burden, therefore, preventive measure and therapies for this disorder are being assiduously pursued [1].

Loss of body weight and depletion of fat free muscle mass are common and serious problems in patients with COPD indifferent of the degree of airflow limitation and are caused by a combination of several physiological and pathophysiological alterations such as systemic inflammation, inadequate energy intake and/or increased energy expenditure [2].

Malnutrition in COPD has been associated with systemic inflammation, cachexia, anorexia, skeletal muscle dysfunction, dyspnea, reduced health status, enhanced risk of exacerbations and increased mortality [3]. Consequently, current COPD guideline recommendations by the Global Initiative for Chronic Obstructive Lung Disease (GOLD) consider nutritional monitoring an important part of routine evaluation of COPD patients [4].

Common definitions of malnutrition include weight less than 90% of predicted value as given by the Metropolitan Insurance Company, and/or body mass index (BMI) of less than 18.4 kg/m$^2$. Often the terms malnutrition and cachexia are used interchangeably and defined by the BMI but here are different states of low weight. In a large study of patients with COPD, Schols *et al.* distinguished three different types of impaired nutrition: semi-starvation (low BMI with normal or above normal fat-free mass [FFM] index), muscle atrophy (low FFM index and normal or above normal BMI) and cachexia (low BMI and low FFM index). Schols observed that patients with muscle atrophy and those with cachexia had similar outcomes. On the other hand, the groups with normal or above-normal BMI (semi-starvation or no impairment) had better survival. Thus the groups with low FFM have greater mortality than the others, and it would appear that low FFM is a better predictor of mortality than low BMI [2].

The prevalence of cachexia is presented to be between 20-40% in the COPD population [5]. The

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significance of cachexia in COPD is strongly evident in the survival curves, as shown by Schols *et al*. [6].

He observed that the median survival was cut almost in half by cachexia from 4 to 2 years. The differences in survival are not due to the differences in severity of COPD by spirometric criteria. What is not known is whether cachexia can be prevented, or even reversed, once developed, and how this will affect survival [2].

Other important consequence of cachexia relates to impaired quality of life. With fatigue and muscle weakness being even greater than for non-cachectic COPD patients, cachexia worsens the quality of life considerably.

## IMPORTANT MECHANISMS OF CACHEXIA IN COPD PATIENTS

A mechanism that would cause cachexia in patients with COPD is the energy imbalance. Due to airways obstruction, the work of breathing is increased. Sergi *et al*. noted that the resting expenditure was found to bet 10% higher in COPD patients than in normal subject of similar age, height and weight [7].

The oxygen cost of exercise has also been noted to be increased than in normal subjects [8].

In addition to this concept, it is well known that there are some fiber-type shifts in skeletal muscle in COPD from type I to type II. Muscle fiber II uses more oxygen per unit work output than type I, thus there is an extra metabolic load due to oxygen-inefficient muscle usage. Another factor is that cachexia itself leads to anorexia, and this leads to a vicious circle, such that the more weight lost, the less food is taken in. Aggressive nutritional augmentation and appetite stimulation have not solved the problem, casting some doubt on the centrality of this mechanism [9].

Another mechanism proposed for cachexia in COPD patients would be the disuse of the muscles that lead to atrophy. Patients with COPD are very inactive thus extreme inactivity may play an important role in cachexia. As with nutritional guidance, instituting a regimen of regular exercise does not cure cachexia per se, but just as with nutrition, physical activity is always encouraged [10].

Latest research has focused on systemic inflammation to be the genesis of cachexia in COPD. There is considerable evidence that this may be important, but there are many gaps in present knowledge that stand in the way of making any definitive conclusions about the role of inflammation in developing cachexia. The most important molecules that are receiving attention are TNF-α, IL-1β, IL-6, C-reactive protein, and reactive oxygen species (ROS) and reactive nitrogen species (RNS) [11].

Oxidative stress is greater in COPD patients than in controls, and the oxidised/reduced glutathione ratio is also increased. Furthermore, exercise training appears to accentuate oxidative stress in COPD. Thus, ROS and/or RNS may play a role in cachexia as molecules capable of incurring tissue damage. However, these authors found that cachectic patients were able to increase their exercise capacity after training [12,13].

In this context, it should not be forgotten that ROS can also serve as signalling molecules in the process of adaptation to exercise. It remains a puzzle whether exercise, both acute and repeated (training), is pro- or maladaptive in these patients, and whether exercise has positive or negative (or no) effect on quality of life and mortality in cachectic COPD patients.

## THE IMPACT OF OBESITY ON COPD PATIENTS

The prevalence of obesity and COPD is increasing at remarkable rate in the Western world, and this has a major negative health and economic ramifications [14].

The clinical consequences of obesity in COPD are diverse and include increased perceived activity related dyspnea, decreased quality of life, sleep disorders and elevated oxygen uptake at peak exercise. Obesity in COPD is known to be associated with reduced self-reported daily activity levels, and this, in turn, may predispose to increased risk for comorbidities, which include skeletal muscle de-conditioning, cardiovascular diseases, insulin resistance and osteoporosis. The mechanisms of activity limitation in obese COPD patients have only recently become the focus of systemic studies [15].

Jones and Nzeku have analyzed the impact of the abnormalities on lung volumes across the stages of obesity and demonstrated an inverse relationship between increasing BMI and decreasing end expiratory lung volume (EELV) and expiratory reserve volume (ERV) in healthy population. Total lung capacity (TLC) declines modestly with mild to moderate obesity [16].

Consequently, inspiratory capacity (IC) increases with higher BMI, reflecting the relative preservation of TLC in the presence of decreased EELV. The reduced EELV in obesity means that airways resistance (Raw) is increased, and expiratory flow limitation may exist during resting breathing [17,18].

The presence of diffuse small airways disease in COPD further amplifies the before mentioned association of increased BMI and higher air resistance in obesity. Although much is known about the respiratory muscles in COPD there has been no large systematic evaluation of respiratory muscle structure and function in obese patients with COPD [19].

Ora *et al.* in a physiological study of patients with COPD and mild to moderate obesity observed that there was no difference in respiratory muscle strength during rest and exercise in the obese group compared with the

normal-weight group. The former had greater static lung recoil and intra-abdominal pressures despite similar lung function impairment by forced expiratory volume in the first second ($FEV_1$) criteria. The factors that predispose to hypercapnic ventilatory failure in patients in whom COPD and morbid obesity coexist have not been systematically studied [20].

Low BMI is associated with increased COPD-related mortality, unrelated to disease severity. Recent studies indicate that the FFM index is an even more important determinant of prognosis in moderate to severe COPD than BMI. Fat mass index was not predictive for prognosis in patients with moderate to severe COPD [2,21].

Besides the presence of chronic airflow obstruction, low-grade systemic inflammation is one of the mechanisms that may be responsible for the increased rate of cardiovascular complications in COPD [22].

The role for smoking-related systemic inflammation is evident in the pathogenesis of COPD and led to the proposal of the term chronic systemic inflammatory syndrome complementary to the diagnosis of COPD [23].

In a recent meta-analysis of longitudinal studies, the presence of the metabolic syndrome was strongly associated with cardiovascular morbidity and mortality. Indeed, some studies suggest the presence of insulin resistance in COPD, especially in normal weight to obese patients and those who are hypoxaemic [24].

## BODY COMPOSITION ASSESSMENT

Surrogate measures such as the well known BMI give no indication of body composition, muscle mass or nutritional state. Thus malnutrition that requires intervention can exist although the individual has a normal BMI. These patients are usually not detected by subjective global assessment of nutritional status. In COPD patients, it has been recognized that FFM further differentiates into body cell mass (BCM) and extra cellular mass (ECM) rather than low BMI that should be considered as a critical parameter of disease severity and prognosis [25].

Monitoring changes in FMM and fat mass (FM) can improve our understanding of disease processes and energy metabolism, leading to the development of more effective nutrition and exercise intervention strategies to counteract the loss of FMM associated with factors such as malnutrition, injury, aging and certain diseases such as COPD.

There are a number of sophisticated but expensive methods that may be used to obtain reference measures of body composition, including magnetic resonance imagery, neutron activation analysis and computerized tomography. Alternatively, hydrometry, dual energy x-ray absorptiometry and densitometry are more commonly used in research settings for measures of body composition.

*Densitometry*

Densitometry refers to the measurement of total densitometry body (Db) and the estimation of body composition from Db. Db is the ratio of body mass to body volume (BV). Body volume is measured by either water displacement or air displacement. For years, the water displacement method, known as hydrodensitometry or hydrostatic weighing, has been considered by some experts as a gold standard method in light of the relatively small technical error associated with the accurate measurement of Db (0.0015 g/cc or approximately 0.7% BF). In order to achieve this degree of accuracy, total body mass, underwater weight, water temperature, and residual lung volume (RV) must be measured precisely (within 0.20 kg for body mass and underwater weight, within 0.0005 degrees Celsius (°C) for water temperature, and within 100 ml for RV). The estimated technical error associated with the RV measurement (0.00139 g/cc) is relatively large compared to the other three sources of error combined (0.0006 g/cc) [26].

*Hydrometry*

Hydrometry, or the measurement of total body water (TBW), is also limited when used singly to derive reference measures of body composition. With this method, the concentration of hydrogen isotopes (deuterium or tritium) in biological fluids (saliva, plasma, and urine) after equilibration is measured and used to estimate TBW [27].

This method assumes that the distribution and exchange of the isotope by the body are similar to the distribution and exchange of water. However, due to the exchange of the isotope with nonaqueous hydrogen in the body, TBW may be overestimated by 1 to 5%. Using this method in conjunction with the two component molecular model to obtain estimates of FFM, it is further assumed that the hydration of the FFM is constant for all individuals (~ 73% of FFM). Because TBW fluctuates widely within and among individuals depending on age, gender, level of obesity, and disease, large errors may result when hydrometry is used with the two component model to derive reference measures of body composition. Siri estimated that biological variability (2%) in the hydration of the FFB would produce a substantial error in the estimation of body fat (2.7% BF) for the general population [28].

*Dual-energy X-ray Absorptiometry*

Dual-energy x-ray absorptiometry (DXA) is a relatively new technology that is gaining recognition as a reference method for body composition research. This method is based on three compartment model that divides the body into total-body mineral, mineral-free lean, and fat tissue masses. The precision of DXA in measuring %BF is estimated to be 1.2%BF [29].

DXA is highly reliable, and there is good agreement (~0.4%BF difference) between %BF estimates obtained by hydrodensitometry (Db adjusted for relative total-body mineral and TBW) and DXA [30].

In addition to obtaining estimates of relative body fat and lean tissue mass, DXA provides segmental and regional measures of body composition. DXA is an attractive alternative to hydrodensitometry as a reference method because it is rapid (a total body scan takes 20 minutes), safe, requires minimal subject cooperation, and, most importantly, takes into account inter-individual variability in bone mineral content. Also, DXA estimates of body composition appear to be less affected by fluctuations in TBW compared to hydrodensitometry and hydrometry.

Kohrt estimated that a 5% difference in the relative hydration of the FFB (78 vs 73% FFB) would produce <0.5 kg error in fat and FFM, suggesting that hydration status has a relatively small effect on soft-tissue estimates obtained via DXA [31].

The majority of the physiologists often use to assess body composition with the methods of skinfolds, anthropometry and bioelectrical impedance analysis (BIA). Given the choice of methods and numerous prediction equations published in the literature, it is often difficult for the clinician to select an appropriate method or prediction equation that accurately assesses the body composition of each patient.

*Skinfold Method*

The skinfold (SKF) is an indirect measure of the thickness of subcutaneous adipose tissue at a specified site. Most SKF equations use two or more SKF measurements to predict either Db or %BF. The accuracy and precision of SKF measurements is highly dependent on technician skill, type of SKF caliper, and client factors. It takes a great amount of time and practice to develop skill as a SKF technician, and standardized procedures must be carefully followed [32].

*Anthropometry*

Anthropometry refers to the measurement of the size and proportions of the human body. Anthropometric prediction equations estimate Db, %BF, or FFM from combinations of body mass, standing height, skeletal diameters, and circumference measures. Compared to SKF measures, these anthropometric techniques are relatively simple, inexpensive, and require less skill and training. The accuracy and precision of anthropometric measures, however, are affected by technician skill and client factors. Circumferences are preferable to SKFs when measuring obese clients for a number of reasons. Regardless of size, circumferences of obese individuals can be measured, whereas, the SKF thickness may exceed the maximum aperture of the caliper, and circumferences require less technician skill, and the difference between technicians is smaller compared to SKF measurements [33].

*Bioelectrical impedance analysis*

The bioelectrical impedance analysis is a commonly used method for estimating body composition. It measures the impedance or resistance to a small electrical current as it travels through the body`s water pool. An estimate of total body water (TBW) is acquired from which total body FFM is calculated using the assumption that 73% of the body`s FFM is water. The resistance to current flow will be greater in individuals with large accounts of body fat given that adipose tissue is a poor conductor of electrical current due to its relatively low water content [34].

This body composition assessment method is simple, inexpensive, quick and non-invasive technique for assessing body composition and its changes over time. BIA is largely used in clinical trial settings and there are a whole series researches regarding the theory and methodology of several different BIA techniques. However, there is a considerable lack of information on the practical aspects of BIA for those primarily interested in learning how to apply this method in practice [35].

Thus, BIA still is an underused and underestimated tool for nutritional assessment in primary care. This can be further explained by the fact that the costs of BIA are currently not always refundable and that there are no guidelines outlining the methods for assessing malnutrition in patients with COPD [36].

In order to ensure the accuracy of the method, the patients must strictly follow each of the BIA testing guidelines. In addition, standardized testing procedures must be followed. Compared to the skinfold method BIA analysis does not require a high degree of technical skill, it is more comfortable and less intrusive for the client and it can be used to estimate body composition of obese individuals [37].

**BIA client guidelines:**

- Urinate within 30 minutes of the test
- No exercise within 12 hours of the test
- No eating or drinking within 4 hours of the test
- No diuretic medications within 7 days of the test
- No alcohol consumption within 48 hours of the test
- No testing of the female clients who perceive they are retaining water during the stage of the menstrual cycle.

The principle of BIA is to determine the electric impedance of an electric current passing through the body [38].

The electrical impedance (Z) consists of two components, resistance (R) and reactance (Xc). Reactance is a measure of body cell mass (BCM) and resistance a measure of TBW. From the determined impedance a number of BIA parameters can be estimated [39].

The BIA analysis determines the following parameters [40]:

- BCM that consists of all cells that have an effect on metabolism (e.g. nervous system, internal organs, muscle); this analysis is the most important when assessing the nutritional status of the patient.
- FFM that represents everything that is not body fat, it consists of BCM and ECM; it is decreased in elderly people and chronic diseases. The Fat mass is indirectly determined from the body weight minus FFM.
- TBW which is elevated in high portion of muscle and water retention such as oedema, and is decreased in situations such as dehydration.
- ECM is mainly extracellular water; it is increased due to extracellular water retention such as oedema and decreased due to water loss e.g. diuretics.

The BIA results are influenced by the following factors [41]:

- Individual characteristics (e.g. skin temperature, race, age, sex)
- Medical conditions and medication that have an impact on the fluid and electrolyte balance; infection and cutaneous disease that may alter the electrical transmission between electrode and skin
- Environmental conditions (e.g. ambient temperature)
- Non-adherence of electrodes, use of wrong electrodes, loosing of cable clip, interchanging of electrodes
- High and weight (should be measured directly by the investigator)
- Position of the body and limbs
- Moderate to intense level of physical activity/exercise before BIA measurements (last exercise at least 12 hours previously)
- Consumption of food and beverages (at least 12 hours previously, fasted state for at least 2 hours)

It has been demonstrated that BIA is a useful body composition measurement in patients with COPD [42]. FFM assessed by BIA is an important factor in these patients in determining maximum exercise performance, along with other pulmonary function parameters [43].

Other study has described that in patients with COPD with acute respiratory failure, BIA estimates nutritional status better than anthropometric parameters or plasma levels of visceral proteins. The marked inflation of the extracellular compartment (extra-/intracellular water volume ratio) that is shown in the BIA analysis in the acute hospitalized COPD patients presumably leads to inaccurate anthropometric result(e.g. overestimation of triceps skinfold thickness and fat mass, and inaccurate measurement of middle-arm muscle circumference). In out-patients with COPD, body composition estimated by BIA analysis presented good reliability and correlation with DXA and the two methods presented satisfactory clinical accuracy [44].

Abbatecola *et al.* observed in a study that investigated the correlations of lean and FM on walking speed and gait speed in patients with COPD that FM was an independent determinant for slower walking speed, while lean mass was not [45]. The importance of mobility limitation using different walking speed distances has been shown to be important even if not attributable to specific disease states in older persons [46].

A community dwelling cohort study of older persons suggested that the absolute amount of FM was negatively associated with physical performance, while lean mass is not significant in absolute terms [47].

A study found that the lean-to-fat ration was associated with greater walking distance in younger men, but not in women with COPD. The same authors showed that higher measures of total adiposity (BMI) and central adiposity were related to functional limitation in both genders [37].

Some authors examined the idea that BIA can predict clinical outcomes, such as mortality and length of hospital stay. An issue that has received little attention is whether the standard procedure for predicting such outcomes using estimates of body composition made using impedance and anthropometry is superior using anthropometry (weight + height) alone or body composition calculated from simple anthropometry alone.

Kyle *et al.* applied a BIA equation (with R, Xc, W and H as predictive variables), derived from 343 healthy subjects whose body composition was assessed with DXA to a wide range of hospitalized patients, including those with cardiovascular disease and COPD. BMI was found to be associated with prolonged length of hospital stay. The study also reported that both low FFM and FM significantly predicted length of stay [48].

In a series of studies involving groups of patients with COPD and a study of mixed groups of patients, it was found that there were variable associations between the FFMI (or lean/fat mass ratio), determined by whole-body BIA and anthropometry, and clinically relevant outcome measures, such as length of hospital stay, mortality and lung function tests. However, when FFMI was not associated with significant relationships, BMI was also not associated with significant relationships. When FFMI was associated with significant relationships, BMI was generally also associated with significant relationships. An exception is a COPD study in which some of the outcomes (dyspnea and lung function tests $FEV_1$% predicted and FEV1/forced vital capacity) were significantly related to FFMI and not BMI [25].

## CONCLUSIONS

Bioelectrical impedance analysis is non-invasive, relatively inexpensive, does not expose to ionizing radiation, has very limited variations, can be performed in almost any subject and provides a viable opportunity for evaluating body composition in COPD patients.

Our understanding of the mechanism underlying malnutrition and subsequent muscle dysfunction in COPD patients has increased significantly. In addition to BMI, determination of FFM is crucial to adequately detect malnutrition in patients with COPD.

There is a high prevalence of malnutrition in hospitalized COPD patients. Given the usefulness of BIA in determining body compartments and being a quick and simple method, its use could become generalized and constitute another instrument of measurement in COPD patients. There is a relationship between $FEV_1$ and the distance covered in the six minutes walking test, number of hospitalization day and readmissions and the FFM index. Furthermore, low FFM index measured by BIA is an independent predictor of mortality in COPD patients.

Further validation of BIA is necessary to understand the mechanisms for the changes in acute illness, extreme heights and body shape abnormalities.

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## MĂSURAREA COMPOZIȚIEI CORPORALE LA PACIENȚII CU BPOC

**REZUMAT**

Bronhopneumopatia obstructivă cronică (BPOC) este o boală ce poate fi prevenită şi tratată, cu efecte semnificative extrapulmonare, care pot contribui la complicaţii semnificative la anumiţi pacienţi. Componenta sistemică rezultă în pierderea în greutatem corporală, slăbiciune musculară, amândouă putând contribui la progresia bolii şi la scăderea calităţii vieţii. Malnutriţia este una din cele mai investigate caracteristici extrapulmonare la pacienţii cu BPOC. Pierderea greutăţii corporale şi depleţia masei fără grăsimi sunt factori de risc comuni şi severi pentru mortalitatea în BPOC.

Măsurători surogate cum ar fi bine cunoscutul indice de masă corporală (IMC) nu dau indicaţii în privinţa compoziţiei corporale, masei musculare sau a stării nutriţionale. Diferite metode sunt utilizate pentru evaluarea nutriţională dincolo de IMC, cum ar fi analiza de impedanţă bioelectrică (BIA). BIA este simplă, ieftină, având o tehnică rapidă şi non-invazivă de evaluare a compoziţiei corporale şi a schimbărilor în timp. Având în vedere utilitatea BIA în determinarea diferitelor compartimente corporale şi fiind o metodă simplă şi rapidă, utilizarea acestuia ar putea deveni generalizată şi constituie încă un instrument de măsurare a compoziţiei corporale la pacienţii cu BPOC.

**Cuvinte cheie:** BPOC, compoziția corporală, malnutriție, cașexie

# THE EFFECTS OF TWO LIPID-LOWERING DRUGS ON MITOCHONDRIAL RESPIRATION IN H9C2 CELL LINE

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**ABSTRACT**
Pravastatin (PR), a HMG-CoA reductase inhibitor, and nicotinic acid (NA), a water-soluble vitamin B, are widely prescribed lipid-modifying drugs. Controversy exists about the effects of these agents on mitochondrial function. The present study was purported to characterize the acute effects of PR and NA (10 μM) on mitochondrial respiration in H9c2 cardiomyoblasts. Mitochondrial respiration of permeabilized cells was assessed by high-resolution respirometry using the Oxygraph-2k in the presence of complex I and complex II substrates (glutamate/malate and succinate) and, of the successive addition of the electron transport chain inhibitors: rotenone, oligomycin, FCCP, and antimycin A, respectively. Our preliminary data show an inhibitory effect on mitochondrial respiration in the presence of pravastatin, whereas nicotinic acid stimulated respiration in H9c2 cardiomyoblasts.
**Keywords:** pravastatin, nicotinic acid, mitochondrial respiration, H9c2 cardiomyoblasts

## INTRODUCTION

In the 21st century the persistence of a high prevalence of cardiovascular diseases worldwide is driven by the dual burden of obesity and diabetes, both conditions being largely associated with dys- or hyperlipidemia. According to guidelines, statins represent the most widely prescribed hypocholesterolemiant drugs for both primary and secondary prevention of cardio-metabolic disorders [1] and are currently accepted as a safe therapy view their pleiotropic beneficial effects [2, 3]. However, statins have also been associated with skeletal muscle symptoms that may progress to toxic myopathy [4] and require the association of non-statin therapies in order to attain the recommended cholesterol levels [5]. Among the mechanisms proposed to underlie statins-induced myopathy an important contributor is represented by the impairment of mitochondrial function [6, 7].

Pravastatin (PR), one of the firstly developed hydrophilic statins, was found already back to 1998 to accelerate the age-dependent impairment of the electron transport chain complex I function in rat muscle cells [8]. However, later studies reported that PR was less myotoxic as compared to the lipophilic statins both in a rat skeletal muscle cell line and after *in vivo* administration in rats [9, 10]. Recently, Bonifacio et al. showed that simvastatin is toxic to the H9c2 rodent cardiomyocyte cell line by decreasing mitochondrial respiration, the activity of enzyme complexes I and IV of the respiratory chain, and the ATP content [11]. Whether PR interferes with mitochondrial function in cardiomyocytes it is not known.

Nicotinic acid (niacin) is one of the oldest known anti-atherosclerotic water-soluble drug, mainly indicated for its HDL cholesterol-elevating effect, the reduction of lipoprotein *a* (whose level is not improved by statins) along with its anti-lipolytic properties; similarly to statins, lipid independent-beneficial effects (anti-inflammatory, vasculo-protective, immuno-modulatory) have also been described [12]. Interestingly, a recent study demonstrated that NA increased the total cellular respiratory capacity in fibroblast cell lines [13].

In line with the above mentioned data, the aim of the present study was to assess the effects of PR and NA on mitochondrial respiration in H9c2 cardiomyoblasts.

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## MATERIALS AND METHODS

### Reagents

All chemicals were supplied by Sigma-Aldrich.

### Cell culture

The rat cardiomyoblast H9c2 cell line was purchased from the European Collection of Cell Cultures. Cells were grown in Dulbecco's modified Eagles high-glucose medium (4.5g/l) containing 1 mM sodium pyruvate, 5 mM HEPES, 10 % fetal bovine serum (FBS), and 100 U/ml penicillin-streptomycin. Cells were kept in 5% $CO_2$ at 37°C and grown for 4 days to achieve the optimum confluence.

### Experiment protocol

#### *1. Preparation of permeabilized cell line H9C2*

2 ml cell suspension (containing 1 million cells/ml) in MIR05 buffer was added to each O-2K chamber. Digitonin (8.1 µM final concentration) was used to permeabilize cell membranes in the respiratory chamber (5 minutes of incubation) before adding the substrates and then the inhibitors [16-18]. Data are expressed in pmol $O_2$ $s^{-1}mg^{-1}$.

#### *2. Measurement of OXPHOS*

Mitochondrial respiration was measured at 37°C by high-resolution respirometry using the Oxygraph-2K (Oroboros Instruments, Austria) with 2 chambers, each containing the respiration buffer MIR05 (0.5 mM EGTA, 1 g/l BSA, 3 mM Mg Cl2 6H2O, 20 mM taurine, 10 mM KH2PO4, 110 mM sucrose, 60 mM K- lactobionate, and 20 mM HEPES, pH 7.1). Respiratory rates were measured in the presence of complex I (glutamate/malate) or complex II (succinate) substrates according to the Substrate-Uncoupler-Inhibitor Titration (SUIT) protocol described by Gnaiger [14] and adapted from Duicu et al [15]. Addition of CI/CII-substrates is a step referred as basal respiration (the classical state 2) whereas addition of ADP to activate oxidative phosphorylation (OXPHOS) is referred to as the active respiration (the classical state 3) or the OXPHOS state. After measuring the basal and the active respiration, oligomycin (an inhibitor of ATP synthase) was added in order to induce the LEAK state, followed by the titration of the classic uncoupler FCCP (0.5 µM steps) in order to obtain the maximum mitochondrial respiration or the electron transfer system (ETS) capacity. The integrity of the outer mitochondrial membrane was assessed by addition of cytochrome c. Respiratory control ratio (RCR) was calculated as the ratio between the OXPHOS capacity (state 3) and the LEAK flux (state 2) [16, 17].

### Statistical Analysis

All values are presented as mean ± SEM. Group comparisons were performed by one-way analysis of variance (ANOVA) and Dunnett multiple comparison test (GraphPad Prism version 5.0). Values for $p < 0.05$ were considered statistically significant.

## RESULTS AND DISCUSSION

Statin therapy, albeit being the cornerstone of treatment in conditions associated with dys- and hyperlipidemia, has been unequivocally related to mitochondrial distress. Since mitochondria are mostly abundant in tissues with high energy demand (such as the myocardial tissue), cardiomyocytes should be firstly affected. Paradoxically, opposite effects of lipophilic statins (simvastatin, atorvastatin) on skeletal and cardiac muscle (albeit both striated muscles) have been reported in the literature, with mitochondrial impairment of skeletal muscle whereas mitochondrial respiratory function in the heart was either not affected or increased [20, 21]. With reference to pravastatin, it has also been reported that chronic administration (40 mg/kg/day) of pravastatin for 3 weeks in mice induced marked alterations of skeletal muscle mitochondria while the ultrastructure of the heart in the treated animals appeared normal [22].

In the present study the classic parameters of mitochondrial respiration were assessed in order to characterize the acute effects of two lipid-modifying drugs, PR and NA (10 µM) in H9c2 cardiomyoblasts. In particular, the following respiratory parameters were measured: State 2 (basal respiration) measured after the addition of complex I (glutamate/malate) or complex II substrate/inhibitor (succinate/rotenone), OXPHOS state (ADP-stimulated respiration), LEAK state, and ETS (the maximal mitochondrial respiratory capacity), respectively - whereas the RCR was calculated.

Our preliminary data show that, in cells respiring on complex I dependent-substrates (glutamate/malate), PR (but not NA) elicited a generalized reduction in all respiratory parameters (Fig. 1). Thus, both basal (State 2) and active (OXPHOS) respiratory rates were significantly decreased by PR (**$p < 0.01$ *vs.* Ctrl), leading to an obvious decrease in the RCR (Fig. 1). Similarly, both ETS (**$p < 0.01$) and the LEAK state (*$p < 0.05$) displayed low values when cells were incubated with PR as compared to the non-treated controls (Fig. 1). The measurement of ETS by progressively adding FCCP reflects how a system reacts to an increased ATP demand. A diminished maximal respiration is the case of cells treated with PR, suggests the risk for an energetic crisis.

At variance, an improved cellular respiratory capacity was obtained when cells were incubated with the same

concentration of NA. The most important increase was found for the basal respiration (***$p$ < 0.001) but also active and uncoupled respiration were higher than the ones of the non-treated cells respiring on glutamate/malate (**$p$ < 0.01 vs. Ctrl) - Figure 1.

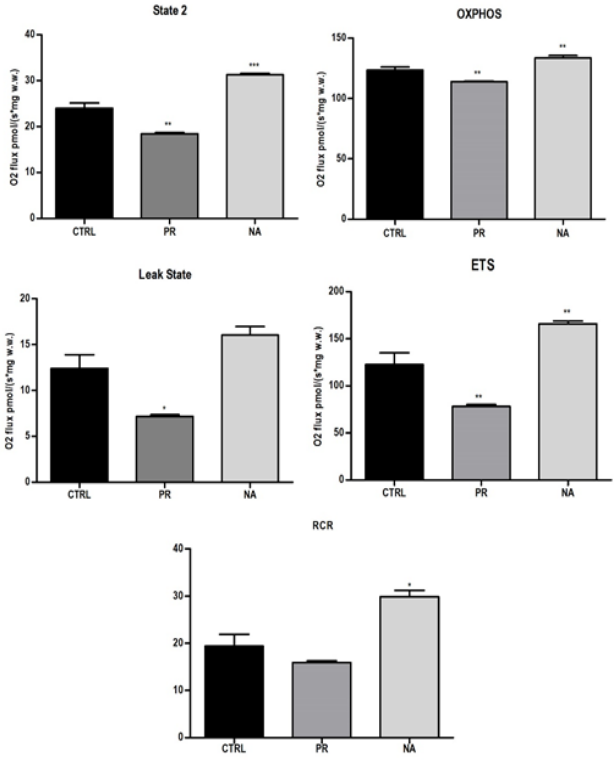


**Fig. 1. Respiratory rates and RCR in cells treated with PR ad NA in presence of Complex I-dependent substrates.**
(*$p$ < 0.05, **$p$ < 0.01, ***$p$ < 0.001 vs Ctrl.)

Interestingly, the effects of the lipid-lowering drugs on the active and uncoupled respiration, respectively, were completely very different for complex II-supported respiration as compared to complex I. Accordingly, an important decrease in both OXPHOS and ETS in cells respiring on succinate (plus rotenone to prevent the retrograde flow of electrons toward complex I) was recorded when myoblasts were incubated with the statin (PR) while no effect was elicited in the presence of niacin (Figure 2).

It is known that NA plays an important role in the electron transport chain via the conversion in nicotinamide adenine dinucleotide to form the reduced dinucleotide that in turn plays a vital role in providing the reducing equivalents to fuel the oxidative phosphorylation [24]. We can speculate that the changes elicited by NA in complex I-supported respiration I (Fig. 1) are related to the fact that NADH is the substrate for Complex I (and has no effect on Complex II-supported respiration, which has as substrate FADH) - Fig. 2.

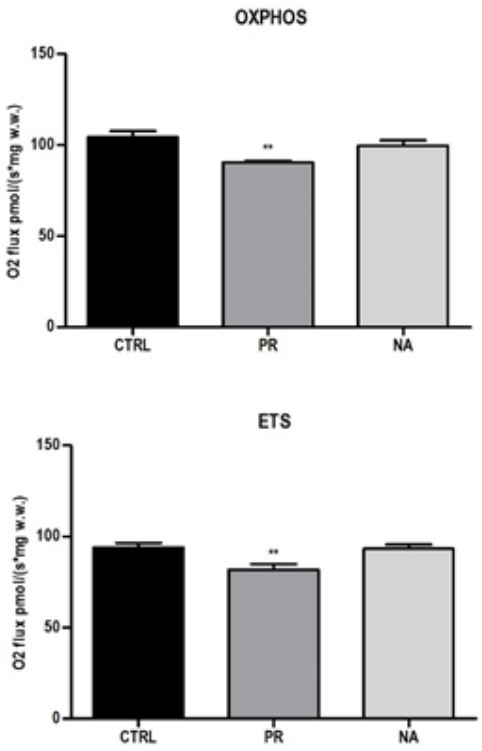


**Fig. 2. Respiratory rates in cells treated with PR ad NA in presence of Complex II-dependent substrates.**
(**$p$ < 0.01 vs Ctrl.)

## CONCLUSION

In conclusion, in H9c2 cells, our preliminary data show an inhibitory effect of a low dose of pravastatin on complex I-dependent mitochondrial respiration whereas nicotinic acid exerts a stimulatory effect in the same conditions. Moreover, PR also decreased oxidative phosphorylation in the presence of complex II substrate whereas NA did not interfere with succinate-supported cellular respiration. Further experiments are required to elucidate whether these results are dose dependent and common for other liposoluble statins.


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## EFECTELE A DOUĂ MEDICAMENTE HIPOLOPEMIANTE ASUPRA RESPIRAȚIEI MITOCONDRIALE LA NIVELUL LINIEI CELULARE H9C2

**REZUMAT**

Pravastatinul (PR), un inhibitor al HMG-CoA reductazei și acidul nicotinic (AN), o vitamină B hidrosolubilă, sunt prescrise pe scară largă ca medicamente cu rol reglator al lipidelor circulante. Efectele acestor compuși la nivelul funcției mitocondriale sunt controversate. Scopul prezentului studiu a fost de a caracteriza efectele acute ale PR și AN (10 μM) asupra respirației mitocondriale la nivelul cardiomioblaștilor H9c2. Respirația mitocondrială la nivelul celulelor permeabilizate a fost realizată prin tehnica respirometriei de înaltă rezoluție cu ajutorul oxigrafului-2k (Oroboros Instr., Austria) în prezența substratelor complexului I și II ale lanțului respirator (glutamat/malat, și succinat) și respectiv, a adiției succesive a inhibitorilor clasici ai complexelor acestuia: rotenonă, oligomicină, FCCP și antimicina A. Rezultatele preliminare arată un efect inhibitor asupra respirației mitocondriale în cazul pravastatinului, în timp ce acidul nicotinic a stimulat respirația cardiomioblaștilor H2c9.

**Cuvinte cheie**: pravastatin, acid nicotinic, cardiomioblași H2c9, respiratia mitocondrială

# PULMONARY EMBOLISM: TO BE OR NOT TO BE CASE REPORT

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**ABSTRACT**

**Background.** Pulmonary artery angiosarcoma are rare to find and diagnosis is a great challenge for the clinician, especially before surgery, due to the very unspecific signs.

**Case presentation.** We present the case of a 62 years old male who was admitted in hospital for evaluation of moderate dyspnea that progressively aggravated in the last 3 months, with progressive alteration of the general status. On admission in the Timisoara Cardiovascular Diseases Institute, in September 2014, the examination revealed an overweight patient (BMI= 27 kg/m² BM), with minor central cyanosis. The patient also presented varicose veins on both lower limbs, with moderate peripheral edema, but without signs of post-thrombotic syndrome. Hepatomegaly 5 cm below the right costal margins and hepatojugular reflux were noticed. On pulmonary auscultation, the patient had diminished vesicular murmur on the level of the inferior left hemithorax, without any rales and with a spontaneous arterial oxygen saturation of 90%. Cardiac examination showed rhythmic heart sounds, with no murmurs, with a ventricular rate of 92 beats/minute and an arterial blood pressure of 110/80 mmHg. Transthoracic echocardiography revealed a left ventricle with volumetric dimensions in normal range, conserved ejection fraction, and no kinetic abnormalities, while the right ventricle was enlarged. Doppler continuous flow investigation showed severe secondary pulmonary hypertension. Angiocoronarographic evaluation showed coronary arteries without significant angiographic lesions. Right heart catheterization revealed increased pressures in the right ventricle and also in the pulmonary artery, with increased vascular pulmonary resistance, but with no left to right shunts. First conclusion was that the patient has post-embolic secondary pulmonary hypertension, with repeated pulmonary embolism episodes, with indication to undergo pulmonary thromboendarterectomy. The surgical team found, on the level of the right pulmonary artery, a large tumor mass, almost occlusive, with a friable macroscopic aspect, a mass that infiltrated the vascular wall and was also present at the level of the left pulmonary artery, while the macroscopic aspect of the tumor mass was suggestive for a pulmonary artery sarcoma.

**Conclusion.** The differential diagnosis of pulmonary vascular diseases are a great challenge for the clinician. Due to the non-specific symptoms, the first diagnosis taken into account is of pulmonary embolism, conducting to thromboendarterectomy, or even surgery. In case of tumors, the prognosis is poor and survival is of approximately one year after surgical tumor mass removal.

**Key words:** tumor mass, thromboendarterectomy, pulmonary embolism, pulmonary hypertension

## BACKGROUND

Pulmonary artery angiosarcoma are rare to find and diagnosis is a great challenge for the clinician, especially before surgery, due to the very unspecific signs.

## CASE PRESENTATION

We present the case of a 62 years old male, admitted for clinical evaluation in Timisoara Cardiovascular Diseases Institute in September 2014. The patient presented moderate dyspnea that progressively aggravated in the last 3 months, with progressive alteration of the general status of the patient.

The patient, a former smoker until 12 years before admission, first presented an episode of hemoptysis in August 2013. At that time, he underwent a pulmonary computer tomography examination, that revealed multiple intra-arterial pulmonary thrombosis and the diagnosis of pulmonary embolism was established (Figure 1). Since September 2013 the patient was under anticoagulant

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treatment with acenocumarol, with an INR usually in therapeutic range (with a value between 2 and 3). However, he presented two more hemoptysis episodes, one in December 2013 and the last one in April 2014.

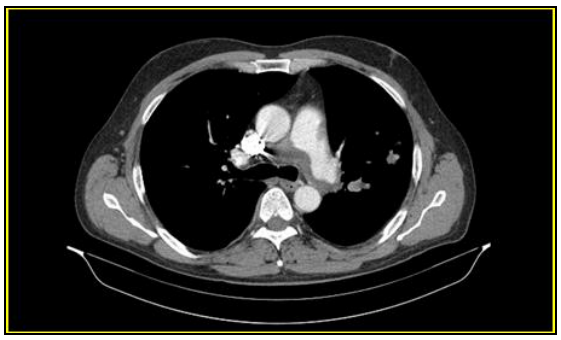

**Fig. 1.** Computer Tomographic acquisition showing a lacunary image on the level of the arterial pulmonary trunk crossing

On admission in the Timisoara Cardiovascular Diseases Institute, in September 2014, the examination revealed an overweight patient (BMI= 27 kg/m² BM), with minor central cyanosis. The patient also presented varicose veins on both lower limbs, with moderate peripheral edema, but without signs of postthrombotic syndrome. Hepatomegaly 5 cm below the right costal margins and hepatojugular reflux were noticed. On pulmonary auscultation, the patient had diminished vesicular murmur on the level of the inferior left hemithorax, without any rales and with a spontaneous arterial oxygen saturation of 90%. Cardiac examination showed rhythmic heart sounds, with no murmurs, with a ventricular rate of 92 beats/minute and an arterial blood pressure of 110/80 mmHg.

Biological samples were in normal range, with an INR of 2.45 and the EKG of the patient showed a sinus rhythm, VA=72 bpm, QRS axis+ 40 degrees, without any repolarization abnormal features. We completed the evaluation of the patient with a transthoracic echocardiography that revealed a left ventricle with volumetric dimensions in normal range (end-diastolic volume of 110 ml), with conserved ejection fraction (EF=50-55%, planimetric determination) and no kinetic abnormalities. However, the right ventricle was enlarged, with a diameter in parasternal long axis view of 4.6 mm. Using Doppler continuous flow, a gradient of 78 mm Hg on the level of the tricuspid valve was observed, with the calculation of a systolic pressure in the pulmonary artery of 85 mmHg (severe secondary pulmonary hypertension). Still, the systolic function of tight ventricle was not altered (TAPSE=28 mm). The inferior vena cava was dilated (7 mm), with inspiratory collapse and the acceleration time measured while superimposing pulsed Doppler flow on the lever of the pulmonary valve was shortened (60 msec).

The evaluation of the patient continued with a peripheral lower limbs veins Doppler echocardiography that showed no signs of intravascular thrombosis, with a normal venous flow. Spirometric evaluation of the patient revealed normal parameters of respiratory function.

The next investigation was an invasive angiocoronarographic evaluation that evidenced coronary arteries without significant angiographic lesions. Right heart catheterization revealed increased pressures in the right ventricle and also in the pulmonary artery, with increased vascular pulmonary resistance, but with no left to right shunts.

At this point of the patient evaluation, taking into consideration the clinical manifestations and also the results of the paraclinical ones, we concluded that we were in front of a patient with progressively altered postembolic secondary pulmonary hypertension, with repeated pulmonary embolism episodes, a patient that, in our opinion had the indication to undergo pulmonary thromboendarterectomy.

Subsequently, September the 5th 2014, a bilateral pulmonary artery thromboendarterectomy, in profound hypothermia was performed. To our surprise, the operating team found, on the level of the right pulmonary artery, a large tumor mass, almost occlusive, with a friable macroscopic aspect, a mass that infiltrated the vascular wall. The tumor mass was also present on the level of the left pulmonary artery (Figure 3) and the macroscopic aspect of the tumor mass was suggestive for a pulmonary artery sarcoma. However, the final diagnosis was to be established by the histopathological examination.

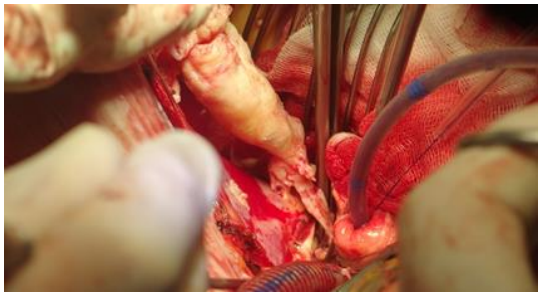

**Fig. 2.** Right pulmonary artery mass removal

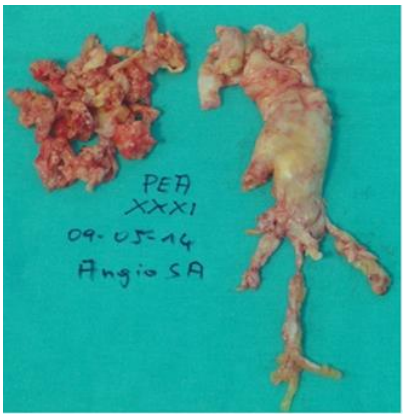


**Fig. 3.** Bilateral pulmonary artery tumor mass

In postoperatory care, the evolution of the patient was favourable, and the transthoracic ecocardiography performed one day after surgery revealed dilated right heart cavities but with a value of the pulmonary artery systolic pressure of 44 mmHg.

Unfortunatelly, the result of the histopathological exam confirmed our suspicion, and diagnosed a malignant mesenchymal tumor proliferation (taken into account chondrosarcoma, mixoid chondrosarcoma or osteosarcoma. For further histological differentiation, immunohistochemical tests would have been useful, but were unfortunatelly unavailable.

When discharged, approximately two weeks after surgery, the patient was on anticoagulant treatement with acenocumarol, diuretic and calcium channel blocker (Diltiazem 3x60 mg/day). He was further refered for oncological treatment and survey, and was unfortunately lost to our follow-up.

## DISCUSSION

The differential diagnosis of pulmonary vascular diseases are a great challenge for the clinician. Out of those, the neoplasms of arterial pulmonary trunc are very rare diseases, with an incidence of 0,001% up to 0,03% in the general population [1].

Due to the non-specific symptoms (dyspnoea, cough, hemoptysis, thoracic pain), the first diagnosis taken into account is the one of pulmonary embolism [2,6].

In most cases, the diagnosis is established while conducting further investigations previous to thromboendarterectomy, or even during surgery (as was our case) [3].

The prognosis is poor, given the fact that, despite their reduced metastatic proliferation, survival after diagnosis is for only a few months, that is 2 months without surgery and approximately one year after surgical tumor mass removal [4,5].

## CONSENT

Written informed consent was obtained from the patient for publication of this case report and any accompanying images. A copy of the written consent is available for review by the Editor-in-Chief of this journal.

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## EMBOLISMUL PULMONAR: A FI SAU A NU FI


**REZUMAT**

**Introducere.** Angiosarcomul arterei pulmonare este o tumoră rar întâlnită și diagnosticată și reprezintă o adevărată provocare pentru clinician, în special înainte de intervenția chirurgicală, datorită semnelor extrem de nespecifice ale acesteia.

**Prezentarea cazului.** Prezentăm cazul unui bărbat de 62 de ani, care a fost internat în spital pentru evaluarea dispneei moderate, care s-a agravat progresiv în ultimele 3 luni, cu alterarea progresivă și a statusului general al pacientului. La internarea în Institutul de Boli Cardiovasculare Timisoara, în septembrie 2014, examenul fizic a arătat că pacientul este supraponderal (BMI= 27 kg/m² BM) și prezenta cianoză centrală minoră. Pacientul avea de asemenea varice venoase la nivelul ambelor membre inferioare, cu edem periferic moderat, dar fără semne de sindrom post-trombotic. Au fost remarcate de asemenea hepatomegalia la 5 cm sub nivelul marginii costale

drepte și refluxul heptojugular. La auscultația plmonară, pacientul prezenta murmur vezicular diminuat la nivelul hemitoracelui ingerior stâng, fără raluri și cu saturație arterială spontană a oxigenului de 90%. Examinarea cardiacă a arătat zgomote cardiace ritmice, fără murmur, cu o frecvență ventriculară de 92 bătăi/min. și tensiune arterială de 110/80 mmHg. Ecocardiografia transtoracică a arătat un ventricul stâng cu dimensiuni volumetrice în limite normale, fracție de ejecție păstrată și fără anomalii kinetice, chiar dacă ventriculul drept era mărit. Investigația Doppler cu flux continuu a arătat hipertensiune pulmonară secundară severă. Evaluarea angiocoronarografică a arătat că arterele coronare nu prezentau leziuni semnificative. Cateterizarea inimii drepte a arătat presiuni crescute la nivelul ventriculului drept și arterei pulmonare, cu o creștere a rezistenței vasculare pulmonare, dar fără existența șunturilor stânga-dreapta. Prima concluzie a fost că pacientul prezintă hipertensiune pulmonară secundară post-embolică, cu episoade repetate de embolism pulmonar, având indicația de trombendarterectomie pulmonară. Echipa chirurgicală a găsit la nivelul arterei pulmonare drepte o masă tumorală mare, aproape ocluzivă, cu un aspect macroscopic friabil, o masă care infiltra peretele vascular și era prezentă și la nivelul arterei pulmonare stângi, aspectul macroscopic al tumorii fiind sugestiv pentru sarcom al arterei pulmonare.

**Concluzii.** Diagnosticul diferențial al bolilor vasculare pulmonare reprezintă o provocare pentru clinicieni. Datorită simptomelor nespecifice, primul diagnostic considerat este cel de embolism pulmonar, având indicație de trombendarterectomie sau chiar de intervenție chirurgicală. In cazul tumorilor, prognosticul este rezervat, iar rata de supraviețuire este de aproximativ 1 an după îndepărtarea chirurgicală a masei tumorale.

**Cuvinte cheie:** masă tumorală, trombendarterectomie, embolism pulmonar, hipertensiune