UNIVERSITATEA DE MEDICINĂ ȘI FARMACIE
“VICTOR BABEȘ” TIMIȘOARA
FACULTATEA DE MEDICINĂ GENERALĂ

TEZĂ DE ABILITARE

CONTRIBUȚII LA APLICAREA METODELOR HISTOCHIMICE ȘI IMUNOHISTOCHIMICE ÎN PROCEDURILE DIAGNOSTICE ȘI CU POTENȚIAL IMPACT TERAPEUTIC

Domeniul MEDICINĂ

OVIDIU ALEXANDRU MEDERLE

TIMIȘOARA
2019

CUPRINS

A. REZUMATUL TEZEI DE ABILITARE

Subsemnatul, **MEDERLE Ovidiu Alexandru**, Doctor în Științe Medicale, domeniul Medicină, Conferențiar la disciplina Histologie si Citologie, din cadrul Facultății de Medicină Timișoara, candidat la obținerea titlului științific de **Doctor abilitat** (dr. habil.), prezint teza de abilitare intitulată *Contribuții la aplicarea metodelor histochimice și imunohistochimice în procedurile diagnostice și cu potențial impact terapeutic.*

Sunt cadru didactic al Facultății de Medicină, din cadrul UMF „Victor Babeș" Timișoara, începând cu anul 1994, când am debutat ca preparator. Ulterior, am ocupat, prin concurs, posturile de asistent și șef de lucrări. În prezent, activez pe postul didactic de Conferențiar. Cei 25 de ani de carieră didactică universitară i-am dedicat disciplinelor de Histologie și Urgențe medico-chirurgicale. Sunt doctor în Științe Medicale, domeniul Medicină, prin Ordinul Ministrului Educaţiei Naţionale nr.4090/1988. Sunt absolvent al Facultății de Medicină, din cadrul UMF „Victor Babeș" Timișoara, promoția 1991, cu media generală de licență 10.

Rezultatele activității de cercetare, desfășurată din 1994 până în prezent, se regăsesc în finalizarea tezei de doctorat, publicarea unui număr de 115 lucrări științifice în reviste cotate ISI, indexate în baze de date internaționale sau prezentate la conferințe și simpozioane naționale și internaționale, trei cereri de brevet de invenție înregistrate la OSIM, două contracte de cercetare în calitate de director și de un produs lansat pe piața comercială, componenta principală a unui kit destinat tratării unei ectoparazitoze. Indicele Hirsh: Google Scolar 8, Web of Science 6 și Scopus 6.

Studiile derulate în perioada elaborării tezei de doctorat intitulată "Cercetări morfohistochimice şi imunohistochimice pe puncţii biopsii în hepatopatia cronică postvirală" au reprezentat startul activității de cercetare care a continuat și după finalizarea tezei.

Direcțiile principale ale activității de cercetare postdoctorale se concretizează în: aplicarea metodelor histochimice și imunohistochimice în procedurile diagnostice și cu potențial impact terapeutic în neoplasme, studii experimentale, boli cronice (osteoporoza, fibroza pulmonară) și în câteva afecțiuni particulare (actinomicoza, hipersensibilitatea

pulmonară); aplicarea metodelor histochimice și imunohistochimice în procedurile diagnostice și cu potențial impact terapeutic în boli cu etiologie parazitară și infecțioasă.

Aplicarea metodelor histochimice și imunohistochimice în procedurile diagnostice și cu potențial impact terapeutic au fost studiate în diferite neoplasme: carcinom scuamo-celular al capului și gâtului, cancer ovarian, prostatic, colic si hepatic. Am folosit următorii markeri imonohistochimici: E-caderina, VEGF clona VG1, CD34, chromogranina, PSA, keratina 8,18, vimentina, VEGFmRNA, ki67.

1. Boia, S; Boia, ER; Ceausu, RA; Balica, CN; **Mederle, OA** - HPV18 Associated with E-cadherin Expression in Head and Neck Squamous Cell CarcinomaREV. CHIM, Volume: 69, Issue: 8 Pages 2638-2641, 2018, ISSN: 0034-7752, IF=1,412.

2. Pirtea Laurentiu, Dorin Grigoras, Cristina Secosan, Ioan Sas, Razvan Ilina, Adriana Andreea Jitariu, **Ovidiu Alexandru Mederle** - Clinical and histopathological parameters correlate with microvessel density but not with Vascular Endothelial Growth Factor expression in ovarian cancer, REV.CHIM. (Bucharest), 69, No. 5, 2018, Pages: 1173-1178 ISSN: 0034-7752, IF=1,232.

3. Bocan Elena Viorica, **O. Mederle**, Simona Sârb, R. Minciu, D. Agapie, M. Raica - Correlation between histopathological form and the degree of neuroendocrine differentiations in prostate cancer- Rom J Morphol Embryol 2011, 52(4):1215–1218 ISSN: 1220-0522, IF=0,523.

4. Ceausu Amalia Raluca, Alexandru Ciolofan, Anca Maria Cimpean, Adina Magheti, **Ovidiu Mederle**, Marius Raica- The Mesenchymal–Epithelial and Epithelial–Mesenchymal Cellular Plasticity of Liver Metastases with Digestive Origin-ANTICANCER RESEARCH 38: 811-816 (2018)doi:10.21873/anticanres.11xxxISSN: 0250-7005, IF=1,937.

5. Elena V. Bocan, **Ovidiu Mederle**, Marius Raica Immunohistochemistry of prostate specific antigen in advanced stage prostate carcinoma Rev Arg de Anat Clin; 2010, 2 (3):106-111.

6. Balica Amalia Raluca, Anca Maria Cimpean, Andreea Cioca, Octavian Cretu, **Ovidiu Mederle**, Alexandru Ciolofan, Pusa Gaje, Marius Raica - Endothelial Cell Proliferation and Vascular Endothelial Growth Factor Expression in Primary Colorectal Cancer and

Corresponding Liver Metastases, Asian Pacific journal of cancer prevention: APJCP Volume: 16 Issue: 11 Pages: 4549-53, 2015.

Pe modele experimentale, am studiat și am testat efectele anticorpilor anti-podoplaninei și cromolinei disodice asupra tumorilor derivate din linia celulară BHK21/C13-fibrosarcom dezvoltate pe membrana chorio-allantoidă a embrionului de pui (CAM). Imunofenotipul fibrosarcomului derivat din fibroblaste BHK-21 / C13 demonstrează că aceste fibroblaste reprezintă o linie celulară specială cu fenotipul vimentin + / CD34- / CD117 + / PROX1 + / podoplanin- / EGFR +, sugerând un comportament foarte agresiv bazat pe mai multe particularități moleculare, descrise anterior pentru această linie celulară. Răspunsul eterogen la cromolina disodică, bevacizumab și anti- podoplanina sprijină utilizarea acestor celule pentru evaluarea viitoare a altor terapii noi.

1. Cimpean Anca Maria, Dusan Lalošević, Vesna Lalošević, Pavle Banović, Marius Raica, **Ovidiu Alexandru Mederle**- Disodium Cromolyn and Anti-podoplanin Antibodies Strongly Inhibit Growth of BHK 21/C13-derived Fibrosarcoma in a Chick Embryo Chorioallantoic Membrane Model- In vi vol 32: 791-798 (2018) ISSN: 0258-851X, IF=0,953.

2. Osakwe, H; Nicolescu, C; Nicolescu, L; Hoinoiu, B; **Mederle, O**; Mussuto, E; Popoiu, C; Boia, E -The Impact of Residual Bowel After Extended Bowel Resection on Bacterial Overgrowth and Bacterial Translocation, REV.CHIM, Volume: 69 Issue: 8 Pages: 2121-2128, 2018, ISSN: 0034-7752, IF=1,412

Osteoporoza este cea mai comună boală metabolică a oaselor. Patogenia osteoporozei este complexă și multifactorială, caracterizată prin scăderea densității minerale osoase (procesul decalcificării osoase focale) și prin deteriorarea microarhitecturii osoase (cavități de resorbție profundă, delimitate de lamelele osoase mai subțiri, cu zone cu rezistență redusă, microfracturi osoase). Osteoclastele se dezvoltă din precursorii liniei celulare monocitare-macrofage mononucleare după stimularea cu factorul de stimulare a coloniilor macrofage (M-CSF) și a receptorului factor nuclear activat pentru ligandul kappa (RANKL). Activatorul receptorului factorului nuclear-KB (RANK) este un membru al familiei factorului de necroză tumorală exprimat de osteoclaste și precursorii lor. Osteoprotegerina (OPG) aparține familiei receptorilor de factor de necroză tumorală (familia TNFR) și inhibă diferențierea și fuziunea

celulelor precursoare osteoclastice și blochează activarea osteoclastelor mature. Am urmărit evoluția RANKL în cazul osteoporozei postmenopauzale, marker al creșterii resorbției osoase. OPG este moderat crescut în cazul osteoporozei și este un marker al formării osoase, stimulând turnover-ul osos. Aspectul fibrelor reticulare nu este luat în considerare în clasificările actuale ale fibrozei pulmonare. Analiza cazurilor incluse în studiu a evidențiat mai multe aspecte: depleția fibrelor reticulare este în mod constant asociată cu stadiile avansate de fibroză; această schimbare majoră poate explica ireversibilitatea procesului fibrotic și absența eficacității tratamentului asupra recuperării funcției pulmonare.

1. Gurban, Camelia Vidica; **Mederle, O**. The OPG/RANKL system and zinc ions are promoters of bone remodeling by osteoblast proliferation in postmenopausal osteoporosis, ROMANIAN Journal Of Morphology And Embryology, Volume: 52 Issue: 3 Supplement: S 2011 Pages: 1113-1119, 2011, ISSN: 1220-0522 IF=0,523

2. **Mederle, OA**; Balas, M; Ioanoviciu, SD; Gurban, CV; Tudor, A; Borza, C. - Correlations between bone turnover markers, serum magnesium and bone mass density in postmenopausal osteoporosis, Clinical Interventions In Aging, Volume: 13 Pages: 1383-1389, 2018, DOI: 10.2147/CIA.S170111

3. Djeska, IS; Ceausu, RA; Gaje, PN; Cimpean, AM**; Mederle, O**; Nicodin, A; Tudorache, V; Raica, M - The Reticular Network Contributes To The Staging Of Idiopathic Lung Fibrosis, Archives Of Biological Sciences, Volume: 65, Issue: 4 Pages: 1599-1604, 2013, DOI: 10.2298/ABS1304599D.

Actinomicoza abdominală continuă să fie o tulburare greu diagnosticată datorită cursului insidios, a simptomelor nespecifice și a markerilor de laborator. Atât investigațiile moderne de imagistică (CT, RMN cu contrast), cât și examinarea histochimică a probelor de biopsie, ajută la stabilirea diagnosticului de actinomicoză abdominală. Terapia cu antibiotice combinată cu intervenția chirurgicală reprezintă o alternativă promițătoare pentru tratarea pacienților cu actinomicoză abdominală.

1. Gurban Camelia Vidita, Florina-Maria Andrica, Cosmin Citu, Elena Hogea, Iosif Marincu, Marioara Cornianu, **Ovidiu Alexandru Mederle**- Combined Therapies in Abdominal Actinomycosis, REV.CHIM., 67, No.1, 2016, Pages: 171-173 ISSN: 0034-7752, IF=1,232

2. Prodea, M; Boia, ER; Ceausu, RA; Librimir, C; Iovanescu, G; **Mederle, OA**- Lung Delayed Hypersensitivity A case with particular features, REV. CHIM, Volume: 69, Issue: 8 Pages: 2071-2073, 2018, ISSN: 0034-7752, IF=1,412

O direcție principală de activitate este concentrată în sfera acariozelor (demodicoza), a zoonozelor parazitare (criptosporidioză, toxoplasmoză, giardioză, tricofiție, cheiletieloză), microsporidiozelor (nosemoză) și a bacteriozelor (infecția cu *Lawsonia spp., Salmonella spp*.).

Ectoparazitoză ce afectează pielea omului și a animalelor și determină apariția leziunilor depilante, alopecice, eritematoase, care, deseori pot atrage complicații bacteriene sau micotice, demodicoza este produsă de specii ale genului *Demodex*, acarieni care parazitează în foliculii pilosebacei.

Demonstrarea transmiterii speciei *Demodex canis* la om a reprezentat scopul studiilor preliminare realizate în 2007, respectiv, 2014. A fost realizat un studiu în care s-a urmărit importanța examenelor complementare care pot fi un indicator paraclinic important în diagnosticul demodicozei (valorile serice ale proteinelor totale, albuminelor, globulinelor totale și imunoglobulinelor G, M, A, E). Prelevarea prin puncție/biopsie a pielii parazitate cu acarianul *Demodex* și realizarea de preparate histologice și imunohistochimice au oferit, pe de o parte, o confirmare a diagnosticului clinic și microscopic și, pe de altă parte, o imagine a reacțiilor tisulare și imunohistochimice, determinate în pielea câinilor, de prezența acarianului. Efectuarea de studii multicentrice, cu implicarea Universității de Medicină și Farmacie „Victor Babeș" Timișoara, a disciplinei de Parazitologie a FMV Timișoara, SC PRIMOSAL București, Institutul de Cercetare - INCDT, București au condus la realizarea, formularea, caracterizarea și testarea pe animalele parazitate a unui gel constituit din extracte naturale, cu absorbție cutanată rapidă, eficient în remisia leziunilor demodecice și fără efect iritant asupra pielii parazitate. Rezultatul cercetărilor este înregistrat la OSIM ca cerere de brevet – A 00075 din 1.02.2016 (Patent no 131619-A0).

1. Gartner Andreea, **Mederle O**., Mederle Narcisa, **2014**, *Demodex Folliculorum* and *D. Brevis*, a cause of facial dermatitis and blepharitis, J. Of Biotech., 0168-1656, 185S, S100.

2. Mederle N., Dărăbuș Gh., Oprescu I., Morariu S., Ilie M., Indre D., **Mederle O**., **2010,** Diagnosis of Canine Demodicosis, Scientia Parasitologica, 11 (1), 20-23, ISSN: 1582-1366.

3. Mederle Narcisa, **Mederle, O**., **2017**, Evaluation of Serum Values of Total Protein (PT), Albumin, Total Globulin and Immunoglobulin (Ig) G, A, M, And E in Canine Demodicosis. Lucrări Științifice- Universitatea De Științe Agricole a Banatului Timișoara, Medicină Veterinară, 50, 1:125-129;

4. Negrescu Adina, **Mederle O.**, Milovanov Cornelia, Ahmadi Mirela, Darabus Gh., Morariu S., Mederle Narcisa, **2017**, The diagnostic value of hematology and blood biochemistry in piodemodicosis, New Front. Chem. Former: Ann. West Univ. Timișoara – Series Chem. Volume 26, Number 2 ISSN: 1224-9513.

5. Milovanov C., Mederle N, Ahmadi-Khoie M, Herman V, **Mederle O A**, Morariu F, Morariu S, Popescu G, Radulov I., Hair-And Nails-Regenerative Composition, Patent Number(S): Ro131851-A0

6. Radbea Narcisa, Dărăbuş Gh, Oprescu I., Ilie M., Nicola Alina, **Mederle O., 2005**, Aspecte histopatologice în demodicoza generalizată la câine, Sci. Parasitol. Vol. 6, 1-2, 100-103.

7. Radbea, N.; **Mederle, O**.; Dărăbuş, G.; Oprescu, I.; Morariu, S.; Ilie, M., **2006**, Immunohistochemical results in canine piodemodicosis, Lucrări Ştiinţifice Medicină Veterinară, USAMV "Ion Ionescu de la Brad" Iași, 49, 8, 404-406.

8. Mederle Narcisa; **Mederle, O**., Raica, M., **2007**, Assessment of the *Demodex Folliculorum* in human cutaneous lesions, Virchows Archiv, 451: 449-450.

9. Horablaga A., Ahmadi-Khoie M., Horablaga N. M., Mederle N., **Mederle O.** A., Milovanov C., Morariu F., Morariu S., Popescu G., Moisturizing body and face cream based on *Oenothera Biennis* comprises mixture of natural oils of evening star, Patent Number(S): Ro132235-A0

10. Mederle N, Dărăbuș G, Ilie M S, **Mederle O,** Morariu F, Morariu S, Negrescu I A, Oprescu I, Gel for treatment of dry wounds in canine Demodicosis gel consists, in mass percentage, of 25% honey, Patent Number(S): RO131619-A0

11. Mederle, Narcisa; Marin, S; Marin, MM; Danila, E; **Mederle, O**; Kaya, MGA; Ghica, AV,**2016**, Innovative Biomaterials Based on Collagen-Hydroxyapatite and

Doxycycline for Bone Regeneration, Advances In Materials Science And Engineering, Article Number: 3452171, DOI: 10.1155/2016/3452171.

Studiile epidemiologice, clinice, terapeutice și de biologie moleculară în nosemoza albinelor susțin o altă direcție de cercetare postdoctorală.

1. Mederle Narcisa, Dărăbuș Gh., Morariu S., **Mederle O.,** Herman V., Oprescu I., Ilie M., Imre Mirela, Motoc Marilena, Chiș Codruța, **2018**, Therapeutic efficacy testing of plant dietary supplement used for prevention and control of nosemosis in bees, Conference of Life Sciences, USABMV Timișoara, Epub Ahead of Print.

2. Mederle Narcisa, Balint, A., Morariu, S., Hora, F.Ş., **Mederle, O**., Marincu, I., Dărăbuș, Gh., **2015**, Research on the prevalence of honey bees nosemosis in Arad County, J. of Biotechnology, 208, S5-S 120.

3. Mederle Narcisa, Maria Luisa Lobo, Sorin Morariu, Florica Morariu, Gheorghe Darabus, **Ovidiu Mederle**, Olga Matos - Microscopic and Molecular Detection of Nosema ceranae in Honeybee Apis mellifera L. from Romania Status on pathogen worldwide distribution, REV.CHIM.(Bucharest), 69, No.12, 2018, ISSN: 0034-7752, IF=1,412

Importanța socială a protozoarelor (*Criptosporidium spp., Giardia intestinalis, Toxoplasma gondi*), a dermatofiților (*Tricophyton spp*.) și a unor acarieni (*Cheyletiella spp*.) rezidă din caracterul zoonotic al acestor endo și ectoparaziți, expresiile clinice și lezionale la om având un mare impact în viața acestora. Contribuții la stabilirea diagnosticului și instituirea unui protocol terapeutic integrat planului de control parazitologic au fost remarcate pe o perioadă de zece ani și s-au materializat în diferite publicații științifice indexate ISI și BDI.

1. Mederle, N; Darabus, G; Oprescu, I; Morariu, S; Hora, FS; Lighezan, R; **Mederle, O** - Identification of potential zoonotic parasitic elements in parks and playgrounds for children in Timisoara, Revista Romana de Medicina Veterinara, Volume: 26 Issue: 4 Pages: 23-26, 2016

2. Darabus, G; Olariu, RT; **Mederle, O**; Mederle, N.; Hotea, I; Sorescu, D; Imre, K; Imre, M - Zoonotic protozoosis in Romania (giardiasis, cryptosporidiosis, toxoplasmosis, sarcocystosis): epidemiological aspects, Revista Romana de Medicina

Veterinara,Volume: 26 Issue: 4 Pages: 47-53,Published: 2016.

3. Vieira, PM; Mederle, N; Lobo, ML; Imre, K; **Mederle, O**; Xiao, LH; Darabus, G; Matos, O - Molecular characterisation of Cryptosporidium (Apicomplexa) in children and cattle in Romania, Folia Parasitologica,Volume: 62, Article Number: 002, 2015, DOI: 10.14411/fp.2015.002

4. Dărăbuş, Gh., **Mederle, O**., Popovici, E.D., Imre, K., Ilie, M., Oprescu, I., Morariu, S., Mederle Narcisa, Hotea Ionela, Olariu, R.O., Baditoiu Luminita Mirela *Prevalenţa criptosporidiozei la animale şi oameni în vestul României* – date preliminare Rev. Rom. de Parazitologie, Vol.XVIII, supliment, 2008 ISSN 1221-1796 (Google Scholar)

5. Florin Ș. Hora, Gheorghe Dărăbuș, Corina Badea, **Ovidiu Mederle**, Narcisa Mederle, *Epidemiological, clinical and therapeutic aspects of the infestation with Trichophyton spp*. (Fungi: Eurotiomycetes: Arthrodermataceae), 2015, Scientia Parasitologica 16 (1-2), 28-32.

6. Gh. Dărăbuş, **O. Mederle**, Narcisa Mederle, T.R. Olariu ,I.Oprescu, S.Morariu, K. Imre, M. Ilie, Ionela Hotea *The study of some biochemical parameters in cryptosporidium experimental infection in broiler chickens* Lucrări ştiintifice Medicină veterinară vol. XL, 2008, Timişoara 344

7. Imre, K., **Mederle, O**.; Mederle Narcisa.; Ilie, M. S.; Hotea, I.; Imre, M.; Indre, D.; Balint, A.; Sorescu, D. *Molecular characterization of human Cryptosporidium isolates in Banat region*, Romania Scientific Works - Series C, Veterinary Medicine, Bucharest(ISSN 1222-5304, 56, 1, 91-96, 2010, CABI

8. Mederle N., Dărăbuş G., Oprescu I., Morariu S., Ilie M., Imre K., Hotea I., **Mederle O.**, 2008 - *Correlation between histological, serological and epidemiological investigations in human toxoplasmosis*, Lucrări Științifice Medicină Veterinară Timișoara, 41, 356-360, ISSN 1221-5295. Recenzată şi indexată: CAB Internațional, England, categorie B+, cod CNCSIS 259;

9. Mederle Narcisa, Dărăbuş, Gh., Ionela Hotea, **Mederle, O.** *Surse şi modalităţi de infestare în toxoplasmoză* Rev. Rom.Parazitologie, vol. XVII, supliment, 2007(Google Scholar)

10. Mederle, Narcisa.; Darabus, Gh.; Oprescu, I.; Morariu, S.; **Mederle, O.** *Epidemiological research in canine dermatophytoses* Revista Scientia Parasitologica ,(ISSN: 1582-1366, 7, 3-4, 2006, CABI)

11. Gartner Andreea Ionela, Mederle Narcisa Geanina, Darabus Gheorghe, Marincu Iosif, **Mederle Ovidiu** Alexandru - *A case report of Cheyletiella blakei infestation in an asymptomatic cat and skin lesions of her owner Journal of Biotechnology* 2016 231 Supplement S107

12. Mederle Narcisa, **Mederle A. O**., Darabus Gh., Gartner Andreea, Ioanoviciu S.- Immuno-fluorescence and immuno-histochemistry methods in diagnose of human cryptosporidiosis, Journal of Biotechnology, 0168 - 165 6, 256, 2017 https://www.sciencedirect.com

Implicațiile speciilor bacteriene din genul *Salmonella*, *Lawsonia* sau *Escherichia* în etiologia complexului enteric suin și rolul acestora în dezvoltarea proceselor morbide au fost urmărite în studii histologice și imunohistochimice realizate pe fragmente de ileon și jejun cu adenomatoză.

Anca Sofiana Surpat (Hulea), Viorel Herman, Iosif Marincu, Narcisa Mederle, **Ovidiu Alexandru Mederle,** *Immunohistochemical method for identification of Lawsonia intracellularis infection in pigs*, Journal of Biotechnology, Volume 208, Supplement, 20 August 2015, Pages S100.

Sunt coautor a trei cereri de brevet de invenție:

- Gelul pentru tratarea leziunilor uscate din demodicoza canină. Patent nr A 00075/1.02.2016. Autori Mederle Narcisa, **Mederle Ovidiu**, Morariu Sorin, Morariu Florica, Darabus Gheorghe, Oprescu Ion, Ilie Marius, Negrescu Adina.

- Compoziție regeneratoare pentru păr și unghii. Patent nr A 00621/25.11.2016. Autori Milovanov Cornelia, Mederle Narcisa, Ahmadi Mirela, Morariu Sorin, Popescu Gabriela, Morariu Florica, Herman Viorel, Radulov Isidora, **Mederle Ovidiu.**

- Cremă pentru hidratantă piele și ten pe bază de Oenothera biennis. Patent nr A 000269/08.05. 2017. Autori Horablaga Adina, Milovanov Cornelia, Ahmadi Mirela,

Mederle Narcisa, Morariu Sorin, Morariu Florica, Horablaga Marinel, Popescu Gabriela, **Mederle Ovidiu.**

De-a lungul carierei universitare au fost depuse propuneri de proiecte în calitate de director sau responsabil, dintre care s-au materializat două.

1. Efectul degranulantelor mastocitare şi al inhibitorilor degranulării asupra angiogenezei tumorale - CNCSIS A/ 759/2006 - 2006-2007.

2. Lanţuri epidemiologice posibile şi modalităţi de control în criptosporidioza la animale şi om - PN II 51-034/2007 - 2008-2010

Realizările profesionale și academice sunt reliefate prin descrierea competențelor didactice, activitatea publicistică (trei cărți prim autor și zece, coautor), coordonarea lucrărilor de licență și prin implicarea doctoranzilor în echipa de cercetare a proiectelor pe care le-am directorat, dar și implicarea acestora în derularea studiilor de cercetare care susțin obținerea celor trei cereri de brevet de invenție, precum și în derularea experimentelor și în interpretarea rezultatelor concretizate în publicarea de lucrări științifice, iar această colaborare a continuat și după finalizarea tezei de doctorat.

Am participat și am organizat împreună cu studenții și doctoranzii, diferite evenimente științifice: Simpozionul Științific internațional dedicat tinerilor cercetători, Simpozionul Științific Internațional FMVT, EuroConferința de Zoonoze Parazitare Timișoara, Conferința Internațională Balkan Fungus, Saloanele Naționale de Invenții: Pro Invent Cluj Napoca, „Traian Vuia" Timișoara, EuroInvent Iași. Am îndrumat studenţi pentru realizarea de lucrări la sesiunile de comunicări studenţeşti: Medis, 1997, 1998, 2003. În 2018 și 2019 am susținut, în cadrul sesiunii științifice studențești MEDIS, workshop-urile cu tema ALS și BLS. În 2018, am susținut în cadrul HEART, workshop-ul cu tema FAST in trauma. În 2019, am coordonat în cadrul GALMED, șase workshop-uri.

Activitatea medicală începe în anul 1991, ca medic stagiar la Spitalul Județean - Secția Urologie. Urmează o perioadă în care am lucrat ca medic în localitățile Seleuș și Pâncota, județul Arad. Între 1994 și 1996, am activat ca medic rezident (Medicină De Urgență), iar din anul 1996, mi-am exercitat profesia ca medic specialist, Medicină de urgență, la Spitalul Clinic Municipal Timișoara, unde, din anul 2001, până în prezent, sunt medic primar, Medicină de urgență.

Recunoașterea profesională, științifică și academică sunt argumentate prin apartenența la comisii și asociații profesionale și științifice, prin organizarea de evenimente științifice și cursuri postuniversitare și prin premiile naționale și internaționale, dintre care, cele mai reprezentative sunt medaliile de aur și argint obținute la Saloanele de Invenții și inovații Geneva și Barcelona.

Planul de dezvoltare al carierei profesionale, științifice și academice este inclus în ultima parte a tezei și conține diferite componente viitoare ale activității didactice, de cercetare științifică și medicală.

A. SUMMARY OF THE HABILITATION THESIS

Ovidiu Alexandru **Mederle**, M.D., Ph D., Associate Professor at the Discipline Histology and Cytology, University of Medicine and Farmacy 'Victor Babes' Timisoara, candidate for the scientific title of Dr. Habil., I'll present the empowerment thesis entitled *Contributions to the application of histochemical and immunohistochemical methods in diagnostic procedures and with potential therapeutic impact.*

I have been teaching at the Faculty of Medicine of the "Victor Babes" since 1994, when I started as an Assistant Professor. Later, I worked as a Lecturer, being currently the Associate Professor. The 25 years of University teaching I've dedicated to the disciplines of Histology and Medical-Surgical Emergencies. I am a Doctor in Medical Sciences, Medicine, by Order of the Minister of National Education no.4090 / 1988. I am a graduate of the University of Medicine at UMF "Victor Babeş" Timişoara, 1991, with the general average of 10.

The results of the research activity carried out since 1994 to date include the completion of the PhD thesis, the publication of 115 Scientific Papers in ISI-rated journals, indexed in international databases or presented at national and international conferences and symposiums, three applications registered patent at OSIM, two research contracts as director and a product launched on the commercial market, the main component of a kit intended to treat an ectoparasite. The Hirsh Index: Google School 8, Web of Science 6 and Scopus 6.

The studies carried out during the elaboration of the Ph.D. Thesis entitled "*Morphohistochemical and immunohistochemical researches on biopsy puncture in postviral chronic hepatopathy*" represented the beginning of the research activity that continued after the thesis was finalized.

The main directions of the postdoctoral research activity are: the application of histochemical and immunohistochemical methods in diagnostic procedures and with potential therapeutic impact in neoplasms, experimental studies, chronic diseases (osteoporosis, pulmonary fibrosis) and in some particular conditions (actinomycosis, pulmonary hypersensitivity); application of histochemical and immunohistochemical methods in diagnostic procedures and potential therapeutic impact in diseases with parasitic and infectious etiology.

The application of histochemical and immunohistochemical methods in diagnostic procedures and with potential therapeutic impact has been studied in various neoplasms: squamous cell carcinoma of the head and neck, ovarian, prostatic, colonic and hepatic cancer. We used several immunohistochemical markers: E-cadherin, VEGF clone VG1, CD34, chromogranin, PSA, keratin 8,18, vimentin, VEGFmRNA, ki67.

1. Boia, S; Boia, ER; Ceausu, RA; Balica, CN; **Mederle, OA** - HPV18 Associated with E-cadherin Expression in Head and Neck Squamous Cell CarcinomaREV. CHIM, Volume: 69, Issue: 8 Pages 2638-2641, 2018, ISSN: 0034-7752, IF=1,412.

2. Pirtea Laurentiu, Dorin Grigoras, Cristina Secosan, Ioan Sas, Razvan Ilina, Adriana Andreea Jitariu, **Ovidiu Alexandru Mederle** - Clinical and Histopathological Parameters Correlate with Microvessel Density but Not with Vascular Endothelial Growth Factor Expression in Ovarian Cancer, REV.CHIM. (Bucharest), 69, No. 5, 2018, Pages: 1173-1178 ISSN: 0034-7752, IF=1,232.

3. Bocan Elena Viorica, **O. Mederle**, Simona Sârb, R. Minciu, D. Agapie, M. Raica - Correlation between histopathological form and the degree of neuroendocrine differentiations in prostate cancer- Rom J Morphol Embryol 2011, 52(4):1215–1218 ISSN: 1220-0522, IF=0,523.

4. Ceausu Amalia Raluca, Alexandru Ciolofan, Anca Maria Cimpean, Adina Magheti, **Ovidiu Mederle**, Marius Raica- The Mesenchymal–Epithelial and Epithelial–Mesenchymal Cellular Plasticity of Liver Metastases with Digestive Origin- ANTICANCER RESEARCH 38: 811-816 (2018)doi:10.21873/anticanres.11xxxISSN: 0250-7005, IF=1,937.

5. Elena V. Bocan, **Ovidiu Mederle**, Marius Raica Immunohistochemistry of prostate specific antigen in advanced stage prostate carcinoma Rev Arg de Anat Clin; 2010, 2 (3):106-111.

6. Balica Amalia Raluca, Anca Maria Cimpean, Andreea Cioca, Octavian Cretu, **Ovidiu Mederle**, Alexandru Ciolofan, Pusa Gaje, Marius Raica- Endothelial Cell Proliferation and Vascular Endothelial Growth Factor Expression in Primary Colorectal Cancer and Corresponding Liver Metastases, Asian Pacific journal of cancer prevention: APJCP Volume: 16 Issue: 11 Pages: 4549-53, 2015.

In experimental models, we studied and tested the effects of anti-podoplanin and disodium chromium antibodies on BHK21 / C13-fibrosarcoma-derived tumors developed on chicken embryo chorio-allantoic membrane (CAM). BHK-21 / C13 fibroblast fibroblast immunophenotype demonstrates that these fibroblasts are a special cell line with the vimentin + / CD34- / CD117 + / PROX1 + / podoplanin- / EGFR + phenotype, suggesting very aggressive behavior based on several molecular particularities, previously described for this cell line. The heterogeneous response to disodium cromolyn, bevacizumab and anti-podoplanin supports the use of these cells for the future evaluation of other new therapies.

1. Cimpean Anca Maria, Dusan Lalošević, Vesna Lalošević, Pavle Banović, Marius Raica, **Ovidiu Alexandru Mederle**- Disodium Cromolyn and Anti-podoplanin Antibodies Strongly Inhibit Growth of BHK 21/C13-derived Fibrosarcoma in a Chick Embryo Chorioallantois Membrane Model- In vi vol 32: 791-798 (2018) ISSN: 0258-851X, IF=0,953.

2. Osakwe, H; Nicolescu, C; Nicolescu, L; Hoinoiu, B; **Mederle, O**; Mussuto, E; Popoiu, C; Boia, E -The Impact of Residual Bowel After Extended Bowel Resection on Bacterial Overgrowth and Bacterial Translocation, REV.CHIM, Volume: 69 Issue: 8 Pages: 2121-2128, 2018, ISSN: 0034-7752, IF=1,412

Osteoporosis is the most common metabolic bone disease. The pathogenesis of osteoporosis is complex and multifactorial, characterized by lower bone mineral density (focal bone decalcification) and deterioration of bone microarhitectomy (deep resorption cavities, delimited by thinner bones, low-resistance areas, bone microfractures). Osteoclasts develop from the mononuclear macrophage cell line precursors after stimulation with macrophage colony stimulating factor (M-CSF) and activated kappa ligand receptor (RANKL). The nuclear factor-KB receptor (RANK) receptor activator is a member of the tumor necrosis factor family expressed by osteoclasts and their precursors. Osteoprotegerin (OPG) belongs to the tumor necrosis factor receptor family (TNFR family) and inhibits the differentiation and fusion of osteoclast precursor cells and blocks the activation of mature osteoclasts. We followed the RANKL progression in postmenopausal osteoporosis, a marker of bone resorption growth. OPG is moderately elevated in osteoporosis and is a marker of bone formation, stimulating bone turnover. Appearance of crosslinked fibers is not considered in current classifications of

pulmonary fibrosis. The analysis of the cases included in the study highlighted several aspects: cross-linking fiber depletion is consistently associated with advanced stages of fibrosis; this major change can explain the irreversibility of the fibrotic process and the absence of treatment efficacy on lung function recovery.

1. Gurban, Camelia Vidica; **Mederle, O**. The OPG/RANKL system and zinc ions are promoters of bone remodeling by osteoblast proliferation in postmenopausal osteoporosis, ROMANIAN Journal of Morphology and Embryology, Volume: 52 Issue: 3 Supplement: S 2011 Pages: 1113-1119, 2011, ISSN: 1220-0522 IF=0,523

2. **Mederle, OA**; Balas, M; Ioanoviciu, SD; Gurban, CV; Tudor, A; Borza, C. - Correlations between bone turnover markers, serum magnesium and bone mass density in postmenopausal osteoporosis, Clinical Interventions in Aging, Volume: 13 Pages: 1383-1389, 2018, DOI: 10.2147/CIA.S170111

3. Djeska, IS; Ceausu, RA; Gaje, PN; Cimpean, AM**; Mederle, O**; Nicodin, A; Tudorache, V; Raica, M - The Reticular Network Contributes to The Staging of Idiopathic Lung Fibrosis, Archives of Biological Sciences, Volume: 65, Issue: 4 Pages: 1599-1604, 2013, DOI: 10.2298/ABS1304599D.

Abdominal actinomycosis continues to be a poorly diagnosed disorder due to the insidious course, nonspecific symptoms and lab markers. Both modern imaging investigations (CT, MRI with contrast) and histochemical examination of biopsy samples helps to establish the diagnosis of abdominal actinomycosis. Combination of antibiotic therapy with surgery is a promising alternative for the treatment of patients with abdominal actinomycosis.

1. Gurban Camelia Vidita, Florina-Maria Andrica, Cosmin Citu, Elena Hogea, Iosif Marincu, Marioara Cornianu, **Ovidiu Alexandru Mederle**- Combined Therapies in Abdominal Actinomycosis, REV.CHIM., 67, No.1, 2016, Pages: 171-173 ISSN: 0034-7752, IF=1,232

2. Prodea, M; Boia, ER; Ceausu, RA; Librimir, C; Iovanescu, G; **Mederle, OA**- Lung Delayed Hypersensitivity A case with particular features, REV. CHIM, Volume: 69, Issue: 8 Pages: 2071-2073, 2018, ISSN: 0034-7752, IF=1,412

A main direction of activity is concentrated in the sphere of mites (demodicosis), parasitic zoonoses (cryptosporidiosis, toxoplasmosis, giardiasis, trichophytosis, cheiletielosis), microsporidiosis (nosemosis) and bacteriosis (Lawsonia spp., Salmonella spp.).
Ectoparasitosis that affects the skin of humans and animals and causes depilation, alopecia, erythematous lesions, which can often cause bacterial or fungal complications. Demodicosis is caused by *Demodex species*, mites that parasite in hair follicles.

Demonstration of Demodex canis transmission in humans was the aim of the preliminary studies conducted in 2007 and 2014 respectively. A study that looked at the importance of complementary examinations that may be an important paraclinical indicator in the diagnosis of demodicosis (serum values of total proteins, albumin, total globulins and immunoglobulins G, M, A, E) was conducted. The puncture / biopsy sampling of parasite skin with *Demodex* mite and the development of histological and immunohistochemical preparations provided, on the one hand, confirmation of the clinical and microscopic diagnosis and, on the other hand, an image of the tissue and immunohistochemical reactions determined in the dog's skin, the presence of the mite. Conducting multicenter studies with the involvement of the University of Medicine and Pharmacy "Victor Babeş" Timişoara, of the discipline of Parasitology FVM Timişoara, SC PRIMOSAL Bucharest, Research Institute - INCDT, Bucharest have led to the elaboration, formulation, characterization and testing of parasitic animals a gel consisting of natural extracts with fast cutaneous absorption, effective in remission of demodecal lesions and without irritant effect on parasite skin. The result of the investigations is filed with OSIM as a patent application A 00075 of 1.02.2016 (Patent no 131619-A0).

1. Gartner Andreea, **Mederle O**., Mederle Narcisa, **2014**, *Demodex Folliculorum* and *D. Brevis*, a cause of facial dermatitis and blepharitis, J. Of Biotech., 0168-1656, 185S, S100.

2. Mederle N., Dărăbuș Gh., Oprescu I., Morariu S., Ilie M., Indre D., **Mederle O**., **2010,** Diagnosis of Canine Demodicosis, Scientia Parasitologica, 11 (1), 20-23, ISSN: 1582-1366.

3. Mederle Narcisa, **Mederle, O**., **2017**, Evaluation of Serum Values of Total Protein (PT), Albumin, Total Globulin and Immunoglobulin (Ig) G, A, M, And E in Canine Demodicosis. Lucrări Științifice- Universitatea De Științe Agricole a Banatului Timișoara, Medicină Veterinară, 50, 1:125-129;

4. Negrescu Adina, **Mederle O.**, Milovanov Cornelia, Ahmadi Mirela, Darabus Gh., Morariu S., Mederle Narcisa, **2017**, The diagnostic value of hematology and blood biochemistry in piodemodicosis, New Front. Chem. Former: Ann. West Univ. Timișoara – Series Chem. Volume 26, Number 2 ISSN: 1224-9513.

5. Milovanov C., Mederle N, Ahmadi-Khoie M, Herman V, **Mederle O A**, Morariu F, Morariu S, Popescu G, Radulov I., Hair-And Nails-Regenerative Composition, Patent Number(S): Ro131851-A0

6. Radbea Narcisa, Dărăbuş Gh, Oprescu I., Ilie M., Nicola Alina, **Mederle O., 2005**, Aspecte histopatologice în demodicoza generalizată la câine, Sci. Parasitol. Vol. 6, 1-2, 100-103.

7. Radbea, N.; **Mederle, O**.; Dărăbuş, G.; Oprescu, I.; Morariu, S.; Ilie, M., **2006**, Immunohistochemical results in canine piodemodicosis, Lucrări Ştiinţifice Medicină Veterinară, USAMV "Ion Ionescu de la Brad" Iași, 49, 8, 404-406.

8. Mederle Narcisa; **Mederle, O**., Raica, M., **2007**, Assessment of the *Demodex Folliculorum* in human cutaneous lesions, Virchows Archiv, 451: 449-450.

9. Horablaga A., Ahmadi-Khoie M., Horablaga N. M., Mederle N., **Mederle O.** A., Milovanov C., Morariu F., Morariu S., Popescu G., Moisturizing body and face cream based on *Oenothera Biennis* comprises mixture of natural oils of evening star, Patent Number(S): Ro132235-A0

10. Mederle N, Dărăbuș G, Ilie M S, **Mederle O,** Morariu F, Morariu S, Negrescu I A, Oprescu I, Gel for treatment of dry wounds in canine Demodicosis gel consists, in mass percentage, of 25% honey, Patent Number(S): RO131619-A0

11. Mederle, Narcisa; Marin, S; Marin, MM; Danila, E; **Mederle, O**; Kaya, MGA; Ghica, AV,**2016**, Innovative Biomaterials Based on Collagen-Hydroxyapatite and Doxycycline for Bone Regeneration, Advances in Materials Science and Engineering, Article Number: 3452171, DOI: 10.1155/2016/3452171.

Epidemiological, clinical, therapeutic and molecular biology studies in honeybees nosemosis support another direction of postdoctoral research.

1. Mederle Narcisa, Dărăbuș Gh., Morariu S., **Mederle O.,** Herman V., Oprescu I., Ilie M., Imre Mirela, Motoc Marilena, Chiș Codruța, **2018**, Therapeutic efficacy testing of plant dietary supplement used for prevention and control of nosemosis in bees, Conference of Life Sciences, USABMV Timișoara, Epub Ahead of Print.

2. Mederle Narcisa, Balint, A., Morariu, S., Hora, F.Ş., **Mederle, O**., Marincu, I., Dărăbuș, Gh., **2015**, Research on the prevalence of honey bees nosemosis in Arad County, J. of Biotechnology, 208, S5-S 120.

3. Mederle Narcisa, Maria Luisa Lobo, Sorin Morariu, Florica Morariu, Gheorghe Darabus, **Ovidiu Mederle**, Olga Matos - Microscopic and Molecular Detection of Nosema ceranae in Honeybee Apis mellifera L. from Romania Status on pathogen worldwide distribution, REV.CHIM.(Bucharest), 69, No.12, 2018, ISSN: 0034-7752, IF=1,412

The social importance of protozoa (*Criptosporidium spp., Giardia intestinalis, Toxoplasma gondi),* dermatophytes (*Tricophyton spp*.) and mites (*Cheyletiella spp*.) resides in the zoonotic character of these endo and ectoparasites, the clinical and lesional expressions in humans having a great impact on their lives. Contributions to establishing the diagnosis and establishing a therapeutic protocol integrated into the parasitic control plan have been noted over a ten-year period and have finalized in various scientific publications indexed by ISI and BDI.

1. Mederle, N; Darabus, G; Oprescu, I; Morariu, S; Hora, FS; Lighezan, R; **Mederle, O** - Identification of potential zoonotic parasitic elements in parks and playgrounds for children in Timisoara, Revista Romana de Medicina Veterinara, Volume: 26 Issue: 4 Pages: 23-26, 2016

2. Darabus, G; Olariu, RT; **Mederle, O**; Mederle, N.; Hotea, I; Sorescu, D; Imre, K; Imre, M - Zoonotic protozoosis in Romania (giardiasis, cryptosporidiosis, toxoplasmosis, sarcocystosis): epidemiological aspects, Revista Romana de Medicina Veterinara,Volume: 26 Issue: 4 Pages: 47-53,Published: 2016.

3. Vieira, PM; Mederle, N; Lobo, ML; Imre, K; **Mederle, O**; Xiao, LH; Darabus, G; Matos, O - Molecular characterisation of Cryptosporidium (Apicomplexa) in children

and cattle in Romania, Folia Parasitologica,Volume: 62, Article Number: 002, 2015, DOI: 10.14411/fp.2015.002

4. Dărăbuş, Gh., **Mederle, O**., Popovici, E.D., Imre, K., Ilie, M., Oprescu, I., Morariu, S., Mederle Narcisa, Hotea Ionela, Olariu, R.O., Baditoiu Luminita Mirela *Prevalenţa criptosporidiozei la animale şi oameni în vestul României* – date preliminare Rev. Rom. de Parazitologie, Vol.XVIII, supliment, 2008 ISSN 1221-1796 (Google Scholar)

5. Florin Ș. Hora, Gheorghe Dărăbuș, Corina Badea, **Ovidiu Mederle**, Narcisa Mederle, *Epidemiological, clinical and therapeutic aspects of the infestation with Trichophyton spp*. (Fungi: Eurotiomycetes: Arthrodermataceae), 2015, Scientia Parasitologica 16 (1-2), 28-32.

6. Gh. Dărăbuş, **O. Mederle**, Narcisa Mederle, T.R. Olariu ,I.Oprescu, S.Morariu, K. Imre, M. Ilie, Ionela Hotea *The study of some biochemical parameters in cryptosporidium experimental infection in broiler chickens* Lucrări ştiintifice Medicină veterinară vol. XL, 2008, Timişoara 344

7. Imre, K., **Mederle, O**.; Mederle Narcisa.; Ilie, M. S.; Hotea, I.; Imrie, M.; Indre, D.; Balint, A.; Sores, D. *Molecular characterization of human Cryptosporidium isolates in Banat region*, Romania Scientific Works - Series C, Veterinary Medicine, Bucharest(ISSN 1222-5304, 56, 1, 91-96, 2010, CABI

8. Mederle N., Dărăbuş G., Oprescu I., Morariu S., Ilie M., Imre K., Hotea I., **Mederle O.**, 2008 - *Correlation between histological, serological and epidemiological investigations in human toxoplasmosis*, Lucrări Științifice Medicină Veterinară Timișoara, 41, 356-360, ISSN 1221-5295. Recenzată şi indexată: CAB Internațional, England, categorie B+, cod CNCSIS 259;

9. Mederle Narcisa, Dărăbuş, Gh., Ionela Hotea, **Mederle, O.** *Surse şi modalităţi de infestare în toxoplasmoză* Rev. Rom.Parazitologie, vol. XVII, supliment, 2007(Google Scholar)

10. Mederle, Narcisa.; Darabus, Gh.; Oprescu, I.; Morariu, S.; **Mederle, O.** *Epidemiological research in canine dermatophytoses* Revista Scientia Parasitologica ,(ISSN: 1582-1366, 7, 3-4, 2006, CABI)

11. Gartner Andreea Ionela, Mederle Narcisa Geanina, Darabus Gheorghe, Marincu Iosif, **Mederle Ovidiu** Alexandru - *A case report of Cheyletiella blakei infestation in an asymptomatic cat and skin lesions of her owner Journal of Biotechnology* 2016 231 Supplement S107

12. Mederle Narcisa, **Mederle A. O**., Darabus Gh., Gartner Andreea, Ioanoviciu S.- Immuno-fluorescence and immuno-histochemistry methods in diagnose of human cryptosporidiosis, Journal of Biotechnology, 0168 - 165 6, 256, 2017 https://www.sciencedirect.com

The implications of bacterial species of *Salmonella*, *Lawsonia* or *Escherichia* in the etiology of the enteric swine complex and their role in the development of morbid processes have been investigated in a histological and immunohistochemical study performed on ileum and jejune with adenomatosis.

1. Anca Sofiana Surpat (Hulea), Viorel Herman, Iosif Marincu, Narcisa Mederle, **Ovidiu Alexandru Mederle,** *Immunohistochemical method for identification of Lawsonia intracellularis infection in pigs*, Journal of Biotechnology, Volume 208, Supplement, 20 August 2015, Pages S100.

I am co-author of three Patent applications:

- Gel for the treatment of dry lesions from canine demodicosis. Patent No. A 00075 / 1.02.2016. Authors Mederle Narcisa, Mederle Ovidiu, Morariu Sorin, Morariu Florica, Darabus Gheorghe, Oprescu Ion, Ilie Marius, Negrescu Adina.

- Regenerating hair and nail composition. Patent nr A 00621 / 25.11.2016. Authors Milovanov Cornelia, Mederle Narcisa, Ahmadi Mirela, Morariu Sorin, Popescu Gabriela, Morariu Florica, Herman Viorel, Radulov Isidora, Mederle Ovidiu.

- Skin and skin moisturizing cream based on Oenothera biennis. Patent No. A 000269 / 08.05. 2017. Authors Horablaga Adina, Milovanov Cornelia, Ahmadi Mirela, Mederle

Narcisa, Morariu Sorin, Morariu Florica, Horablaga Marinel, Popescu Gabriela, Mederle Ovidiu.

Throughout my university career, project proposals have been submitted as a director or responsible, of which two have materialized.

1. Effect of mast cell degranulates and degranulation inhibitors on tumor angiogenesis - CNCSIS A / 759/2006 - 2006-2007.

2. Possible epidemiological chains and control modalities in cryptosporidiosis in animals and humans - PN II 51-034 / 2007 - 2008-2010

The professional and academic achievements are highlighted by the description of didactic competences, the journalistic activity (three first author books and ten, co-author), the coordination of the bachelor's work and the involvement of PhD students in the research team of the projects that I manage, conducting the research studies that support the obtaining of the three patent applications, as well as in carrying out the experiments and interpreting the results materialized in the publication of scientific papers, and this collaboration continued after the doctoral dissertation.

I've participated and organized with the students and PhD students various scientific events: International Scientific Symposium Young People and Veterinary Medicine Researcher, FVM Timisoara International Scientific Symposium, Euro conference of Parasitological Zoonosis Timisoara, Balkan Fungus International Conference, National Invention Salons: Pro Invent Cluj Napoca, Traian Vuia "Timisoara", EuroInvent Iasi.

I guided students to work at the students communication sessions: Medis, 1997, 1998, 2003. In 2018 and 2019 I held the workshops on ALS and BLS at the MEDIS students' scientific sessions. In 2018, I've supported in the HEART, a workshop on FAST trauma. In 2019, we coordinated six workshops within GALMED.

I've started my medical activity in 1991 as a trainee at the County Hospital - Urology Department. There was a period when I worked as a doctor in the villages of Seleus and Pincota, Arad County. Between 1994 and 1996, I worked as a physician trainee, since 1996 I have been a specialist physician, Emergency Medicine, at the Municipal Clinical Hospital of Timişoara, where, since 2001, until now, I'm a primary physician, Emergency Medicine.

Professional, scientific and academic recognition is substantiated by my membership in professional and scientific committees and associations, by organizing scientific events and postgraduate courses and by national and international awards, among which the most representative are the gold and silver medals obtained at the Inventions Salons and Geneva and Barcelona innovations.

The career development plan for the professional, scientific and academic career is included in the last part of the thesis and contains various future components of didactic, scientific and medical research.

B. REALIZĂRI ȘTIINȚIFICE ȘI PROFESIONALE

Subsemnatul, **MEDERLE Ovidiu Alexandru**, Doctor în Științe Medicale, domeniul Medicină, Conferențiar la disciplina Histologie si Citologie, din cadrul Facultății de Medicină Timișoara, candidat la obținerea titlului științific de **Doctor abilitat** (dr. habil.), prezint teza de abilitare intitulată *Contribuții la aplicarea metodelor histochimice și imunohistochimice în procedurile diagnostice și cu potențial impact terapeutic.*

1. RELEVANȚA ȘI IMPACTUL REZULTATELOR ȘTIINȚIFICE

1.1. CARIERA PROFESIONALĂ

♣ Cadru didactic al Facultății de Medicină, din cadrul UMF „Victor Babeș" Timișoara, începând cu anul 1994, când am debutat ca preparator. Ulterior, am ocupat, prin concurs, posturile de asistent și șef de lucrări. În prezent, activez pe postul didactic de Conferențiar. Cei 25 de ani de carieră didactică universitară i-am dedicat disciplinelor de Histologie și Urgențe medico-chirurgicale.

♣ Doctor în Științe Medicale, domeniul Medicină, prin Ordinul Ministrului Educaţiei Naţionale nr.4090/1988.

♣ Absolvent al Facultății de Medicină, din cadrul UMF „Victor Babeș" Timișoara, promoția 1991, cu media generală de licență 10.

1.2. REZULTATELE ȘTIINȚIFICE

Rezultatele activității de cercetare, desfășurată din 1994 până în prezent, se regăsesc în finalizarea tezei de doctorat, publicarea unui număr de 115 lucrări științifice în reviste cotate ISI, indexate în baze de date internaționale sau prezentate la conferințe și simpozioane naționale

și internaționale, trei cereri de brevet de invenție înregistrate la OSIM, două contracte de cercetare în calitate de director. Indicele Hirsh: Google Scolar 8, Web of Science 6 și Scopus 6 (Anexa 2).

Studiile derulate în perioada elaborării tezei de doctorat intitulată ”Cercetări morfohistochimice şi imunohistochimice pe puncţii biopsii în hepatopatia cronică postvirală”, conducător științific: Prof. Dr. Univ. Drăgan Maria, au reprezentat startul activității de cercetare care a continuat și după finalizarea tezei. Am studiat morfohistochimia și imunohistochimia parenchimului hepatic normal și în condiții de injurie virală, în special cu virus B. Am evidențiat, pentru prima dată la noi în țară, celulele Ito-Nemoto, cu anticorpi monoclonali anti actină-muşchi neted. Rezultatele privind studiul acestor celule au fost prezentate şi publicate în revista Romanian Journal of Morphology and Embriology și în revista Timișoara Medicală. Am studiat împreună cu Prof. Dr. Univ. Marius Raica implicațiile structurilor normale în patogenia bolilor hepatice, elementele de histoprognostic care au cu adevărat valoare pentru evoluția și tratamentul bolnavului. În urma acestei colaborări am editat (coautor) monografia “Interpretarea biopsiilor hepatice”, Ed. Mirton, Timișoara, 1998. În această perioadă, am evaluat microscopic afecţiunile cronice hepatice care impun stadierea şi gradarea. Gradarea s-a realizat prin efectuarea scorului Knodell, care atenţionează asupra unor cazuri cu risc crescut pentru evoluţia spre hepatită cronică agresivă şi ciroză. Stadierea informează asupra prezenţei şi extensiei fibrozei hepatice. Gradarea şi stadierea trebuie să se completeze reciproc pentru a obţine o imagine cât mai apropiată de momentul evolutiv al hepatopatiei cronice. Este prima aplicare a coloraţiei tricrome Gomöri modificată pe ficatul normal şi patologic. Evaluarea dinamică a aspectelor microscopice ale biopsiilor hepatice reprezintă modalitatea cea mai exactă de apreciere a evoluţiei afecţiunii cronice hepatice.

Relevanța și aplicabilitatea practică (diagnostică și terapeutică) a cercetărilor științifice post-doctorale, descrise în prezenta teză de abilitare, reprezintă recunoașterea muncii de cercetare, susținută de 115 publicații științifice indexate în bazele de date internaționale sau prezentate la congrese și simpozioane internaționale, trei brevete de invenție, două contracte de cercetare în calitate de director și de un produs lansat pe piața comercială, componenta principală a unui kit destinat tratării unei ectoparazitoze.

Direcțiile principale ale activității de cercetare postdoctorale se concretizează în:

- Aplicarea metodelor histochimice și imunohistochimice în procedurile diagnostice și cu potențial impact terapeutic în neoplasme, studii experimentale, boli cronice (osteoporoza,

fibroza pulmonară) și în câteva afecțiuni particulare (actinomicoza, hipersensibilitatea pulmonară)

- Aplicarea metodelor histochimice și imunohistochimice în procedurile diagnostice și cu potențial impact terapeutic în boli cu etiologie parazitară și infecțioasă

I. APLICAREA METODELOR HISTOCHIMICE ȘI IMUNOHISTOCHIMICE ÎN PROCEDURILE DIAGNOSTICE ȘI CU POTENȚIAL IMPACT TERAPEUTIC ÎN NEOPLASME, STUDII EXPERIMENTALE, BOLI CRONICE (OSTEOPOROZA, FIBROZA PULMONARĂ) ȘI ÎN CÂTEVA AFECȚIUNI PARTICULARE (ACTINOMICOZA, HIPERSENSIBILITATEA PULMONARĂ)

I.1. APLICAREA METODELOR HISTOCHIMICE ȘI IMUNOHISTOCHIMICE ÎN PROCEDURILE DIAGNOSTICE ȘI CU POTENȚIAL IMPACT TERAPEUTIC ÎN NEOPLASME

1. Boia, S; Boia, ER; Ceausu, RA; Balica, CN; **Mederle, OA** - HPV18 Associated with E-cadherin expression in head and neck squamous cell carcinoma, REV. CHIM, Volume: 69, Issue: 8 Pages 2638-2641, 2018, ISSN: 0034-7752, IF=1,412

2. Pirtea Laurentiu, Dorin Grigoras, Cristina Secosan, Ioan Sas, Razvan Ilina, Adriana Andreea Jitariu, **Ovidiu Alexandru Mederle** - Clinical and Histopathological parameters correlate with Microvessel Density but not with Vascular Endothelial Growth Factor expression in ovarian cancer, REV.CHIM. (Bucharest), 69, No. 5, 2018, Pages: 1173-1178 ISSN: 0034-7752, IF=1,232

3. Bocan Elena Viorica, **O. Mederle**, Simona Sârb, R. Minciu, D. Agapie, M. Raica - Correlation between histopathological form and the degree of neuroendocrine differentiations in prostate cancer- Rom J Morphol Embryol 2011, 52(4):1215–1218 ISSN: 1220-0522, IF=0,523

4. Ceausu Amalia Raluca, Alexandru Ciolofan, Anca Maria Cimpean, Adina Magheti, **Ovidiu Mederle**, Marius Raica- The Mesenchymal–Epithelial and Epithelial–Mesenchymal cellular plasticity of liver metastases with digestive origin- ANTICANCER RESEARCH 38: 811-816(2018) doi:10.21873/anticanres. 11xxxISSN: 0250-7005, IF=1,937

5. Elena V. Bocan, **Ovidiu Mederle**, Marius Raica - Immunohistochemistry of prostate specific antigen in advanced stage prostate carcinoma, Rev Arg de Anat Clin; 2010, 2 (3):106-111

6. Balica Amalia Raluca, Anca Maria Cimpean, Andreea Cioca, Octavian Cretu, **Ovidiu Mederle**, Alexandru Ciolofan, Pusa Gaje, Marius Raica- Endothelial cell proliferation and Vascular Endothelial Growth Factor expression in primary colorectal cancer and corresponding liver metastases, Asian Pacific journal of cancer prevention: APJCP Volume: 16 Issue: 11 Pages: 4549-53, 2015

Aplicarea metodelor histochimice și imunohistochimice în procedurile diagnostice și cu potențial impact terapeutic au fost studiate în diferite neoplasme: carcinom scuamo-celular al capului și gâtului, cancer ovarian, prostatic, colic si hepatic. Am folosit mai mulți markeri imonohistochimici: E-caderina, VEGF clona VG1, CD34, chromogranina, PSA, keratina 8,18, vimentina, VEGFmRNA, ki67.

O corelație semnificativă între HPV18, imunoexpresia E-caderinei și gradul moderat diferențiat a fost găsită în cancerul laringian. Cazurile negative HPV18 de rino, hipofaringe și laringe au prezentat o scădere a valorilor E-cadherinei pentru tipurile moderate, slab și nediferențiat. Valoarea maximă pentru E-cadherina, independent de statusul HPV18, a fost asociată cu tumorile orofaringiene.

Absența celulelor endoteliale proliferate la jumătate dintre studiile experimentale, cazurile cu tumori primare și metastazele hepatice în carcinomul moderat diferențiat sugerează un fenomen de mimică vasculară. Neconcordanța dintre numărul total de vase și proliferarea endoteliului în tumorile primare indică existența deja a unei rețele vasculare funcționale sau existența unor mecanisme influențate de alți factori angiogenici.

Rezultatele concluzionează că MDV (micro-densitate vasculară) poate fi considerat un indicator util pentru progresia tumorilor locale și poate explica cel puțin parțial comportamentul angiogen și diseminarea la distanță a celulelor carcinoamelor ovariene. Cu

toate acestea, cancerele ovariene sunt boli extrem de heterogene care necesită descoperirea unor biomarkeri prognostici și terapeutici mai bine precizați. MDV și VEGF reprezintă doar o mică parte din numeroșii factori care influențează creșterea tumorală, angiogeneza, diseminarea locală și metastazele.

Am identificat imunohistochimic diferențierea neuroendocrină a cancerului de prostată cu chromogranina A. În adenocarcinomul prostatic au fost stabilite două grade de diferențiere neuroendocrină prin corelarea cu scorul Gleason: slab (3-5) și moderat (5-8). În cazul carcinomului cu celule mici, scorul Gleason a fost de 8-10 și diferențierea neuroendocrină a fost intensă. Am demonstrat că diferențierea neuroendocrină, detectată cu chromogranina A, este asociată cu scorul Gleason, boala agresivă și rata scăzută de supraviețuire. Chromogranina este un marker prognostic al bolii și este superior diagnosticului patologic standard.

În concluzie, imunohistochimia pentru PSA este extrem de utilă pentru a detecta invazia perineurală în specimene luate prin biopsie de bază și rezecție transuretrală. Am investigat carcinoamele prostatei care au relevat valoarea PSA, pentru diagnosticul diferențial. Reacția a fost pozitivă în 88,2% din cazuri, și a fost negativă în carcinoamele uroteliale și mici. Rezultatele noastre au arătat o expresie intensă în multe cazuri cu un scor ridicat Gleason. Imunohistochimia pentru PSA crește semnificativ rata de detectare a invaziei perineurale și confirmă originea prostatică a metastazelor nodulilor limfatici ai site-ului primar aparent necunoscut. Acest aspect sugerează existența unei corelații semnificative între prezența celulelor fenotipice hibride nediferențiate în tumorile primare și celulele fenotipice hibride diferențiate în metastazele hepatice ale cancerului colorectal, aspecte care susțin ipoteza de plasticitate EMT/MET (tranziție epitelio-mezenchimală) și MET/EMT a unor celule tumorale în metastaze hepatice. Existența unei corelații semnificative între celulele cu fenotip hibrid nediferențiat și modelul de creștere histologică în cancerul colorectal și gastric indică potențialul unei strategii de direcționare cu MET în combinație cu chimioterapia convențională pentru tratamentul metastazelor hepatice de origine digestivă.

HPV18 Associated with E-cadherin Expression in Head and Neck Squamous Cell Carcinoma

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HPV is an important oropharyngeal cancer cause, but it may have a role in other head and neck cancers? HPVpositive head and neck squamous cell carcinoma (HNSCC) epithelial-mesenchymal transition role is unclear. We included 38 cases: 20 laryngeal, 3 corresponding lymph nodes; 5 oropharyngeal, 5 hypopharyngeal, 2 rhynopahryngeal, 2 pharyngolaryngeal and 1 naso-sinusal case. Immunoreactivity was positive in nuclear expression cells, accordingly: score 1 (10-30%), 2 (30-50%) and 3 (>50%). HPV18 immunoexpression appeared in 18 cases (47.36%), (11 laryngeal, 4 oropharyngeal, 1 hypopharyngeal, 1 pharyngolaryngeal and 1 naso-sinusal). The score was 1 in larynx well differentiated type. The score was between 1 and 3 in larynx moderately differentiated types, and a significant correlation HPV18/E-cadherin was found (p= 0.031). HPV18+ /E-cadherin low values were noticed in larynx, oropharynx, pharyngo-larynx and naso-sinusal well and moderately differentiated types. HPV18-/E-cadherin low values were present in larynx, hypo and rhyno-pharynx moderately and poorly differentiated and larynx well differentiated types. Larynx presented HPV18/E-cadherin and moderately differentiated type significant correlation. Rhyno, hypo-pharyngeal and laryngeal presented HPV18–/E-cadherin low values association for moderately, poorly and undifferentiated types. The oropharyngeal location was associated with E-cadherin maximum values, independently of HPV18 status.

Keywords: HPV18, head and neck squamous cell carcinoma, mesenchymal epithelial transition

Characterized by phenotypic, biological, aetiological, and clinical heterogeneity, HNSCCs represented the sixth most common cancer worldwide, affecting 600.000 new patients each year [1].

Human papillomaviruses (HPVs) are a heterogeneous group of small non-enveloped epitheliotropic DNA viruses belongs to the Papillomaviridae family and targeting the basal cells of stratified epithelia at either mucosal or cutaneous sites. The HPV types 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59 were classified by the IARC (International Agency for Research on Cancer) Working Group as carcinogenic and 68 type as probably carcinogenic to humans [2].

HPV was found in 25% of all HNSCC [3, 4]. HPV represented the major cause of oropharyngeal (tonsillar and tongue base) cancer in developed countries, detected in 45–90% of cases, in a smaller subset of laryngeal (24%) and oral cavity cancers (23%) [5-8]. The demonstrated role of HPV18, until now, is minor compare to HPV16. Using HPV16 and 18 type-specific PCR, Quintero et al. showed that 20% of primary HNSCCs analysed cases were HPV positive. Among these 82% were HPV16 and 18% were HPV18 positive cases [9].

While HPV is an important cause of oropharyngeal cancer, it is unclear whether HPV may have a role in head and neck cancers with other location.

E-cadherin, a calcium-dependent cell-surface protein, characterized by long cytoplasmic and extracellular domains, is the main protein of adherents junctions that anchor oral epithelial cells to each other. Regarding the involvement of E-cadherin in HNSCCs there is some major direction debate in the literature. Such as, it was showed that aberrant E-cadherin expression was associated with a poor prognosis in patients with HNSCC [10]. Loss or sequestration of E-cadherin in the nucleus releases β-catenin, which translocates to the nucleus to induce transcription of EMT genes [11]. It was found that HPVpositive oropharyngeal squamous cell carcinoma tissues were significantly associated with EMT-induction, progression of lymph node metastasis and better prognosis than HPVnegative cases [12]. Some studies demonstrated the lowest E-cadherin levels in poorly differentiated type of HNSCC, the hypermethylation of the E-cadherin gene and a similar expression between primary tumors and metastases, perhaps because of MET in metastatic tumors [13, 14]. It was shown the involvement of E-cadherin reduced production in the inhibition of protective immune responses in HNSCC as a result of the activation of migration and function of Langerhans and dendritic cells [15].

The aim of this work was to describe the possible correlations between HPV18 and E-cadherin expression for different location of head and neck cancer.

Experimental part

Material and method

We included in the present study 38 cases of HNSCC with different localisation: larynx (20 cases) and 3 corresponding lymph nodes, oropharynx (5 cases), hypo-pharynx (5cases), rhynopahrynx (2cases), pharyngo-larynx (2 cases) and naso-sinusal (1 case). Immuno-histo-chemical techniques included heat-induced epitope retrieval with Novocastra Bond Epitope Retrieval Solution 2, a ready-to-use, *p*H 9.0 solution (Leica Biosystems,

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Newcastle Ltd, Newcastle UponTyne NE 12 8EW, U.K.) for 20 minutes. Endogenous peroxidase blocking was realised with 3% hydrogen peroxide for 5 minutes. E-cadherin (monoclonal, clone 36B5, ready to use, Leica Biosystems, Newcastle UponTyne, U.K., 30 minutes incubation time) and HPV 18 (clone BF7, dilution 1:50, Novus Biologicals, Cambridge, U.K. CB4 0FQ, 30 minutes incubation) were used as primary antibodies. The Bond Polymer Refine Detection System was used for visualisation. As chromogen 3, 3 diamino-benzidine dyhidrochloride was applied for 10 minutes and hemotoxylin for 5 minutes, as counterstain. The entire immunohistochemical procedure was performed with Leica Bond- Max (Leica Biosystems, Newcastle uponTyne, U.K.) autostainer.

Immunoreactivity was estimated as positive in the cells with nuclear expression, according to the following score: score 1 (10-30%), score 2 (30-50%) and score 3 (> 50%) positive cells. Microscopic evaluation and image acquisition was performed with Axiocam 506 color, Zeiss, Jena, Germany.

Results and discussions

Histopathological evaluation indicated the presence of well (6 cases), moderately (27 cases), poorly (4 cases) and undifferentiated (1 case) types.

The immunoexpression of HPV 18 was noticed in 18 cases, with the following distribution: larynx (11 cases), oropharynx (4cases), hypopharynx (1 case), pharyngo-larynx (1 case) and naso-sinusal (1 case). From the 3 lymph node corresponding to larynx cases, 2 were positive and one negative for HPV18.

In the category of laryngeal HNSCC well differentiated cases, 3 cases were HPV18 positive and one case was negative. We noticed a score value of 1 (10-30% positive tumor cells) in all of these cases. As a particular aspect, the stromal cells with cytoplasmic expression was present in one irradiated case (fig. 1a).

The corresponding lymph node was HPV18 positive. The case score value was 2, with heterogeneous distribution in the tumor area, the highest nuclear intensity cells disposed in tumor area periphery, in lymphoid tissue vicinity (fig. 1b).

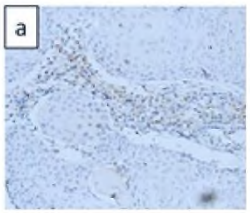

Fig. 1a. Stromal cells HPV18 immunoexpression, magnification X200.

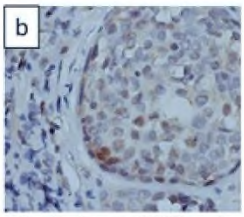

Fig. 1b. Larynx SCC lymph node metastasis, the most intensely HPV18 positive cells in tumor area periphery, magnification X400.

Moderately differentiated carcinomas were evaluated in 13 cases, 6 cases being HPV18 positive. The score values were 1 (3cases), 2 (2 cases) and 3 in 1 case. The distribution pattern was heterogeneous, with tumor area center and periphery highest nuclear expression cells (fig. 1c).

The HPV18 positive stromal cells were found. One of the corresponding lymph nodes was positive and one negative. The positive one presented a score value of 1 and tumor area isolated positive cells, without a predominant disposition to the tumor area periphery.

One of the two poorly differentiated cases with laryngeal origin was HPV18 positive. The score value was 1, with the most intense reaction in the cells nucleus, from the tumor area center. In the quasinormal larynx, the HPV18 nuclear immunoexpression was noticed in basal, intermediate and superficial layers cells (fig. 1d).

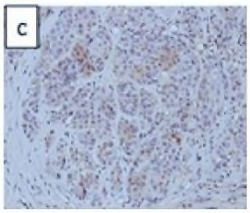

Fig. 1c. Larynx moderately differentiated SCC, heterogeneous distribution pattern, magnificationX100.

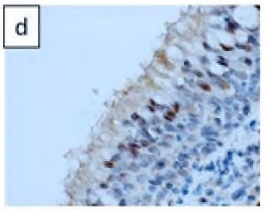

Fig. 1d. Basal, intermediate and superficial layers quasinormal larynx cells HPV18 nuclear immunoexpression, magnification X400.

At the pseudostratified and stratified epithelium junction, HPV18 expression remained in the basal cell nuclei and in suprabasal layer (fig.1e).

In the 5 cases of oropharyngeal HNSCC the tumor grading was G2. In 2 out of 5 cases we encountered the highest score value of 3 and the immunoexpression extended in the surface epithelium full height and the most intense reaction was found in tumor area both center and periphery (fig. 1f).

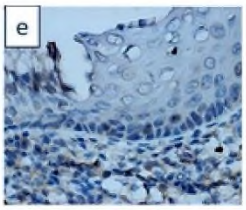

Fig. 1e. The border between pseudostratified and stratified epithelium, larynx poorly differentiated SCC – cell's nuclei HPV 18 expression in the basal and suprabasal layers, magnification X 1000.

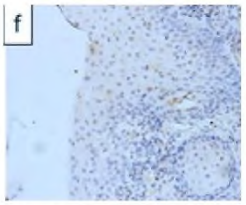

Fig. 1f. Surface epithelium and tumor area HPV18 immunoexpression (oropharynx moderately differentiated SCC), magnification X200.

The value score of 2 (1 case) and 1(1 case) were noticed, as well.

Besides oropharynx, the score value of 3 was present in naso-sinusal region (G3 type). The distribution pattern was heterogeneous, with tumor area periphery highest intensity.

The hypo-pharynx was characterized by the score value of 2. The positive cells were found in the tumor area, with highest intensity in the periphery.

The maximum E-cadherin immunoexpression score value was 3.

HPV18 positive cases, presented this value as follow: larynx, well differentiated type (2 cases), moderately differentiated type (5 cases), poorly differentiated type (1 case), oropharynx (2 cases) and hypo-pharynx (1 case).

The E-cadherin score value of 3 was found in a few HPV18 negative laryngeal cases, well differentiated type (1case), moderately differentiated type (1 case), 1 oropharyngeal case, 1 hypopharyngeal case, 1 pharyngo-laryngeal case and 1 rhyno-pharyngeal case.

The E-cadherin score value of 2 was noticed in the HPV18 positive cases, according to the histological grade: larynx well differentiated (1 case) and moderately differentiated type (6 cases), oropharynx (1 case),

pharyngo-larynx (1 case) and 1 poorly differentiated naso-sinusal HNSCC.

The HPV18 negative cases were not associated to E-cadherin immunoexpression score value decrease well differentiated type, only in moderately, poorly and undifferentiated types of HNSCC. Six laryngeal HNSCC cases diagnosed as G2 and 3 hypo-pharyngeal HNSCC cases (G2, G3) were characterized by the HPV18 absence and of and E-cadherin score value of 2.

Two laryngeal and hypo-pharyngeal HNSCC poorly differentiated cases and one rhyno-pharyngeal un-differentiated case presented HPV18 negative and E-cadherin score value of 2.

In cases of laryngeal lymph node metastases the HPV18 positive and negative had the same E-cadherin score value of 3. A significant correlation between the HPV18 and E-cadherin immunoexpression was noticed in moderately differentiated laryngeal HNSCC cases (p= 0.031). The E-cadherin immunoexpression was noticed in the tumoral islands (fig. 1g) and covering epithelium, in suprabasal and intermediary layers (fig. 1h).

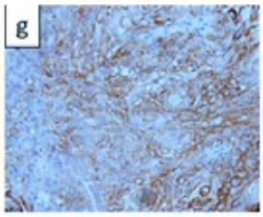

Fig. 1g. Tumor islands Ecaderin immunoexpression, magnification X200

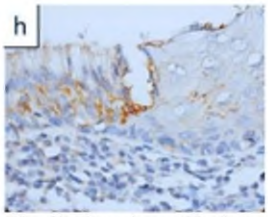

Fig. 1h. Covering epithelium Ecadherin immunoexpression, larynx poorly differentiated SCC, magnification X1000

The HPV16 and HPV18 are among the high risk classified types, of both, genital and non-genital tract. Together with alcohol and tobacco consumption, they represent the main risk factors for the development of HNSCCs, especially with tonsils and tongue base localization.

Some data [3, 16] showed the role of HPV in different head and neck cancer such as larynx and oral cavity, but in smaller proportion, compare with oropharyngeal cancer. It was noticed that the distribution of HPV in HNSCCs may vary with site. The rare presence of HPV18 in the oropharynx was confirmed, heaving a special tropism for glandular tissue [3]. Other study showed a 17% of HPV18 positive and a 69.2% for HPV16 in the laryngeal carcinoma cases [17]. In our study, 47.36% of cases were HPV18 positive, with the following distribution: larynx (28.94%), oropharynx (10.52%), hypo-pharynx (2.63%), pharyngo-larynx (2.63%), naso-sinusal (2.63%) and rhyno-pharynx (0%).

Literature data report a favourable prognosis in patients with HPV-positive HNSCCs [18-20]. On the contrary, some studies showed no association between HPV positivity and patient prognosis [21-23]. The others underlined that the HPV-positive subgroup correlates with a higher risk of recurrence or developing a second primary tumor [24, 25]. In our study we noticed in a case, which received treatment, tumor and stromal cells HPV18 presence.

The HPV18 positive stromal cells were also found in our study in the moderately differentiated type of larynx HNSCC, and a HPV18/E-cadherin immunoexpression significant correlation was identified.

Kim et al. [26], demonstrated in their study that the E-cadherin negative group has more moderate and poor differentiation type than the higher E-cadherin expressing group. In our study, we noticed that the HPV18 negative cases were associated with the lower score values of E-cadherin immunoexpression in the larynx, hypo-pharynx, rhyno-pharynx moderately, poorly and undifferentiated type.

In a study which used the p-16 positive CERV196 and p-16 negative HNSCC22B SCC cell lines, Umbreit et al.[27], found a strong expression of beta-catenin and E-cadherin in both SCC lines, independently of HPV status, but the last one, positive influenced by treatment time with EGF and EGF/TGF beta 1. We found a maximum value of E-cadherin score, to the positive and negative HPV18 cases localized only in oropharynx.

Conclusions

A significant correlation between HPV18, E-cadherin immunoexpression and moderately differential grade was found in the laryngeal cancer. The rhyno, hypo-pharyngeal and laryngeal HPV18 negative cases presented a decrease of E-cadherin values for the moderately, poorly and undifferentiated types. The maximum value for E-cadherin, independently of HPV18 status was associated with the oropharyngeal sub-sites.

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Clinical and Histopathological Parameters Correlate with Microvessel Density but Not with Vascular Endothelial Growth Factor Expression in Ovarian Cancer

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Ovarian cancer malignancies have the worst prognosis among all gynecological malignancies. As angiogenesis represents a key step for tumor progression, vascular endothelial growth factor (VEGF) is one of the most discussed pro-angiogenic factors. VEGF expression was investigated in 62 cases of ovarian carcinomas. Microvessel density (MVD) was evaluated by correlating the results with clinical and histopathological parameters. Because of the controversial results reported in other studies, VEGF was assessed together with MVD. Our results suggest a more complex angiogenic mechanism in ovarian cancer based on the discrepancies between VEGF expression, microvessel density and their correlation with clinical parameters. The conflicting data arising from this study supports the implications of different growth factors, others than VEGF in ovarian cancer. This hypothesis is sustained by the lack of correlation between VEGF and clinical parameters, and by the significant correlation between microvessel density and clinicopathological parameters. Thus, further studies are needed for a complete evaluation of angiogenesis in ovarian cancer.

Keywords: VEGF, MVD, ovarian cancer, angiogenesis, growth factors

Among all gynecologic malignancies, ovarian cancer has the worst prognosis, and represents the fifth leading cause of death due to malignant diseases in women. Despite standard treatment, cytoreductive surgery followed by platinum/paclitaxel-based chemotherapy, the overall survival rate in ovarian malignancies is only 35% [1]. The high mortality rate in ovarian cancer is due to the difficulty of detecting this malignancy at an early stage and the lack of effective therapeutic strategies in advanced stages. For a better understanding of ovarian cancer pathogenesis, the use of reliable early diagnostic markers and novel therapeutic targets is necessary.

A lot of data support the importance of angiogenesis in ovarian cancer progression. It has been shown that VEGF over-expression in ovarian cancer stimulates not only the formation of new blood vessels, but also induces malignant transformations in the normal epithelial cells of the ovarian surface.

As the most studied and the most effective pro-angiogenic factor, VEGF is known to induce endothelial cell proliferation, migration and survival. VEGF has been identified in a large variety of human malignancies, several evidences supporting its involvement in tumor angiogenesis. In most cases VEGF level of expression correlates not only with MVD but also with clinicopathological prognostic parameters. These observations have generated extensive laboratory studies, which led to the development of numerous specific inhibitors, out of which the humanized monoclonal antibody known as bevacizumab is the most renowned.

Approval of this antiangiogenic substance by the Food and Drug Administration prompted the initiation of several clinical trials, most of them being focused on ovarian cancer. Despite the promising results in early stages of ovarian cancer, advanced stages of this disease resulted in treatment failure without a plausible explanation. It seems that most of these clinical trials did not consider the angiogenic profile of primary tumors and/or peritoneal metastasis as selection criteria when patients were included in the study.

VEGF production in the normal ovarian tissue during the fertile period is accepted by many authors [2]. The detection rate for VEGF is about 7% in postmenopausal women, but is increased up to 42% in ovarian cancer patients [3]. However, no direct connection has been found between VEGF and microvessel density (MVD), nor between VEGF and the heterogeneous pattern of vascularization [4].The complexity of this issue is due to the presence of VEGF165 and 121 in both normal and malignantly transformed ovarian tissue at the same levels as VEGF [5].

The rather low detection rate for VEGF in ovarian carcinomas is unable to explain the high values of MVD for immature vessels. A possible explanation could be related to the presence of VEGF- B (167 and 186 forms) that is capable of stimulating angiogenesis and tumor progression [6]. The correlation between increased VEGF detection rate and ovarian tumor progression seems more accurate in clear cell carcinomas. Based on these findings, many authors advocate for adjustment of therapy to the angiogenic profile of each patient individually [7-10].

MVD evaluation is the first and probably the most useful method for assessing tumor angiogenesis. MVD evaluation techniques were applied in many scientific papers for

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almost all types of human and experimental tumors and, for almost 10 years period of time, it was the only method that generated mathematical results, thus making statistical analysis possible. In a large number of studies a statistically significant relation between the number of vessels (calculated using the method proposed by Weidner et al 1993), local tumor progression and the risk of distant metastasis was found [11]. However, MVD especially draws attention only on the number of vessels at a certain point in the evolution of the tumor and not on the angiogenic profile of the tumor cells. Under these circumstances, the controversial results published by different authors are explainable, even after the standardization of this procedure in terms of working methodology.

In the normal and malignantly transformed ovary this issue becomes significantly more complicated, mainly due to the impact of ovarian hormones on the number and distribution of vessels. In this regard, hormonal therapy, that aims to reduce the levels of circulating gonadotropins, may prolong remission in ovarian cancer by extending the dormant feature of the tumor [12]. These observations suggest that certain hormonal combinations may have either inhibiting or stimulatory effects on angiogenesis in ovarian tumors. MVD analysis in primary ovarian tumors and in metastatic peritoneal tumors showed no correlation between this parameter and clinicopathological features such as the age of the patient, tumor stage, histological type, preoperative CA125 levels and survival rate [13].

Our study aims to evaluate MVD by correlating the results with the patient's age (with special reference to menopausal status), tumor stage, histological type and degree of differentiation, that has been very poorly studied until now in terms of MVD, and VEGF expression.

Experimental Part

Material and methods

Patient selection.

62 female patients diagnosed with ovarian carcinomas were retrospectively selected during a four-year period of time. All patients had complete clinicopathological and postsurgical evaluation data. The ovarian carcinomas were accurately characterized regarding local and distant invasion and surgical protocols applied for each patient. A signed informed consent was obtained from each patient prior to their inclusion in the study.

All procedures were carried out according to the principles embodied in the Declaration of Helsinki and were approved by the Institutional Review Board of Victor Babes University of Medicine and Pharmacy, Timi'oara, Romania.

Description of specimens and primary histopathological processing methods

Tumor specimens were surgically removed and carefully selected by retrieving the most representative parts, including both the tumor area and the normal adjacent ovarian tissues. Tumor areas containing necrosis and extensive hemorrhage were avoided. 10x10x3 mm tumor tissues biopsies were washed in saline solution followed by 10% buffered formalin fixation for 24 h. Tissue specimens were then paraffin embedded. 5µm serial sections were taken from each paraffin embedded specimen and mounted on silanized slides. One slide from each case was stained using routine haematoxylin and eosin method for histopathologic evaluation and case selection for immunohistochemical procedures. DAKO LSAB2/HRP system was used for immunohistochemical evaluation and Bond Polymer Refine Detection System (Leica Biosystems, Newcastle uponTyne, UK) was used for visualization.

We investigated the immunohistochemical expression of VEGF clone VG1 in the selected cases. The correlation between our results and the clinical and histopathological available data was analyzed. VEGF expression was scored from 0 to 3 by assessing positive tumor cells and the staining intensity. Cases scored between 0 and 2 were considered negative while cases scored between 3 and 6 were considered positive.

MVD was evaluated on CD34 stained sections, based on the fact that CD34 selectively identifies only endothelial cells both in the normal ovarian tissue and in the tumor stroma which facilitates blood vessels counting. The evaluation was performed for intratumoral and peritumoral areas by selecting three fields with maximum vascular density at low magnification. The mean of the three fields was then calculated for each case. The intratumoral area was considered the area containing compactly arranged tumor cells, and vessels were counted only in case they were located within the tumor area. The obtained data was correlated with the histopathological types of ovarian cancer included in the study and to the quasinormal ovarian tissue adjacent to the tumor.

Thrombospondin 1 was assessed using the same immunohistochemical procedure.

Statistical analysis was performed using the commercially available SPSS version 17.0. We applied Student's test and a < 0.05 p index value was considered statistically significant.

Results and discussions

In the specimens containing normal ovarian tissue vessels were identified both in medulla and in the cortex stroma, with similar appearance in all cases. All vessels had well-defined borders, regular lumen with or without luminal content. Constantly, the vessels from the medulla were larger than those located in the cortex. We noticed particular features for the vessels found in the corpus albicans. The corpus albicans presented peripherally located vessels that were similar to those identified in the cortex while the corpus area presented rare, small and irregular vessels. The number of vessels found in normal ovarian stroma ranged between 16 and 35, with a mean of 22.34 vessels.

In ovarian tumors, the lowest MVD values were obtained in Brenner tumors and Sertoli cells tumors. However, the results were not significantly different from those obtained for the normal ovary. Despite being a benign lesion, the Sertoli cells tumor, presented heterogeneously distributed blood vessels with variable caliber only in the peritumoral area, in the connective tissue septa located between the nests of proliferating Sertoli cells. In the other tumor cases, we observed a direct correlation between the types of vessels and the investigated area. Thus, in the peritumoral area, blood vessels were consistently larger, with a wider lumen and a thin, regular wall, occasionally presenting emerging angiogenic sprouts. Unlike the peritumoral area, the intratumoral vessels were smaller, with a narrow lumen, irregular contour and were located among the tumor cells. All investigated tumors presented increased microvessel density values for the peritumoral areas. Serous adenocarcinomas showed a relatively increased variability in both the distribution and density of blood vessels. Intratumoral areas that presented rare vessels were excluded from MVD evaluation. In most areas, however, numerous vessels were present in both the peritumoral and intratumoral areas, ranging from 22 to 68 and from 16 to 44 respectively. The vessels were extremely variable in size and the identification of vascular structures without

an apparent lumen potentially indicates the presence of immature vessels. These features have been evident especially in the intratumoral areas whereas in the peritumoral areas they were rarely found. We noticed a particular aspect in four cases of serous adenocarcinoma, which regards the presence of numerous blood vessels exhibiting a plexiform layout in the peritumoral area. No blood vessels were identified in the intratumoral adjacent area. Higher magnification analysis showed that most vessels were dilated and contained blood elements within their lumen. Immature or intermediate types of vessels were either rare or absent. Due to the difficulties encountered when attempting to evaluate the number of vessels in these areas, we only chose to count the points of emergence. Moreover, we noticed some peculiarities apparently dependent on the histological type of the tumor. Thus, in the proliferating tumor areas with papillary differentiation, the vessels were strictly located within the connective tissue. In the solid tumor area however, the vessels were disposed between the malignant cells. In the clear cell carcinoma type, blood vessels were often situated in direct contact with the malignant cells that were arranged in nests and presented numerous irregular cytoplasmic processes. In the endometrioid carcinoma type, tumor cells were often disposed around fine connective tissue axes which, under low magnification, showed a large number of blood vessels.

The values of MVD statistical analysis associated with clinicopathological prognostic parameters revealed a statistically significant correlation between MVD, tumor stage ($p < 0.00021$) and degree of differentiation ($p < 0.0032$). We found no statistically significant correlation with the patient's age ($p = 0.33$), nor with the histopathological type of ovarian cancer ($p < 0.24$). The associations between MVD values and the histopathological types of ovarian cancer are presented in table 1. Based on these data, we noticed that the values were similar for the classical types of ovarian carcinomas, except for the mucinous carcinoma where the values were slightly lower, but not statistically significant.

Following the surgical procedure, a number of patients presented residual disease. By analyzing the relationship between MVD, residual disease and age, we did not obtain statistically significant correlations, neither in univariate nor in multivariate analysis, as it is shown in table 2. These results could be explained through the evaluation of MVD using specimens taken from the primary tumor and by the fact that during the second-look intervention fragments with uncertain relevance to this type of investigation were taken for processing.

The reaction for thrombospondin1 was positive in 43 cases (69.35%) out of the total number of 62 cases. The results were statistically analyzed in association with MVD values, tumor grade, tumor stage (FIGO), histopathological type and menopausal status. For the statistical analysis we considered both the mean and the maximum MVD values.

When comparing serous and non-serous ovarian tumors, we did not obtain any statistically significant correlation between MVD and TSP-1 expression. Also, we did not obtain statistically significant correlations neither with the

Form / MVD	Peritumoral / stroma	Intratumoral
Normal ovary	22.34 (16-35)	NA*
Serous adenocarcinoma	42.25 (24-68)	33.5 (16-44)
Endometrioid carcinoma	46.76 (36-97)	41.20 (35-48)
Mucinous carcinoma	31.66 (21-49)	26.33 (19-38)
Clear cell carcinoma	43.33 (39-54)	33.67 (24-51)
Undifferentiated carcinoma	44.66 (35-59)	41.00 (28-61)
Brenner tumor	22.5 (15-36)	1.66 (0-6)
Sertoli cells tumors	25.33 (17-29)	0

**NA: not applicable*

Table 1
MVD VALUES IN THE NORMAL OVARY AND IN THE HISTOPATHOLOGICAL TYPES OF OVARIAN CARCINOMAS

		Univariate Analysis			Multivariate Analysis		
Variables	Score	Odds ratio	95% CI	p	Odds ratio	95% CI	p
MVD		1.35	1.01-1.8	0.03	1.37	1-1.89	0.05
Residual disease	0 1	2.4	0.48-11.97	0.2	0.58	00.1-35.44	0.7
Age	0 1	1.5	0.34-6.94	0.5	0.61	0.004-87.1	0.8

Table 2
MVD AND RESIDUAL DISEASE

menopausal status (p = 0.6), nor with the histopathological type of ovarian cancer (p = 0.33). MVD was associated with decreased TSP-1 expression in cases presenting MVD values that were greater or less than 21.7/HPF. An intense TSP-1 expression was evident in cases presenting mean MVD values below 9.

VEGF reaction was negative in the normal ovarian tissue surrounding the tumors, with the specification that most patients were postmenopausal females, and ovarian follicles were no longer identified.

For all mucinous tumor types (n= 5), Brenner tumors (n= 2), Sertoli cells tumor and yolk sack tumors included in our study, VEGF reaction was negative. However, we noticed a positive reaction in 18 out of 62 studied cases (29.03%). In the group of positive cases, 11 cases were scored 3- 4, and 7 were scored 5- 6. No correlation was found between the tumor histopathologycal type and VEGF expression. The relationship between VEGF expression and tumor stage (p< 0.2) and between VEGF expression and tumor grade (p= 0.12) showed a variable pattern of positive reactions. A constant positive reaction pattern was noticed in 5 out of 6 cases of clear cells carcinomas. The staining intensity was strong and was scored + 3 (fig. 1). Clear cells

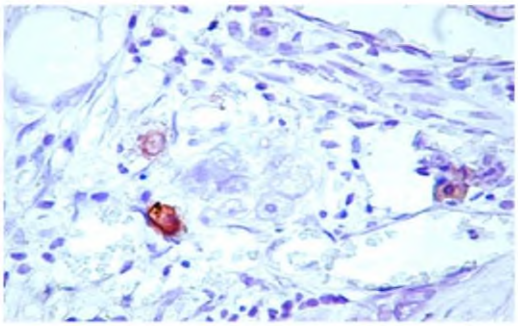

Fig. 1. VEGF positive reaction. Note the VEGF patterns of distribution and the staining intensity found in clear cells carcinomas. x400

were occasionally noticed within the lumen of small vessels.

Most serous adenocarcinomas included in our study showed a weak to moderate cytoplasmic VEGF reaction. We found the same expression pattern in the invasive areas (fig. 2), only the staining was characterized by a linear pattern that outlined the tumors. Surprisingly, VEGF was

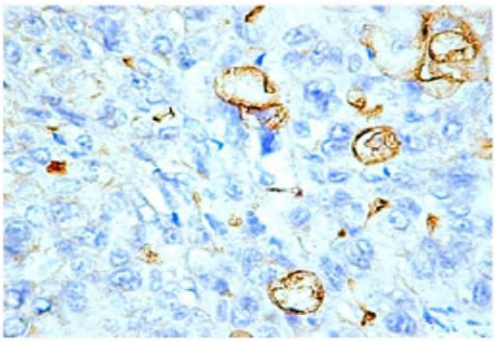

Fig. 2. VEGF positive reaction in the invasion area of ovarian carcinomas. Note the linear pattern of the staining outlining the tumors. x400

intensely expressed and exhibited a diffuse cytoplasmic pattern in 4 poorly differentiated cases of ovarian cancer.

MVD values and correlations with clinicopathological parameters shown by our results are similar to those already published in the literature. Szubert et al. investigated the associations between serum VEGF, bFGF and endoglin levels with microvessel density and expression of pro-angiogenic factors in benign and malignant ovarian tumors and found that MVD values were increased in epithelial ovarian cancer compared to benign ovarian tumors [14]. It seems that serum VEGF levels are a useful predictive marker for ovarian cancer MVD and tumor VEGF expression [14]. Several studies focused on the correlations between microvessel density (MVD) and clinicopathological parameters and found that an increased microvessel density CD34 expression is an independent mortality risk factor in ovarian cancer [15].

Ovarian cancer is usually diagnosed in advanced stages, thus remaining the most lethal gynecological cancer [16-18]. Currently, several pathogenic steps in ovarian carcinogenesis had been revealed, but the complexity of ovarian carcinomas and the numerous mechanisms that lead to malignant transformations in the ovary are still poorly understood. More data is needed in order to completely elucidate these issues along with exhaustive patient selection based on a great range of clearly defined criteria when attempting to improve the efficiency of antiangiogenic treatment strategies in ovarian cancer patients. In 2016, Li et al. performed a meta-analysis that aimed to investigate the effects of angiogenesis inhibitors in the treatment of patients with advanced or recurrent ovarian cancer and found that antiangiogenic therapy showed a clear progression survival free benefit but with the cost of an increased toxicity [19]. The impact of antiangiogenic drugs in overall survival was undefined for ovarian cancer patients [19]. These results support our findings regarding the necessity of proper patient selection when applying antiangiogenic therapeutic strategies in ovarian carcinoma cases.

Despite being the most important antiangiogenic treatment, anti-VEGF based therapy is followed by numerous side effects and is not the only efficient treatment in ovarian cancer. Novel scientific trials have recently pinpointed the direct correlation between SEMA4D and the degree of differentiation in epithelial ovarian cancer [20]. Chen Y et al. have concluded that VEGF along with SEMA4D possess synergistic effects in stimulating angiogenesis in ovarian carcinomas and the SEMA4D signaling pathway may become a potential target in the complex therapeutic management of patients diagnosed with epithelial ovarian cancer [20]. However, further experimental and clinical trials are needed in order to determine whether anti-SEMA4D alone could be sufficient in order to reduce MVD in ovarian cancer or a combined anti-VEGF/anti-SEMA4D would be more beneficial. Also, a proper patient selection based on firmly defined eligibility criteria is needed in order to reduce the degree of toxicity after combined antiangiogenic therapy. Advanced stages of ovarian carcinomas are known to be followed by chemotherapy resistance [21] depending on the cancer associated genetic abnormalities and biological behavior. Besides VEGF, other growth factor molecules are implicated either in promoting or inhibiting tumor angiogenesis in ovarian cancers. A recent study conducted by Pazos et al. shows that PDGF-B exerts an indirect inhibitory effect on the ovarian cancer vasculature [22]. It appears that PDGF-B normalizes the tumor vessels following single administration and favors gamma-secretase inhibitor (DAPT) anticancer action when being co-administrated [22]. However, the scientific data regarding PDGF implications in ovarian cancer remain controversial. Also, the exact interaction, if existent, between VEGF and PDGF, must be further investigated. In this regard, PDGFR-beta and VEGFR-2 are implicated in

promoting resistance to platinum-based chemotherapy in ovarian cancer patients [23]. Also, both PDGFR-beta and VEGFR-2 may become novel predictive biomarkers for therapy resistance and for overall and progression-free survival [23]. Moreover, despite the benefits on antiangiogenic therapy, a complex and properly defined therapeutic management in ovarian cancers may also include AXL receptor tyrosine kinase (AXL-RTK) inhibitors that detain a certified role in suppressing tumor growth and progression [24].

In the past year, a great range of therapeutic substances have emerged following both clinical and experimental trials focused on the different types of ovarian cancers. Besides bevacizumab, paclitaxel and carboplatin based chemotherapy, PARP (poly-ADP ribose polymerase) platinum and even immunotherapy are currently being taken into consideration for the management of patients diagnosed with ovarian cancer [25-28]. Antiangiogenic therapy and immunotherapy seem to become one of the major focuses of future scientific studies concerning ovarian cancers. Lyons et al. have demonstrated that ovarian tumor assoiated macrophages act as pro-angiogenic factors and that macrophage inhibition using CSF1R inhibitors determines the reduction of tumor growth [29]. VEGF is thus not the only factor that promotes ovarian cancer associated angiogenesis, several other molecules and even cells of the imune system being implicated either as independent factors or in association with VEGF. Considering these aspects, vascular endothelial cadherin (VEC) has gained interest in the research field of ovarian cancers due to its role in activating endothelial genes and triggering stability-related genes thus exerting a direct influence on the ovarian carcinoma vasculature [30]. Also, glycodelin is an important promoter of tumor angiogenesis in ovarian cancer and influences the differentiation and function of immune cells such as T and B cells, dendritic cells, macrophages and NK cells [31]. Under these circumstances, glycodelin may become an effective target in antiangiogenic therapy and immunotherapy in ovarian cancers. A potential combined antiangiogenic and immunotherapeutic strategy for patients diagnosed with malignant lesions of the ovary seems promising but is in need of further investigations.

Conclusions

We found a statistically significant correlation between MVD, tumor stage ($p < 0.00021$) and degree of differentiation ($p < 0.0032$). We noticed no statistically significant correlation neither with the patients' age ($p = 0.33$), nor with the histopathological type of ovarian cancer ($p < 0.24$). 43 cases (69.35%) out of the total number of 62 cases were positive for Thrombospondin-1, but the results could not be correlated with MVD values when comparing serous with non-serous tumors. According to these results we conclude that MVD may be regarded as a useful indicator for local tumor progression and may al least partially explain the angiogenic behavior and distant dissemination of ovarian carcinomas cells. However, ovarian cancers are extremely heterogeneous diseases that require the discovery of well defined prognostic and therapeutic biomarkers. As stated above, ovarian tumor associated angiogenesis is a complex and poorly understood phenomenon that needs to be fully comprehended in order to ensure a proper patient management. MVD and VEGF represent only a small part of the numerous factors that influence tumor growth, angiogenesis, local dissemination and distant metastases.

Based on the discrepancies between VEGF expression, microvessel density and their correlation with clinical parameters, our results suggest a more complex angiogenic mechanism in ovarian cancer. The conflicting data arising from this study supports a more elaborate angiogenic process in ovarian cancer, involving other factors than VEGF. This aspect is sustained by the lack of correlation between VEGF and clinical parameters, and a significant correlation between microvessel density and clinicopathological parameters. Also, antiangiogenic treatment in ovarian cancers depending on their angiogenic profile is applicable to primary tumors. As far as we know, no scientific data is available in literature regarding the angiogenic profile of ovarian cancer metastases. Whether the angiogenic profile of the metastasis is different from that of the primary tumor is a controversial issue to be solved through further experimental and clinical trials. From this point of view, further studies may be able o ensure a complete evaluation of angiogenesis in ovarian cancer based on the current data regarding early and advanced stages of this disease. Ovarian cancers are heterogeneous pathological entities that include a wide range of genetic abnormalities and a variable clinical and biological behavior, thus being subjects to individualized and targeted therapies. Also, we support the refinement of the patient selection process in order to reduce the risk of false negative results following the application of novel therapeutic strategies such as antiangiogenic treatment and immunotherapy.

In another papers were studied the correlation between histopatological form and the degree og neuroendocrine differentiations in prostate cancer [32] and the reticular network contributes to the staging of idiopathic lung fibrosis [33].

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Correlation between histopathological form and the degree of neuroendocrine differentiations in prostate cancer

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Abstract

Prostate cancer (PCa) is the most frequent neoplasic condition in males, but only 64–65% of the cases are sensitive to hormone therapy. The aim of this study was to investigate the neuroendocrine component of the prostatic carcinoma, in relation to the histopathological form and the degree of differentiation. Biopsies were obtained through transurethral resection, from 82 patients with prostate cancer. In order to assess the histopathological form and the Gleason score, one section from each case was stained with Hematoxylin–Eosin. Additional sections were stained with chromogranin A. We considered neuroendocrine cell hyperplasia to have a higher value than that observed in benign prostatic hyperplasia (BPH) and normal prostate (over three neuroendocrine cells/gland). The quantification of neuroendocrine differentiation (NED) has been significant; the reaction was considered to be weak (2–10% neuroendocrine cells), moderate (10–20%) and intense (over 50%). Cells positive for chromogranin A have been identified in all the cases, but a larger number than that registered in normal tissue has been noted in 59 patients (71.95%). In most of the cases, the neuroendocrine cells have been distributed in small groups among the neoplasic cells, and rarely isolated. In two cases of small cell carcinoma most of the tumoral cells have been positive for chromogranin A. In conclusion, the study of neuroendocrine differentiation in patients with prostatic carcinoma revealed hyperplasia of positive chromogranin A cells, in 71.95% of cases. Neuroendocrine prostatic differentiation is correlated with the advanced stage of evolution and possibly with the resistance to hormonal treatment.

Keywords: chromogranin A, neuroendocrine differentiation, prostatic adenocarcinoma, small cell carcinoma.

Introduction

Prostate tumors with neuroendocrine differentiation represent a heterogeneous group of entities. There is no accepted definition of NED in PCa. NED is often identified by scattered clusters of differentiated NE cells, among a predominant population of adenocarcinoma cells, except for rare cases of small cell carcinoma or carcinoid [1]. The prostatic small cell carcinoma is a tumor that exhibits a very aggressive behavior. Most of the patients exhibit metastasis at the time of diagnostic, with low chances of survival. The decrease is registered in less than two years after setting the diagnostic. Adenocarcinoma with neuroendocrine differentiation expresses neuroendocrine markers, prostate-specific antigen (PSA) and specific acid phosphatase (PSAP). Establishing the differential diagnostic helps detect malignant prostatic lesions. Neuroendocrine differentiation can be unapparent on the morphological Hematoxylin–Eosin staining, being detectable only in immunohistochemistry. Palapattu GS *et al.* have recently shown [2] that neuroendocrine tumor cells of prostate cancer selectively express CD44 *in vivo* and *in vitro*, leading them to conclude that the presence of such cells is significant for therapy resistance and tumor recurrence. Furthermore, the number of cells co-expressing Oct4, a stemness marker, and chromogranin A or synaptophysin is increased in prostate cancer compared to benign prostate, and these cells represent NE-like prostate cancer cells [3]. AR-independent mechanisms of androgen-independent PCa could be, at least in part, due to the presence of NE differentiation. Malignant NE cells do not express AR, are more resistant to apoptosis, and also express and secrete a number of molecules that can act as anti-apoptotic and growth factors on adenocarcinoma cells [4]. Neuro-endocrine differentiation (NED) and hormone refractory disease seem to be associated phenomena: extensive NED of a tumor renders it androgen-independence, and androgen blockade induces NED. Moreover, the extent of neuroendocrine component in a prostatic tumor is related to Gleason score, thus advanced prostatic cancer, which is the main indication for hormone therapy, already has, in most instances, a significant neuroendocrine component. The aim of this study was to investigate the neuroendocrine component in prostate cancer, correlated with the histopathological form and the degree of differentiation.

Materials and Methods

We have investigated 82 cases of prostate tumors obtained through biopsies. Most of the patients were

aged between 70–80 years; only five cases were aged between 40–50 years. The PSA level was elevated in 39 cases (more than 10 ng/mL), associated with the clinical symptomatology. After 48 hours fixation in 10% buffered formalin, and paraffin embedding, 3 µm sections were performed for each case. To establish histopathological diagnosis and the Gleason score, one slide, from each case, was stained using the standard method Hematoxylin–Eosin. To determine neuroendocrine differentiation, immunohistochemical staining with chromogranin A (CH A) was performed for each case. The dew axed and rehydrated sections were heated in a microwave oven, in pH 6 citrated buffer, for 10 minutes, for antigen retrieval. Endogenous peroxidase was inhibited using 3% oxygenated water for 5 minutes. The slides were incubated with primary antibody (polyclonal rabbit anti-human chromogranin A), 1:400 dilution for 30 minutes. We used EnVision as working system (DAKO Denmark), and the final reaction product was visualized, in brown, with 3,3'-diaminobenzidine. The nuclear staining was performed with Lillie's watery Hematoxylin. We used as positive control the neuroendocrine cells from normal prostate glands and negative control the basal and secretory cells from normal tissue. In normal tissue, we identified 1–2 neuroendocrine cells per gland. We have investigated the neuroendocrine component of the prostatic carcinoma in relation to the histopathological form, and the degree of differentiation. The results were assessed with Nikon Eclipse E600 microscope and images (taken in JPEG format) were captured and processed using Lucia G software system.

Results

Two types of neuroendocrine cells were identified in the normal zone: the closed type and the open type (Figure 1). In neuroendocrine cell hyperplasia (Figure 2), we found up to three times more neuroendocrine cells per gland than in the normal prostate (one or two cells per gland) and benign prostatic hyperplasia. In cases with prostatic adenocarcinoma the positive cells were assessed according to the number of positive cells per gland. In cases with NED, the neuroendocrine cells were organized in clusters in the malignant glands. We have identified 82 cases of prostatic adenocarcinoma two of them being small cell carcinoma using the standard method Hematoxylin–Eosin. The tumors have been identified in the peripheral zone of the prostate. For the 82 cases of adenocarcinoma, the Gleason score varied between 3 and 10; most of the tumors had a lower score (Gleason less than 8), in four cases, Gleason score was 8 or 9, and for the two small cell carcinoma cases the Gleason score was 10. The neuroendocrine differentiation was weak (2–10%) with Gleason score 3–5 (Figure 3) and moderate (10–20%) with Gleason score 5–8 for adenocarcinoma cases, and intense (over 50%) with Gleason score 8–10 (Figure 4) for undifferentiated adenocarcinoma and small cell carcinoma. We have identified positive CH A cells in all cases. A larger number than that registered in normal tissue was identified in 59 patients, representing 71.95%. In the two cases with prostatic small cell carcinoma, the neuroendocrine differentiation was almost complete, most of the tumoral cells being positive for CH A. The two patients with small cell carcinoma underwent cytostatic and hormone treatment, but they died at 8, respectively 11 months from the diagnostic. In the prostatic adenocarcinoma cases, we observed the presence of isolated neuroendocrine cells, moderate (Figure 5) and reduced neuroendocrine differentiation (Figure 6), and also the presence of intratumoral positive CH A cells. Opposed to the small cell carcinoma, where the neuroendocrine differentiation was organized in large, compact groups (Figure 7), in adenocarcinoma neuroendocrine differentiation appears in small groups (Figure 8). Patients' survival was determined by Gleason score; the survival of patients with Gleason score 3–5 was good – 11 of 17 are still alive, four of them being dead of other causes than prostatic carcinoma; with Gleason score 5–8, 44 of 59 are alive, and form those with Gleason 8–10, only one is still live (Table 1).

Table 1 – *Neuroendocrine differentiation (NED) and Gleason score*

NED	Gleason score	No. of cases	%
2–10%	3–5	17	20.73
10–20%	5–8	59	71.95
>50%	8–10	6	7.31

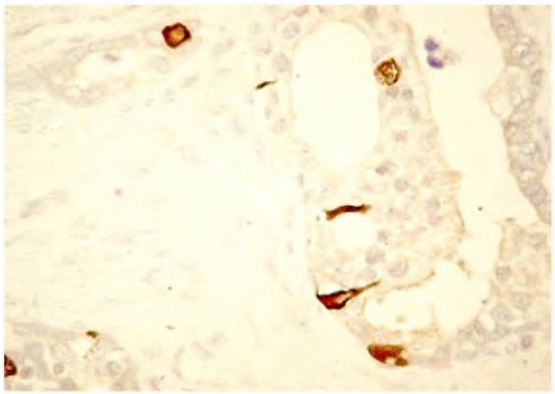

Figure 1 – *Close-type, open-type neuroendocrine cells, CH A positive, 100×.*

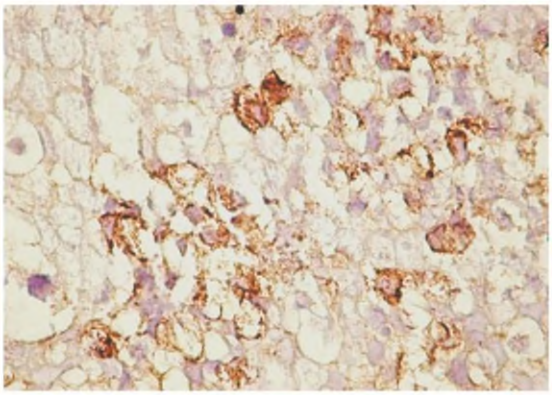

Figure 2 – *Neuroendocrine cell hyperplasia positive CH A, 100×.*

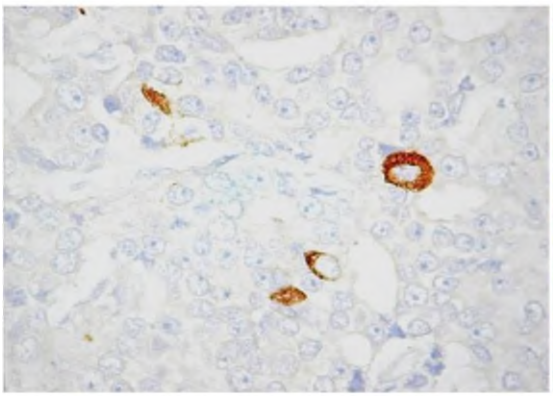

Figure 3 – ***Reduced neuroendocrine differentiation, CH A positive, 100×.***

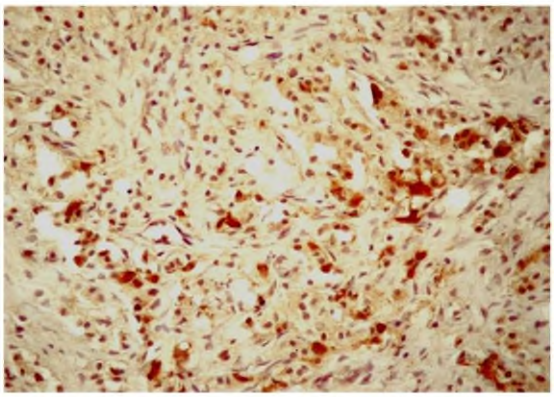

Figure 4 – ***Intense neuroendocrine differentiation, CH A positive, 100×.***

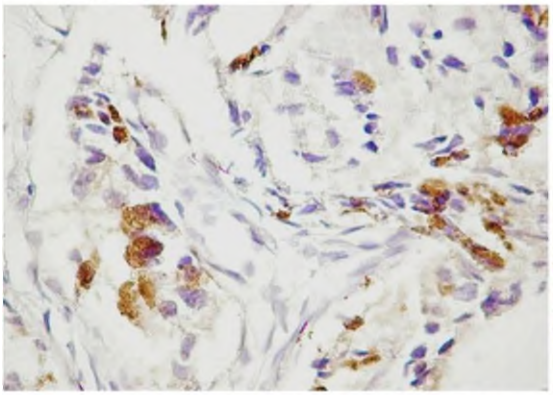

Figure 5 – ***Prostatic adenocarcinoma, small groups, CH A positive, 100×.***

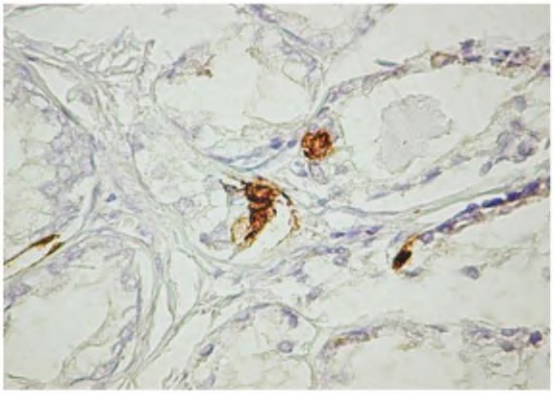

Figure 6 – ***Prostatic adenocarcinoma, isolated cells, CH A positive, 100×.***

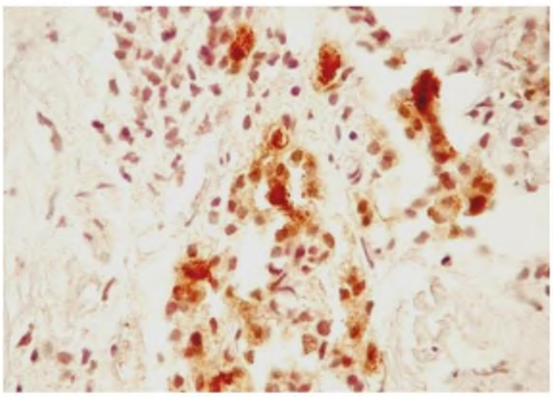

Figure 7 – ***Small cell carcinoma, cells in compact groups CH A positive, 100×.***

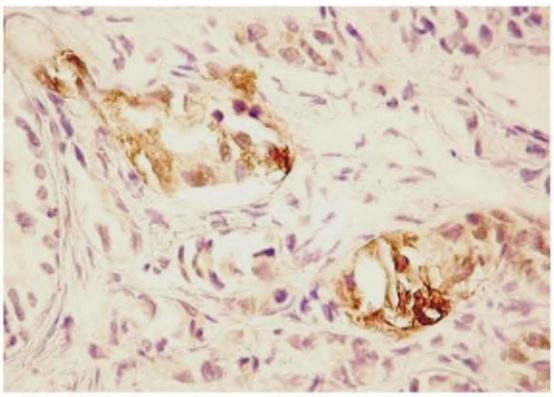

Figure 8 – ***Prostatic adenocarcinoma, intratumoral positive cells, CH A positive, 200×.***

Discussion

The normal neuroendocrine cells and the paracrine-endocrine cells are part of the diffuse neuroendocrine system. In human prostate, these cells have been identified on morphological basis and based on secreted neuroendocrine factors. The origin of NE cells and the molecular mechanism of NE cell enrichment during prostatic carcinoma progression are not fully understood. Recent data suggest that adenocarcinoma cells undergo a transdifferentiation process to become NE-like cells. These cells acquire the NE phenotype and express NE markers, and could be termed 'NE-like PCa cells' [5].

Neuroendocrine differentiation is focally present in all cases of PCa. The number of detected cells in each case varies with fixation, tissue sectioning, antibody detection method, and number of the examined sections. Understanding the role played by NED in prostate cancer is essential because most of the phenotypes are associated with a reserved prognostic and the progression towards independent androgen tumors [6, 7].

Weinstein MH *et al.* [8] studied 104 patients with clinically organ-confined PCa treated only by radical prostatectomy, with the end-point of biochemical

disease progression. Results showed that histological grade and NE differentiation seen in prostatectomy samples predicted progression in multivariate analysis. Moreover, the extent of NE differentiation (more than 70 chromogranin A positive cells per representative section, as revealed by immunohistochemistry) separated patients with tumors of Gleason sum less than, or equal to six, into groups with high and low risk for progression, independent of Gleason sum. The latter observation could provide a basis for stratifying the estimated 85% of newly diagnosed prostate cancers that are organ confined [9]. In our study, neuroendocrine cells identified with chromogranin A were positive in 59 of 82 cases (71.95%).

Neuroendocrine cells have been observed in prostate cancer, their number increasing in accordance with the tumoral stage and degree and particularly with the androgenic deprival. This aspect is associated with the results obtained in the present study, in areas of normal prostate and those of benign prostatic hyperplasia where we identified neuroendocrine cell hyperplasia (more than three cells per gland). At the level of all prostatic adenocarcinoma studied by us, we have observed positive cells for chromogranin A. The study performed by Shimizu S *et al.* in 2007 [10] uses CH A for the identification and quantification of tumoral neuroendocrine cells (NETC); the identified positive cells presented characteristics specific to tumoral cells. In our study we quantified the positive reaction of neuroendocrine as follows: weak (2–10%), moderate (10–20%), and intense (over 50%). Abrahamsson PA *et al.* [11] could not find a correlation between the presence of immunohistochemically positive NE cells and long-term survival. In our study, survival was quantified only in the cases of small cell carcinoma when decease occurred at eight respectively 11 months after the administration of hormone and cytostatic treatment. In these two cases, neuroendocrine differentiation has been quantified as intense (over 50%) associated with a Gleason score of 8–10. The highly aggressive pattern of this tumoral type has been described in a series of studies performed by di Sant'Agnese PA [6]; these tumors are very rare, only 1–2% of the total amount of prostatic tumors being described as part of the conventional adenocarcinoma. The correlation between the Gleason score and the neuroendocrine differentiation was identified in a study performed by Berruti A *et al.* in 2005 [12]. In this case, the relation between the neuroendocrine differentiation and the undifferentiated tumors has been determined. This aspect has also been demonstrated in our study, in the areas of intense neuroendocrine differentiation (over 50%), the Gleason score being over 8.

Conclusions

In the present study, we have immunohistochemically identified the neuroendocrine differentiation of prostate cancer with chromogranin A. In prostatic adenocarcinoma, two degrees of neuroendocrine differentiation were established by correlation with the Gleason score: weak (3–5) and moderate (5–8). In small cell carcinoma, the Gleason score was 8–10 and the neuroendocrine differentiation was intense. We demonstrated that neuroendocrine differentiation, detected with chromogranin A, is associated with Gleason score, aggressive disease and low rate of survival. CH A immunoreactivity is a prognostic marker of disease and is superior to standard pathologic diagnosis.

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The Mesenchymal–Epithelial and Epithelial–Mesenchymal Cellular Plasticity of Liver Metastases with Digestive Origin

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Abstract. *Background: Few data are available regarding the epithelial to mesenchymal transition (EMT) /mesenchymal to epitheilal transition (MET) in the liver metastasis of digestive cancers. The aim of this study was to establish EMT/MET metastatic tumor cell plasticity according with the histological growth pattern of liver metastases. Materials and Methods: Biopsies from 25 patients with liver metastasis (desmoplastic, replacement and pushing type) were evaluated. Double immunostaining of E-cadherin/vimentin, keratin 8,18/vimentin and E-cadherin/ keratin 8,18 were performed. Results: The following cell types were noted: epithelial, mesenchymal, non-differentiated and differentiated hybrid mesenchymal/ epithelial and non-hybrid phenotype. All of the cases had mesenchymal/ epithelial phenotype cells. A significant correlation was found between the non-differentiated hybrid mesenchymal/ epithelial phenotype metastatic cells and histological growth pattern for gastric and colorectal cancer. Conclusion: An MET-targeting strategy, in conjunction with conventional chemotherapy, for treatment of liver metastases may be useful.*

The epithelial to mesenchymal transition (EMT) represents the conversion of epithelial cell phenotype to mesenchymal cell phenotype, which is characterized by elongated, spindle shape cells. The mesenchymal to epithelial transition (MET) is the reverse phenomenon. There are three cell phenotypes described in the EMT/MET process: epithelial, mesenchymal and a hybrid epithelial/mesenchymal (partial or intermediate EMT) phenotype (1).

EMT was described in embryogenesis and organ development in physiological situations (2-4). In malignant lesions, EMT involves cytoskeletal disorders, loss of cell–cell adhesion and apical-basal cell polarity. In colorectal carcinoma, EMT was found in the cells from the invasive front (5). In pancreatic adenocarcinoma, the loss of E-cadherin expression was noted in well- and poorly differentiated ductal adenocarcinoma, and few or none of the undifferentiated carcinomas (6, 7). Fewer than 50% of gastric cancer cells express E-cadherin. It was shown that the diffuse-type gastric cancer is associated with the E-cadherin/N-cadherin switch, whereas the intestinal-type is related to up-regulation of transforming growth factor beta (TGF)β/loss of E-cadherin (8).

Data from the literature show, using murine experimental models of metastasis, the presence of MET phenomenon in liver, lung, and brain metastases (9-11). Liver metastases from prostate cancer showed epithelial morphology in most cases (12). In liver metastases of colorectal adenocarcinoma, the increased expression of E-cadherin and decreased vimentin expression was noted (13, 14). More recently, in an experimental model, the involvement of sciellin as an inducer of MET through the liver metastasis process of colorectal cancer was shown (15). It increased Wnt signaling and favored MET through the sciellin–β-catenin–E-cadherin axis. Thus, in both mouse and human pancreatic ductal adenocarcinoma, metastatic cells appear to re-acquire an epithelial phenotype with increasing lesion size. Immunohistochemical analysis revealed a higher immunoexpression intensity of claudin 7 in primary tumors than in micro-metastases. Increased immunoexpression of fibroblast-specific protein 1 was found in micro-metastases compared to gross metastases and primary tumors (16).

The replacement growth pattern was suggested as being prevalent in liver metastases with pancreatic origin (17) and the pushing type for the gastric origin (18). The following

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types of growth were described for liver metastases of colorectal cancer: replacement, pushing, desmoplastic and mixed. The prognostic role of the histological growth pattern of liver metastasis was shown at diagnosis but also after portal vein embolization in colorectal cancer (19, 20).

The aim of this study was to establish metastatic tumor cell plasticity according to the histological growth pattern of liver metastases with colorectal, pancreatic and gastric origins.

Materials and Methods

The present study included 25 patients operated on between 2009-2016. Liver metastasis was of different origins: colorectal adenocarcinoma in 15 cases, pancreatic in seven cases and gastric adenocarcinoma in three cases. Signed consent was obtained from each patient included in this study.

All procedures were carried out according to the principles of the Declaration of Helsinki and were approved by the Institutional Review Board (no. 7339/22.04.2016). The patients included in the study underwent excisional tumorectomy of the liver metastasis. Metastatic fragments were fixed in 10% buffered formalin for 24 hours and paraffin embedded. Morphological and immunohistochemical staining were performed.

Double immunostaining techniques for keratin 18/vimentin and E-cadherin/vimentin were used. Heat-induced epitope retrieval with Bond Epitope Retrieval Solution 2 (ready-to-use, pH 9.0; Leica Biosystems, Newcastle Ltd, Newcastle upon Tyne, UK) for 20 minutes was the first step. Endogenous peroxidase blocking was achieved with 3% hydrogen peroxide for 5 minutes. The following primary antibodies were used: keratin 8,18 (monoclonal, clone 5 D3, ready to use), E-cadherin (monoclonal, clone 36B5, ready to use), vimentin (monoclonal, clone V9, ready to use). All antibodies used were from Leica Bond Biosystems. The Bond Polymer Refine Detection System and The Bond Polymer Refine Red Detection System were used for visualization. As chromogen, 3,3-diaminobenzidine dyhidrochloride was applied for 10 minutes, and hematoxylin was used as a counterstain for 5 minutes. The entire immunohistochemical procedure was performed with Leica Bond-Max (Leica Biosystems) autostainer.

The immunoreactivity was estimated as following: keratin 8,18/vimentin: red cytoplasmic/brown cytoplasmic, E-cadherin/vimentin: brown membranous/red cytoplasmic and E-cadherin/keratin 8,18: brown membranous/red cytoplasmic. Microscopic evaluation and image acquisition was performed with Axiocam 506 color (Zeiss, Jena, Germany). The mesenchymal/epithelial hybrid phenotype cells were quantified at ×400 magnification in three consecutive areas with highest density. For statistical analyses, SPSS 17 software (IBM Analytics, Armonk, NY, USA) was used; differences with $p=0.05$ were considered statistically significant.

Results

The microscopic evaluation of hematoxylin and eosin-stained liver metastases of digestive origin revealed three histological growth patterns, as following: desmoplastic (28%), pushing (32%) and replacement (40%). All of the liver metastases of pancreatic origin included in the study had a replacement growth pattern, while those with gastric origin had the pushing growth pattern. The colorectal liver metastases exhibited all three histological growth patterns. Most of the liver metastases described above had G2 tumor grade (48%), followed by G3 (44%) and G1 (8%).

Double immunostaining for E-cadherin/vimentin and keratin 8 or 18/vimentin revealed the following cell types for the desmoplastic (seven cases), pushing (five cases) and replacement histological growth pattern of CRCLM (three cases): epithelial phenotype: E-cadherin$^+$/vimentin$^-$, keratin 8,18$^+$/vimentin$^-$ metastatic cells; mesothelial phenotype: E-cadherin$^-$/vimentin$^+$, keratin 8,18$^-$/vimentin$^+$; mesothelial/epithelial or non-differentiated hybrid phenotype: E-cadherin$^+$/vimentin$^+$, keratin 8,18$^+$/vimentin$^+$; and non-hybrid phenotype: E-cadherin$^-$/vimentin$^-$, keratin 8,18$^-$/vimentin$^-$ (Figure 1a). A heterogeneous expression of E-cadherin, keratin 8,18 with values ranged between 1 to 3 for epithelial phenotype metastatic cells was present. The mesothelial/epithelial hybrid non-differentiated phenotype cells were noted inside of the areas with decreased E-cadherin, keratin 8,18 expression. All of the liver metastases presented hybrid phenotype cells. The distribution of hybrid non-differentiated phenotype cells was isolated or in clusters for desmoplastic (Figure 1b, c) and replacement types, and isolated for the pushing type (Figure 1d).

The same heterogeneous patterns of E-cadherin and keratin 8/18 expression were noted in the primary tumors of desmoplastic, pushing and replacement types of colorectal cancer liver metastases. A tendency for non-differentiated hybrid type tumor cells to localize in the basal part of the glands (desmoplastic type) and in the basal (Figure 1e), intermediate and luminal part of the glands (replacement type) was noted. In the primary tumor of replacement model, the luminal hybrid non-differentiated cells (E-cadherin$^+$/vimentin$^+$; keratin 8,18$^+$/vimentin$^+$) had cellular shape changes, such as hybrid ameboid/mesenchymal morphology (Figure 1f). One desmoplastic type corresponding primary tumor was characterized by the absence of hybrid non-differentiated tumor cells.

In the replacement and pushing histological growth pattern of liver metastases and corresponding primary tumors with pancreatic (seven cases) and gastric (three cases) origin, the heterogeneous profile of E-cadherin/vimentin, keratin 8/18 immunoexpression was maintained. The distribution of hybrid phenotype cells was isolated for pushing type liver metastases growth pattern (gastric; Figure 1g) and isolated or clusters for replacement types (pancreatic origin) types.

The relation between the arithmetic averages of hybrid non-differentiated cells the distribution pattern in primary tumors and the histological growth pattern of liver metastases are summarized in the Table I.

The double immunostaining of E-cadherin and keratin 8/18 revealed the expression of keratin 8/18 to be predominant in hepatic-metastatic cells of colorectal,

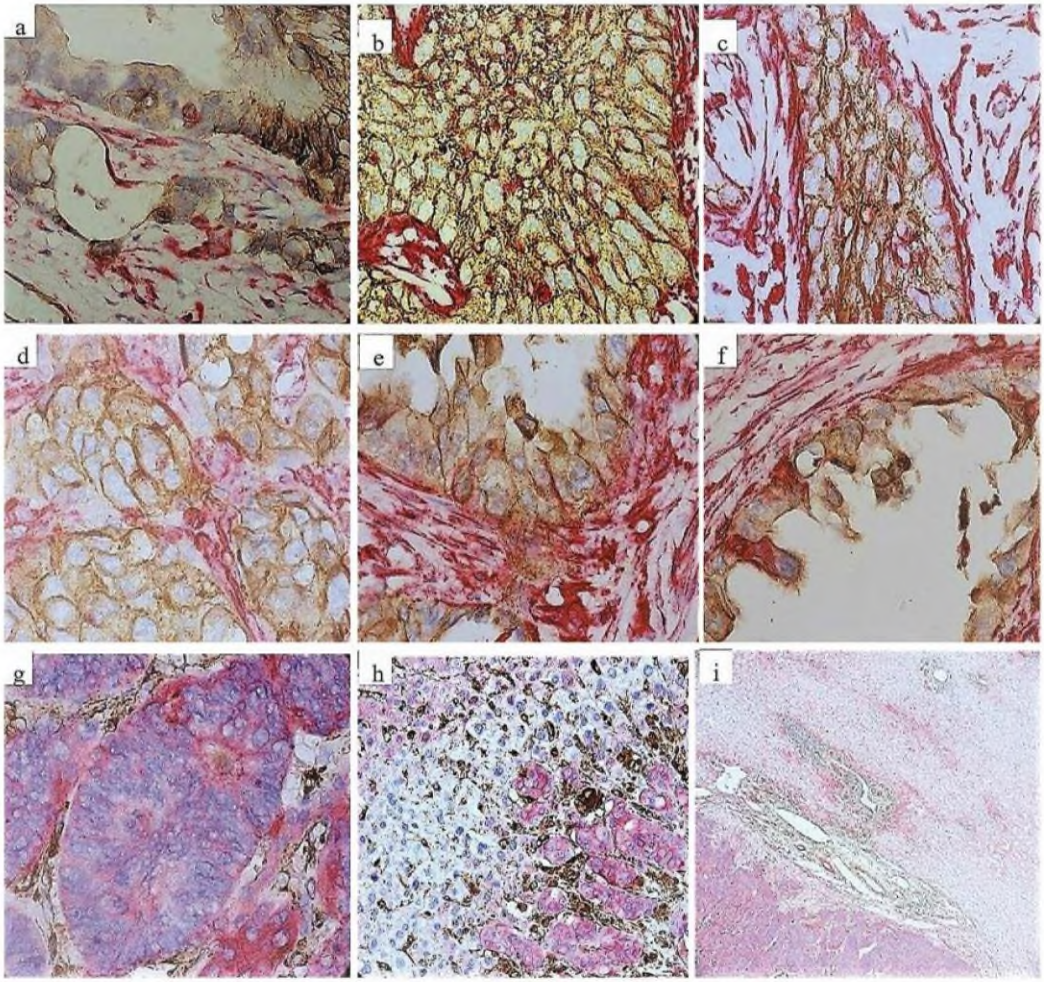

Figure 1. *Different patterns of epithelial to mesenchymal transition in liver metastases of various origins. a: Non-hybrid phenotype cells, E-cadherin−/vimentin−, in the replacement growth pattern of colorectal liver metastases (CRCLM) E-cadherin/vimentin double immunostaining, ×1000 magnification. b: Isolated cell with hybrid phenotype in desmoplastic type of CRCLM, E-cadherin/vimentin double immunostaining, ×400 magnification. c: Clusters of hybrid cells, desmoplastic type of CRCLM, E-cadherin/vimentin double immunostaining, ×400 magnification. d: Isolated hybrid phenotype cells in the pushing type of CRCLM, E-cadherin/vimentin double immunostaining, ×400 magnification. e: Isolated cell basal hybrid phenotype in the replacement type CRCLM, E-cadherin/vimentin double immunostaining, ×400 magnification. f: The amoeboid/mesenchymal morphology of hybrid phenotype cell in the replacement type of CRCLM, E-cadherin/vimentin double immunostaining, ×1000 magnification. g: Isolated hybrid phenotype cell in the pushing type CRCLM, keratin 8,18/vimentin double immunostaining, ×1000 magnification. h: Keratin 8/18 expression in metastatic epithelial phenotype cells and hepatocytes in the replacement type of a pancreatic liver metastasis keratin 8,18/vimentin double immunostaining, ×400. i: Keratin 8/18 immunostaining with intensity value of 3 in hepatocytes close to the portal spaces, keratin 8,18/vimentin double immunostaining, ×200 magnification.*

pancreatic and gastric cancer. The distribution of co-expressing E-cadherin^{+}/keratin 8/18^{+} cells (hybrid differentiated phenotype) was isolated for pushing type (colorectal and gastric) and isolated or clustered cells for replacement and desmoplastic growth pattern of liver metastases (colorectal and pancreatic) types.

Regarding the E-cadherin/vimentin and keratin 8,18/vimentin immunoexpression at the border between metastases and the adjacent liver, all of the cases had higher intensity of E-cadherin and keratin expression in the metastatic epithelial phenotype cell compare with hepatocytes (Figure 1h), with a reduced intensity in the hepatocytes in the immediate vicinity of metastases, but

Table I. *The relation between the arithmetic average of hybrid non-differentiated cells, histological growth pattern of liver metastases and the distribution pattern in primary tumors and liver metastases.*

Histological growth pattern of liver metastases/ primary tumor	Hybrid non-differentiated cells in liver metastases (E-cadherin$^+$/vimentin$^+$/ primary tumor)		Hybrid non-differentiated cells (keratin 8/18$^+$/vimentin$^+$/ primary tumor)		The distribution pattern of hybrid nondifferentiated cells in liver metastases and primary tumors	
CRCLM, desmoplastic type	3.14	2	3.71	2.85	Isolated or cluster	Isolated or clusters, predominantely in the basal region of the glands
CRCLM, pushing type	2.6	2.8	1.2	1.6	Isolated	Isolated
CRCLM, replacement type	4.33	4.66	3.33	6.66	Isolated or clusters	Isolated or clusters; basal, intermediate and luminal part of the glands; hybrid, ameboid/mesenchymal changes
PLM, replacement type	2	4.85	3	5.42	Isolated or clusters	Isolated or clusters
GLM, pushing type	4	7	4	5	Isolated	Isolated

CRCLM: Colorectal liver metastases, PLM: pancreatic liver metastases, GLM: gastric liver metastases.

with increased intensity at a distance from them. In cases with tumor invasion of the portal space, the hepatocytes in close vicinity to the portal space had high intensity of keratin 8/18 and E-cadherin immunoexpression, independent of histological growth pattern of liver metastases (Figure 1i).

For the primary colorectal cancer tumors, significant correlation was found between the presence of non-differentiated hybrid phenotype cells (E-cadherin$^+$/vimentin$^+$; keratin 8,18$^+$/vimentin$^+$) and the histological growth pattern (p=0.003; p=0.025). Significant correlation was noted between the number of non-differentiated hybrid phenotype cells (keratin 8,18$^+$/vimentin$^+$) and differentiated hybrid phenotype (keratin 8,18$^+$/E-cadherin$^+$) in primary tumors, and those in liver metastases: p=0.009 and p=0.044, respectively. A significant correlation was noted between the number of differentiated hybrid phenotype cells (E-cadherin$^+$/keratin 8,18$^+$) and of non-differentiated hybrid phenotype cells (keratin 8,18$^+$/vimentin$^+$, p=0.05; E-cadherin$^+$/vimentin$^+$, p=0.032) in liver metastases. A similar association was present in primary tumor (p=0.050).

For pancreatic cancer, a significant correlation was found between the number of differentiated hybrid phenotype cells and differentiation grade G in primary tumor and corresponding liver metastases (p=0.05).

A significant correlation between the number of non-differentiated hybrid phenotype cells (E-cadherin$^+$/vimentin$^+$) and the histological growth pattern characterized gastric cancer (p=0.005).

Discussion

In normal situations, E-cadherin, a calcium-dependent cell-adhesion molecule is necessary for epithelial histogenesis, tissue stabilization, differentiation and induction of EMT during embryogenesis (21, 22). In pathological situations, abnormal expression of E-cadherin and β-catenin favor the mesenchyme phenotype cell (23).

Keratin 8/18 expression in normal conditions was found in simple epithelium (liver, pancreas, kidney), mixed epithelium (breast, lung) and is involved in embryogenesis (24, 25). In pathological situations, its increased expression was noted in adenocarcinomas and squamous cell carcinoma with different localization (26, 27). The mains roles of keratin 8/18 were: modulation of protein localization, protein targeting and apoptosis (28).

Data from the literature show that MET is a part of the metastatic process, in which the tumor cells regain epithelial properties at their secondary site (29, 30). It was noted that metastatic lesions had the same features of epithelial immunoexpression markers as primary tumors (31, 32). This pattern was found in our study. The hybrid differentiated cell type (E-cadherin$^+$/keratin 8/18$^+$) was found in metastatic cells. Significant correlation between the presence of co-expressing E-cadherin/keratin 8/18 cells in primary tumor and colorectal cancer liver metastases was found also. These observations may support the idea that a constant number of hybrid differentiated cells from primary tumors migrate to the secondary organ and maintain the same phenotype there. The existence of significant correlation between the non-differentiated hybrid phenotype cells in primary tumor and differentiated hybrid phenotype cells in colorectal cancer liver metastases argues for EMT/MET and MET/EMT plasticity of some tumor cells. This hypothesis was sustained by a significant correlation between non-differentiated and differentiated hybrid phenotype cells in both primary tumor and liver metastases of colorectal carcinoma.

Strauss *et al*. showed that some cells with hybrid epithelial/mesothelial phenotype in primary ovarian cultures and tumors in situ can be multipotent, express markers of other lineages, and drive tumor growth *in vivo* by giving rise to another epithelial/mesothelial subset as well as completely differentiated epithelial cells (33). Partial loss of E-cadherin expression was associated with carcinoma progression and unfavorable prognosis. In the final stages of numerous carcinomas, E-cadherin expression appears to be heterogeneous, with E-cadherin$^-$ tumor cells interposed between E-cadherin$^+$ tumor cell areas, suggesting that certain carcinoma cells have EMT properties (34). The same heterogeneous phenotype cells, with E-cadherin$^+$/vimentin$^-$, E-cadherin$^+$/keratin 8,18$^+$, keratin 8,18$^+$/vimentin$^-$ cells between cells without immunoexpression of epithelial markers were noted in the liver metastases and corresponding primary tumors analyzed in the present study. Over 75% of circulating tumor cells in women with metastatic breast cancer were found to co-express epithelial marker, mesenchymal marker *N*-cadherin, and stem cell markers (35).

It was shown for the patients with replacement growth pattern of CRCLM that the hazard of death was 2-2.5 times higher than for patients with pushing growth or mixed growth pattern, and nearly four times higher than for patients with desmoplastic growth pattern. The negative prognostic effect of the replacement growth pattern was even more pronounced after adjusting for tumor size (20). These findings correspond to the highest values of differentiated and non-differentiated hybrid cell types for the replacement histological growth pattern. Significant correlation was found between the histological growth pattern and frequency of cells with non-differentiated phenotype in colorectal and gastric cancer.

EMT was also observed in other cancer types and also in their corresponding metastases (36). Thus EMT has already become a target for several different therapeutic agents tested in experimental or preclinical studies (37, 38).

Conclusion

This study suggests the existence of significant correlation between the presence of non-differentiated hybrid phenotype cells in primary tumor and differentiated hybrid phenotype cells in colorectal cancer liver metastases. These support the hypothesis of EMT/MET and MET/EMT plasticity of some tumor cells in liver metastases. The existence of significant correlation between the cells with non-differentiated hybrid phenotype and the histological growth pattern in colorectal and gastric cancer indicates the potential for an MET-targeting strategy, in conjunction with conventional chemotherapy, for treatment of liver metastases of digestive origin. Onica for their excellent technical support.

Conflicts of Interests

None declared.

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Original Communication

IMMUNOHISTOCHEMISTRY OF PROSTATE SPECIFIC ANTIGEN IN ADVANCED STAGE PROSTATE CARCINOMA

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RESUMEN

El cáncer de próstata es la malignidad más frecuente en los seres humanos en la actualidad. Los estudios post mortem hacen una estimación que el carcinoma de próstata (PCa) se propagará en 25% de los hombres con enfermedad establecida histológicamente. *Material y método:* Se ha hecho una investigación retrospectiva sobre la expresión inmune histoquímica de antígeno prostático específico (PSA) en 84 pacientes ingresados en el hospital con sospecha clínica de cáncer de próstata. Fueron examinadas portaobjetos del archivo de biopsia de resección transuretral, biopsia por punción con aguja hueca y cirugía abierta. Portaobjetos tenidos fueron utilizados para el diagnóstico patológico y para la puntuacion de Gleason. Portaobjetos adicionales fueron tenidos para antígeno prostático específico y la reacción final del producto fue estimado en negativo (0), bajo/moderado positivo (+1) y positivo intenso (+2). *Resultados:* Hiperplasia benigna próstata fue encontrado en 14 casos, y todos mostraron una reacción moderada /intensa para el antígeno prostático específico. Hiperplasia asociada basal de células fue siempre negativa. Carcinoma fue encontrado en 68 pacientes. La reacción inmune para antígeno prostático específico fue positiva en 88.2% casos, y encontramos una relación directa entre la intensidad de la reacción y la puntuacion de Gleason. Todas las carcinomas uroteliales y pequeñas fueron negativas. La reacción PSA ha detectado 39.68 % de los casos con invasión perineural en comparación con solamente 23.8% encontrado en los portaobjetos tenidos hematoxilina-eosina (H&E). La expresión inmune antígeno prostático específico no discrimina entre lesiones benignas atípicas y el carcinoma bien diferenciados. *Conclusión*: Se concluye que la reacción inmune antígeno prostático específico ayuda mucho para el diagnóstico diferenciado, detección de la invasión perineural, y el metástasis ganglionar.

***Palabras llave**: Carcinoma próstata, Antígeno prostático específico, Inmune histoquímica diagnóstico, Invasión perineural.*

ABSTRACT

Prostate cancer is the most frequent malignancy in human nowadays. Postmortem studies estimate that prostate carcinoma (PCa) will spread in only 25% of men with histologically defined disease. *Material and method*: It was retrospectively investigated the immunohistochemical expression of prostate-specific antigen (PSA) in 84 patients admitted with clinical suspicion of prostate cancer. Slides were performed from archive biopsies taken by transurethral resection, core biopsy and open surgery. Routine stained slides were used for the pathologic diagnosis and Gleason score. Additional slides were stained for PSA, and the final reaction product was estimated as negative (0), weak/moderate positive (+1), and intense positive (+2). *Results*: Benign prostate hyperplasia was found in 14 cases, and all showed moderate/intense reaction for PSA. Associated basal cell hyperplasia was always negative. Carcinoma was found in 68 patients. The immunoreaction for PSA was positive in 88.2% cases, and we found a direct relationship between the intensity of the reaction and Gleason score. All urothelial and small carcinomas were negative. PSA immunoreaction detected 39.68% cases with perineural invasion as compared with only 23.8% found on hematoxylin-eosin (H&E) stained slides. PSA immunoexpression does not discriminate between atypical benign lesions and well-differentiated carcinoma. *Conclusion*: It is concluded that PSA immunoreaction is helpful for the differential diagnosis, detection of the perineural invasion, and lymph node metastases.

***Key words**: Prostate carcinoma, Prostate specific antigen, Immunohistochemistry diagnosis, Perineural invasion*

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INTRODUCTION

Prostate cancer is the most frequently diagnosed cancer in American men and is the second leading cause of cancer deaths (Jemal et al, 2007). Androgen withdrawal initially induces apoptosis and cell cycle arrest in prostate cancer (CaP); however, CaP eventually loses its dependence on androgens and progresses to an androgen independent state.
Postmortem studies estimate that CaP will spread in only 25% of men with histologically defined disease. This is why the early diagnosis is extremely important for the practice, and allows the surgeon to perform the radical prostatectomy. Usually, an increased level of serum prostate specific antigen (PSA) is found in over 78% of patients with prostate cancer. The detection and quantization of different forms of PSA, such as free PSA, can improve the specificity for the use of PSA as a CaP biomarker (De Angelis et al, 2007; Loeb and Catalona, 2007). Our purpose was to investigate, the expression of PSA in different pathologic types of prostate carcinoma, and the possibility to increase detection of perineural invasion with special reference to the relationship between PSA immunohistochemical expression and Gleason score.

MATERIAL AND METHODS

Eighty-four patients with clinical suspicion of prostate carcinoma were investigated. All cases with prostate carcinoma were T3 or T4, and only one had pelvic lymph node dissection. Biopsies were taken by transurethral resection, core biopsy and open surgery. Archive paraffin blocks were used to perform step-sections. Routine haematoxylin-eosin method was made in all cases, for the pathologic diagnosis and Gleason score. Additional sections were stained for PSA, using the monoclonal anti-human PSA antibody (clone ER-PR8, IgG1 Kappa isotype), LSAB2 system (DAKO, Denmark), and the final reaction product was visualized with diaminobenzidine dihidrochlorid (DAB). The final reaction product was stained in brown and nuclei in pale-blue with Mayer's mild haematoxylin. The evaluation of slides stained for PSA consisted in grading the intensity (0 – negative, 1 – weak or moderate positive, and 2 – intense positive). The pattern of distribution was appreciated as homogeneous or heterogeneous, granular or non-granular.

RESULTS

Examination of haematoxylin-eosin stained slides showed the presence of 16 benign lesions and 68 carcinomas. We took in study only cases with prostates carcinoma. Pathologic analysis revealed the high incidence of cases with mix pathological features (32 cases, 48.4%). Frequently, there were associated two or even three patterns (glandular and undifferentiated being the most frequently found). There were detected foci of small cell carcinoma associated to conventional carcinoma in 14 cases. The distribution of cases related to the dominant pattern of the prostate carcinoma is shown in Table 1.

Carcinoma (n=68)	Nr of cases	%
Glandular	16	23.52
Undifferentiated	41	60.29
Cribriform	1	1.47
Ductal	3	4.41
Small cell	3	4.41
Urothelial	2	2.94

Table 1. Distribution of cases considering the dominant pathologic pattern

From each case two slides were stained for PSA, and all were reviewed twice for the intensity of PSA immunoreaction. The intraobserver results overlapped in 64 from 68 cases that mean an accuracy of 94.11%. All urothelial carcinomas (n=2), small cell carcinomas (n=3) and foci of small carcinoma within conventional adeno-carcinoma were PSA-negative. Overall, the reaction for PSA was positive in 60 from 68 cases with prostate carcinoma (88.2%) (Table 2).

PSA intensity	Number of cases	%
Negative (0)	8	11.76
Weak/moderate (1)	19	27.94
Intense (2)	41	60.29

Table 2. Results on the intensity of PSA immunoreaction in prostate carcinoma

In conventional adenocarcinoma the PSA reaction was positive as a fine granular final reaction product that occupies the cytoplasm. The reaction pattern was granular and rarely diffuse (Fig.1). A particular feature is related to the clear cell carcinoma (hypernephroid) and areas with signet cells, where the reaction product is always located at the periphery of the cytoplasm, but all cells are intensely stained. In some cases the reaction was positive for all glandular cells (homogeneous pattern) and in others only isolated cells or clusters were intensely stained (heterogeneous pattern). The last aspect was noticed especially in cribiform carcinoma (Fig. 2).

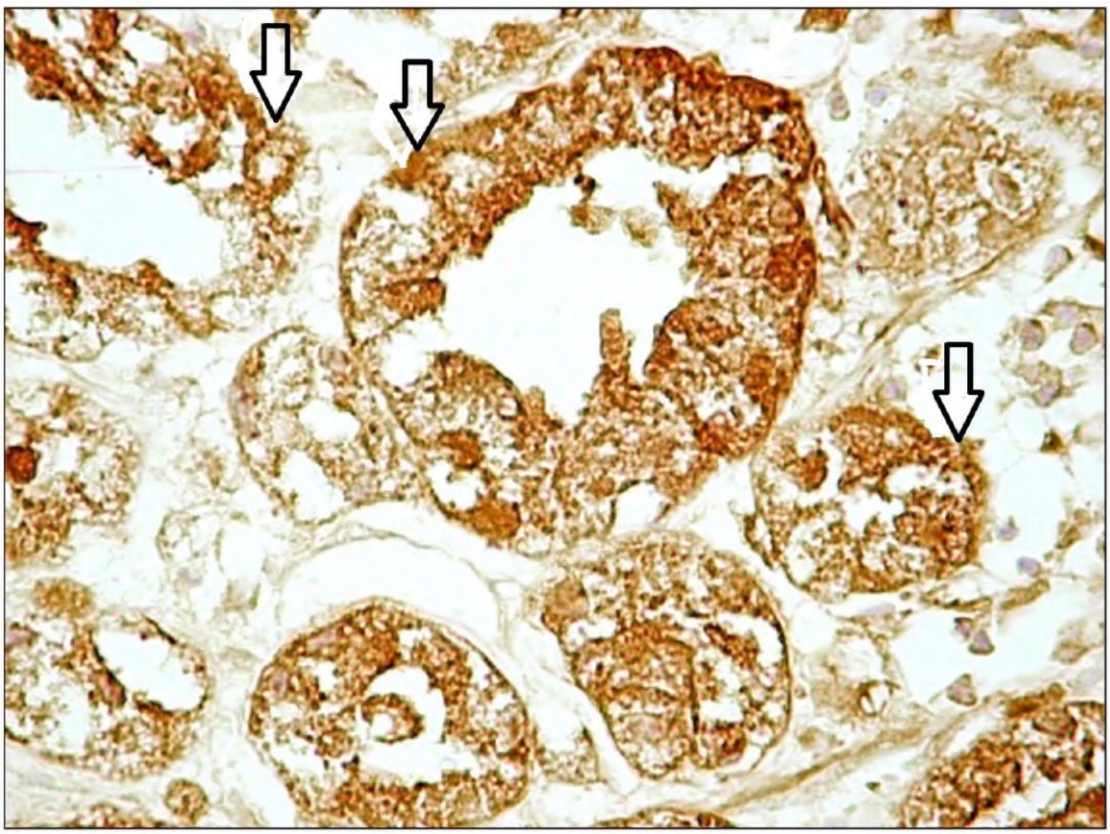

Figure 1. Conventional adenocarcinoma, anti-PSA. Intense positive immunoreaction, granular and homogeneous ob. 20X

In well-differentiated adenocarcinoma (Gleason score 3 to 5, n=6) the reaction was positive in all but one case. The large majority of cases were characterized by weak reaction, and only isolated cases were appreciated as +2. Often, the intensity of the reaction was significantly weaker than in benign prostate hyperplasia. All cases with Gleason score from 6 to 8 (n=18) were positive. There were found 41 cases with undifferentiated carcinoma, with high Gleason score: 32 were intense positive (+2) with homogeneous pattern (78%), 7 weak/moderate positive (17.07%), and 2 were negative (4.87%). In cases with high Gleason score prevailed the heterogeneous pattern of PSA, and in cases with Gleason score less than 8 there were not found significant differences.

Detection of the perineural invasion is extremely important for the management of the patient because it represents the main route for the progression of the tumor. There were investigated for this aspect only 63 cases because small cell and urothelial carcinomas were excluded. They are both negative, and therefore, the method cannot bring new information about the perineural invasion.

Overall, we found perineural invasion on usual stained slides in only 15 cases (23.8%). Malignant cells were isolated, or arranged in small clusters, or even forming large glands that occupy the entire epineurium. On slides stained for PSA perineural invasion was found in 25 cases (39.68%) (Fig. 3). For both methods, perineural invasion was rarely noticed in specimens taken by core biopsy, but even in this case our results were better using anti-PSA immunohistochemistry.

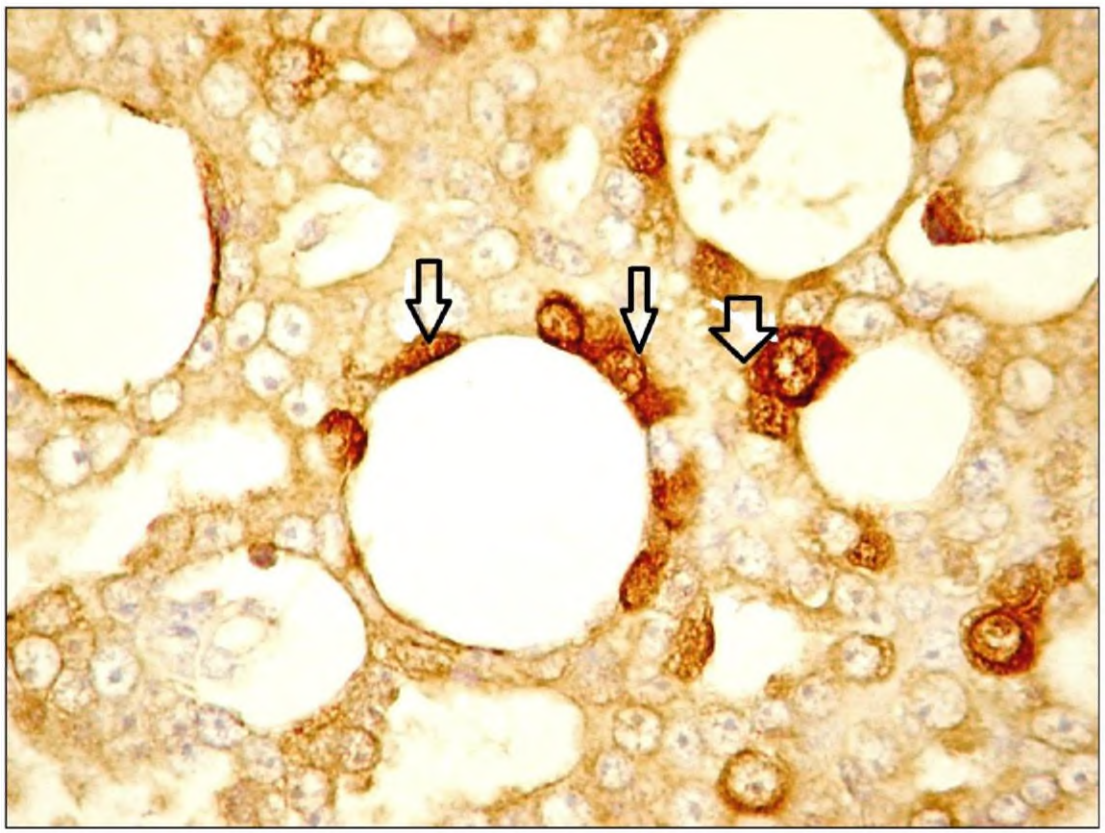

Figure 2. Cribriform carcinoma with heterogeneous reaction for PSA. The presence of scattered and intensely stained malignant cells around the pseudo-lumens ob. 20X

DISCUSSION

Many problems that occur in the current pathologic diagnosis are related to undifferentiated tumors developed at the bladder neck, and lymph node metastasis of unknown primary. In the case of prostate cancer, identification of prostate specific antigen (PSA) is extremely helpful. PSA is highly but not completely specific. Monoclonal as well as polyclonal antibodies stain secretory cells of the benign tissue and malignant cells of glandular origin (excepting neuroendocrine cells). In prostate cancer the expression of PSA is found in more than 95% of cases, but urothelial, squamous, and small cell carcinomas are always negative. Our data correspond with literature; in small cell carcinoma PSA was negative in all 3 cases but undifferentiated form that mimics urothelial carcinoma was positive for PSA (Table 1).

PSA levels, Gleason score and TNM staging are established and considered essential to the prognostic of prostate cancer, when analyzed separately or jointly. Other factors such as the additional clinical morphologic ones and

molecular markers can also contribute substantially (study by Hernandez and Thompson, 2004). Among the factors related to the biopsy, the percentage of positive fragments has presented a positive correlation with the potential risk of biochemical progression after treatment. In a study by Varma et al (2002) demonstrated that polyclonal anti-PSA has a superior sensitivity and they even recommend the high-grade prostate cancer as external control of PSA reaction. Our results are also supported by a recent study of Martinez-Pineiro et al (2003) that found a strong correlation between PSA RT-PCR and Gleason score; we established that in cases with Gleason score less than 5 only isolated cases were estimated as +2 (intense reaction for PSA) but in cases with high Gleason score the reaction of PSA was intensely positive (Table 2) and was associated with high risk of progression of disease but was not supported by the study of Bostwick et al (1998) that found PSA immunoreactivity declined from benign epithelium to PIN and prostatic adenocarcinoma, suggesting that PSA is regulated differentially and decreased in expression with malignant transformation.

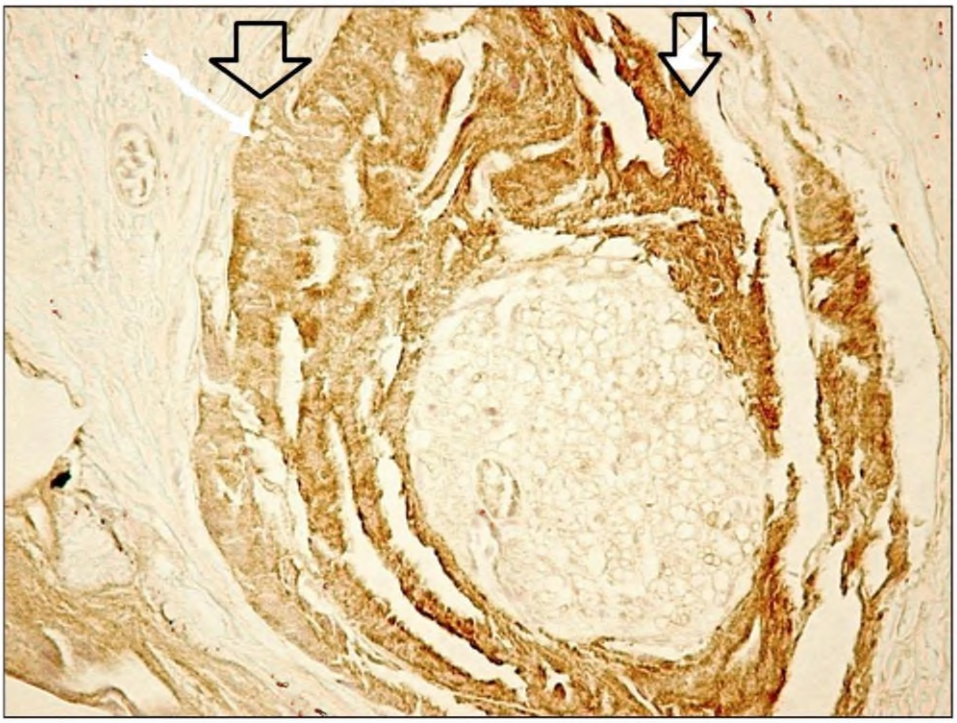

Figure 3. Perineural invasion in prostate carcinoma. PSA positive ob. 20X

PSA expression frequently is heterogeneous; therefore, immunostains may need to be performed on multiple blocks containing tumor when dealing with challenging cases (Shah et al, 2004). We found in cribriform carcinoma a heterogeneous pattern; only isolated cells or clusters (fig. 2) in accordance with literature but in other cases the reaction was positive for all glandular cells (homogeneous pattern); in cases with adenocarcinoma was found a strong granular citoplasmatic reaction and the pattern was granular and rarely diffuse (Fig. 1).

Perineural invasion is the hallmark of invasive prostate cancer, and it is found in more than 70% of cases on specimens of radical prostatectomy. Patients with perineural invasion were twice as likely to progress compared with patients without perineural invasion (Sebo et al, 2002). Before using PSA on routine examination we found perineural invasion only in 23.8% but after using immunohistochemistry we found that rate of

perineural invasion significantly increased to 39.68% but even so is less than in the mentioned study. It is believed that every pathologist will immediately identify a large perineural invasion (as shown in Fig. 3). More hard is to diagnose small groups of malignant cells located close to thin nerve fibers. We may conclude that immunohistochemistry for PSA is extremely useful to detect perineural invasion in specimens taken by core biopsy and transurethral resection.
In this study we investigated the carcinomas of the prostate that revealed the value of the PSA, for the differential diagnosis. The reaction was positive in 88.2% of cases, and it was negative in urothelial and small cell carcinomas. Our results showed intense expression in many cases with high Gleason score. Immunohistochemistry for PSA significantly increases the rate of detection of perineural invasion and confirms the prostatic origin of lymph node metastasis of apparently unknown primary site.

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Endothelial Cell Proliferation and VEGF Expression in Primary Colorectal Cancer and Liver Metastases

RESEARCH ARTICLE

Endothelial Cell Proliferation and Vascular Endothelial Growth Factor Expression in Primary Colorectal Cancer and Corresponding Liver Metastases

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Abstract

Background: . Colorectal carcinoma (CRC) is one of the major causes of cancer death worldwide. Data from the literature indicate differences between the proliferation rate of endothelial cells relative to the morphology growth type, possibly due to origin of specimens (autopsy material, surgery fragments) or quantification methods. Vascular endothelial growth factor (VEGF) is a factor that stimulates the proliferation of endothelial cells. It is expressed in more than 90% of cases of metastatic CRC. Aim: The aim of this study was to evaluate the endothelial cell proliferation and VEGF expression in primary tumors and corresponding liver metastases. Materials and Methods: Our study included 24 recent biopsies of primary tumors and corresponding liver metastases of CRC cases. CD34/ Ki67 double immunostaining and RNA scope assay for VEGF were performed. Results: In the primary tumors analysis of VEGFmRNA expression indicated no significant correlation with differentiation grade, proliferative and non-proliferative vessels in the intratumoral and peritumoral areas. In contrast, in the corresponding liver metastases, VEGFmRNA expression significantly correlated with the total number of non-proliferative vessels and total number of vessels. CD34/ Ki67 double immunostaining in the cases with poorly differentiated carcinoma indicated a high number of proliferating endothelial cells in the peritumoral area and a low number in the intratumoral area for the primary tumor. Moderately differentiated carcinomas of colon showed no proliferating endothelial cells in the intratumoral area in half of the cases included in the study, for both, primary tumor and liver metastasis. In well differentiated CRCs, in primary tumors, a high proliferation rate of endothelial cells in the intratumoral area and a lower proliferation rate in the peritumoral area were found. A low value was found in corresponding liver metastasis. Conclusions: The absence of proliferative endothelial cells in half of the cases for the primary tumors and liver metastases in moderately differentiated carcinoma suggest a vascular mimicry phenomenon. The mismatch between the total number of vessels and endothelial proliferation in primary tumors indicate that a functional vascular network is already formed or the existence of some mechanisms influenced by other angiogenic factors.

Keywords: Colon carcinoma - endothelial cell proliferation - metastasis

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Introduction

Normal tissues are characterized by the presence of blood and lymphatic vessels lined by endothelial cells exhibiting a low proliferation rate ranged between 0.1- 3 % of all endothelial cells which turnover daily but this percentage decline with age (Schwartz SM et al., 1973). Tumor blood vessels have an increased endothelial cells proliferation rate of 20-2000 times higher than in normal tissues (Hobson et al., 1984, Zecchin et al, 2015), representing 0.05% from the total number of human tumor proliferating cells (Kendall et al., 1999). Significant differences have been observed concerning endothelial cells proliferation rate between several tumor types, this aspect being already certified in tumors as non inflammatory breast cancer and hepatocellular carcinoma (11% and 35%, respectively) (Colpaert et al., 2003; Kendall et al., 1999; Imura et al., 2004; Quinn et al., 1993).

CRC is one of the major causes of cancer death worldwide, being the third most common diagnosed cancer in men and the second in women (Baena and Salinas, 2015). Metastatic ability of colorectal cancer cells is well certified and it is influenced by heterogeneous factors as individual's age, dietary habits, any complaint of obesity, diabetes, previous history of cancer or intestinal polyps (Rasool et al, 2013) or by histopathologic subtypes

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(Hugen et al, 2014). The liver is the most common target for metastasis in patients with this disease and hepatic metastasectomy increase overall survival rates (Frankel and D'Angelica, 2014). In primary CRC, Vermeulen et al. (1995) have demonstrated that the fraction of cycling endothelial cells was higher in tumor tissue (cell labeling index of 9.9%) as compared with adjacent normal mucosa.

Many factors are involved in the development and progression of liver metastasis. The tumor cell-endothelium interaction influences the organ-specific metastasis. One of the most important angiogenic growth factor is represented by vascular endothelial growth factor (VEGF) (Yu et al. 2012). This factor is involved in the induction of vasodilation, endothelial cells migration, proliferation and vessel assembly (Lamalice et al., 2007; Zhou et al., 2012). The overexpression of VEGF was found to the patients with liver metastasis of colon carcinoma (90.9% of cases) and particularly in well differentiated colon carcinomas (83.3%) (Cao et al. 2009). Data from the literature on endothelial cell proliferation in liver metastases of colon carcinoma shows variable results. Thus, the study of endothelial cell proliferation in relation with the three growth pattern of liver metastasis indicated a significant difference of the proliferating endothelial cells fraction between desmoplastic, replacement (3%) and the pushing growth pattern (11%) (Vermeulen et al. 2001). Eefsen et al. (2012) found elevated proliferation fraction of endothelial cells for the pushing growth pattern, but without a significant difference comparative with desmoplastic or replacement growth pattern.

Based on these considerations, the aim of this study was to evaluate the endothelial cells proliferation and VEGF expression in primary tumors and corresponding liver metastasis.

Materials and Methods

Our study included 24 recent biopsies of primary tumors and corresponding liver metastasis from patients with CRC. Biopsies were fixed in buffered formalin and embedded in paraffin. Sections from each case were stained with hematoxylin-eosin for histopathological diagnosis. Immunohistochemical study included double immunostaining for CD34 and Ki67. Heat-induced epitope retrieval with pH 6.0 solution (Leica Biosystems, Newcastle uponTyne, UK), for 30 minutes was followed by endogenous peroxidase blocking (3% hydrogen peroxide, 5 minutes) and incubation with primary antibody Ki67 (Dako Glostrup Denmark, ready to use, clone MIB-1, 30 minutes). NovoLink Max Polymer Detection System The Leica Biosystems, Newcastle uponTyne, UK, was used as visualization system and 3,3 - diaminobenzidine as chromogen. Immunohistochemical technique continued with endogenous peroxidase blocking with 3% hydrogen peroxide for 5 minutes, incubation with the second antibody CD34 (Dako Glostrup Denmark, mouse monoclonal anti-human, clone 1A4, ready to use, 30 minutes), visualised with Vina Green as chromogen, for 10 minutes (Biocare Medical, LLC, Concord, CA 94520, USA). Nuclear staining was performed with Lille's hematoxylin. The full immunohistochemical procedure was performed with DakoAutostainer Plus (DakoCytomation). The proliferating endothelial cells were defined as cells lining the vessels lumen and coexpressing both CD34 and Ki67. Proliferative and non-proliferating vessels were counted on three consecutive microscopic fields at 200X magnification, in the intratumoral and peritumoral areas. RNA scope assay was performed using RNAScope 2.0 FFPE Reagent Kit (Advanced Cell Diagnostics, Inc., Hayward, CA) according to the manufacturer's instructions. The formalin fixed, paraffin embedded tissue sections were pretreated with heat and protease prior to hybridization with a target probe. A HRP-based signal amplification system was then hybridized to the target probes followed by color development with DAB. VEGF quantification was made according to the following score: score 0 (1 dot/cell), score 1 (1-3 dots/cell), score 2 (4-10 dots/cell), score3 (>10 dots/cell, less than 10% positive cells), score 4 (>10 dots/cell, more than 10% positive cells). Image acquisition and analysis were performed using Nikon Eclipse E 600 microscope and Lucia G software for microscopic image analysis.

The local research ethics committee approved the protocol of the study and informed consent was obtained from all subjects according to the World Medical Association Declaration of Helsinki.

Results

Histopathological evaluation based on routine haematoxylin and eosin method revealed liver metastasis from well (8 cases), moderately (8 cases) and poorly differentiated (8 cases) CRC.

For well differentiated colon carcinoma, in primary tumor, a high proliferation rate with 3 to 15 proliferative endothelial cell in the intratumoral area was noticed. A lower proliferation rate was found in the peritumoral area. In comparison with the primary tumor, in corresponding

Table 1. Values of Proliferating Endothelial Cells According with Grading, Localization, Tumor Area

Grading	Primary tumor		Liver metastasis	
	intratumoral proliferating endothelial cell	peritumoral proliferating endothelial cell	intratumoral proliferating endothelial cell	peritumoral proliferating endothelial cell
well differentiated	52	32	12	4
moderately differentiated	24	10	12	16
poorly differentiated	20	48	38	16

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Table 2. VEGF mRNA Expression in Primary Tumors and Corresponding Liver Metastasis

Primary tumor		Liver metastasis
well differentiated carcinoma	heterogeneous expression, score 3, distinct dots	heterogeneous expression, score 1;
moderately differentiated carcinoma	different intensity of expression in the tumor area, score 4, compact clusters	heterogeneous expression, score 3; expression in the endothelial cells of sinusoides and very rare in the hepatocytes
poorly differentiated carcinoma	low intensity of reaction, score 2, visible dots and small clusters	low expression, score 1, more intense in the adiacent hepatic parenchyma

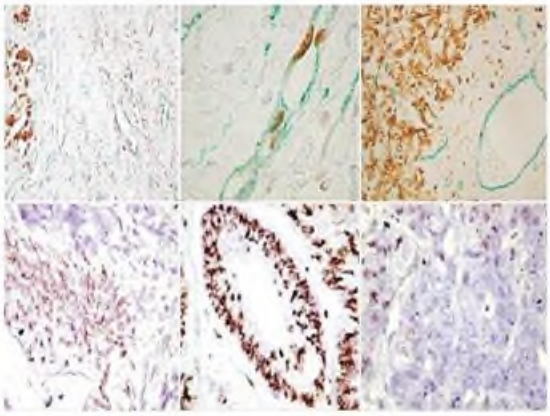

Figure 1. A) High Number of Proliferating Endothelial Cells in the Peritumoral Area and a Low Number in the Intratumoral Area of the Primary Tumor, 20x; B) Intussusception Phenomenon Associate with Endothelial Cell Proliferation, 100x; C) Discontinuous Wall and Proliferating Endothelial Cells in the Centrilobular Vein Adjacent to the Metastasis, 20x; D) VEGFmRNA Expression, Well Differentiated Carcinoma- Score3, 20x; E) VEGFmRNA Expression, Moderately Differentiated Carcinoma- Score4, 20x; F) VEGFmRNA Expression, Poorly Differentiated Carcinoma- Score2, 40x

liver metastasis, a low value was found. Thus, liver metastasis showed values between 1 and 3 for the proliferating endothelial cells in the intratumoral area and the absence of proliferating endothelial cells in the peritumoral area.

The moderate differentiated CRC showed the absence of proliferating endothelial cells in the intratumoral area of half of the evaluated cases, for both primary tumor and liver metastasis. A high rate of proliferating endothelial cells was found in the peritumoral area with close values for primary tumor and corresponding metastasis.

CD34/ Ki67 double immunostaining in the cases with poorly differentiated carcinoma indicated a high number of proliferating endothelial cells in the peritumoral area and a low number in the intratumoral area for the primary tumor (Figure1 A). As a particular aspect, we noticed to these cases the presence of intussusception phenomenon associate with endothelial cell proliferation (Figure 1 B). In the corresponding liver metastasis, the proliferating endothelial cells were present in the peritumoral area and absent in the intratumoral area. The intratumoral blood vessels were small, non proliferative with collapsed lumen. The central vein adjacent to the metastasis showed a discontinuous wall and proliferating endothelial cells (Figure 1 C).

The main values of proliferating endothelial cells and the relations with grading, localization and tumor area are summarized in table 1.

In the intratumoral area of primary tumor, we found a significant correlation between the number of proliferative vessels (CD34+/Ki67+) and intratumoral proliferative endothelial cells (p=0.001). Number of non-proliferative vessels (CD34+/ Ki67-) was significantly correlated with the total number of intratumoral vessels (p=0.001) and with poor differentiated carcinoma. It was noticed that the total number of intratumoral vessels partialy correlate with the differentiation degrees (p= 0.05).

Peritumoral area of primary tumor presented a correlation between non-proliferative vessels and differentiation degrees (moderate and poor differentiated type; p=0.01).

VEGFmRNA expression in primary tumor had variable scores with differentiation degree. In primary tumors, the score distribution was as follows: for well differentiated carcinoma score 3, with heterogeneous expression and distinct dots (Figure 1 D); moderately differentiated carcinoma presented a different intensity of expression in the tumor area, compact clusters and score 4 (Figure 1 E); poorly differentiated carcinoma had low intensity of reaction, visible dots and small clusters with a value of score 2 (Figure 1 F).

Analysis of VEGFmRNA expression in liver metastasis indicated a heterogeneous expression with values from 1 to 3 (Table 2). In the primary tumors no significant correlation between VEGFmRNA expression and pathological type, proliferative and non-proliferative vessels in the intratumoral and peritumoral areas was found. Opposite, in the corresponding liver metastasis, VEGFmRNA expression was significantly correlated with the total number of non-proliferative vessels (p=0.026) and total number of vessels (p=0.036).

Discussion

Tumors had preferential sites for metastasis. Thus, colon cancer has tendency to give rise to liver metastasis. At the time of the diagnosis, 25% of patients presented liver metastasis.

The addition of Bevacizumab to FOLFOX 4 (oxaliplatin, 5-fluorouracil, and leucovorin) indicated an increased of median overall survival and progression free- survival with 2.1 and 2.6 month (Giantonio BJ et al., 2007; Zhu et al., 2014). No major differences (4.7 and 4.2 month increase) were observed between administration of irinotecan, 5-fluorouracil, leucovorin and placebo treatment with foregoing treatment and bevacizumab (Dirican et al., 2014; Hurwitz et al., 2004).

The fraction of endothelial cell proliferation was described as an important factor used in the evaluation of angiogenesis (Vermeulen et al., 2002; Vermeulen et al., 1996). A study on brain metastases from NSCLC indicated a higher proliferation rate and vascular maturity comparative with primary tumors (Benjamin et al., 1999; Jubb et al., 2011). The mature blood vessels are less sensitive to Bevacizumab than immature vessels and a lower efficacy for these patients was showed.

Three distinctive morphological growth patterns were described for liver metastasis: desmoplatic, replacement and pushing (Vermeulen et al 2001). Recently, these growth patterns seems to have a prognostic significance (Nielsen et al., 2014), growth patterns having a direct correlations with reccurence free survival and other prognostic factors (Eefsen et al, 2015). A high angiogenic activity was found in the pushing growth pattern and a lower one has been noticed in a desmoplastic and replacement growth pattern. Elevated values for endothelial proliferative cells in the pushing growth pattern were found, but without a significant difference comparative to the other two types (Eefsen et al., 2012). We found, in liver metastasis values between 1 and 3 for proliferating endothelial cells for the well differentiated carcinoma, the absence of proliferating endothelial cells in half of the cases of the moderately differentiated colon carcinoma in the intratumoral area.

Our results showed a different proliferative index between the tumor blood vessel endothelium from the tumor core and its peripheral areas. This finding could partially explain the ineffective antiangiogenic and/ or antivascular therapy of tumor angiogenesis in liver metastasis.

Lack of CD34 immunostaining in some blood vessels lined by several Ki67-positive endothelial cells, suggests that liver metastasis tumor blood vessels are more permeable than normal blood vessels. Our study suggests that liver metastasis blood vessels are heterogeneous, do not have the same proliferative status or expression of markers concurrently and may respond in a different way to antiangiogenic therapy

In the present study, the total number of vessels did not correlate with endothelial proliferating cells in the primary tumor. From this, it derived two hypotheses: functional intratumoral vascular network is already formed, vessels are stabilized at the moment of diagnosis or involvement of other angiogenic mechanisms dependent on other growth factors that induce the formation of new vessels. The fact that non proliferating intratumoral vessels correlates with the total number of intratumoral vessels reinforces the idea that nonproliferative vessels are already functional, possibly stabilized.

Tokunaga et al. (1998) demonstrated that expression of VEGFmRNA isoforms was correlated with liver metastasis and poor prognosis in colon carcinoma. Choi et al., (2012) obtained different results: no significant relationship between the expression of VEGF, COX 2 and depth of tumor invasion, lymph node metastasis, vessel invasion, perineural invasion and liver metastasis. On the other hand, it has been showed that low VEGF165b / VEGF total ratio may be a predictive marker for bevacizumab in metastatic colorectal cancer, and individuals with high relative levels may not benefit. Initial studies of the phase III clinical trials of bevacizumab showed no predictive value for total VEGF expression or microvessel density (Bates et al., 2012; Jubb et al., 2006), suggesting that it was not the VEGF levels that determine the outcome.

In our study, no significant correlation between VEGFmRNA and pathological type, proliferative and nonproliferative vessels types, in the intratumoral and peritumoral areas of primary tumors was found. But, for the liver metastasis we noticed a correlation between VEGFmRNA expression and non-proliferative and total number of vessels.

In primary tumors the total number of intratumoral vessels was not correlated with endothelial cell proliferation, which can suggest that intratumoral vascular network is formed, stabilized at the moment of diagnosis. In the primary tumors no significant correlation between VEGFmRNA expression and histopathological type, proliferative and non-proliferative vessels in the intratumoral and peritumoral areas was found compared to corresponding liver metastasis in which VEGFmRNA expression was significantly correlated with total number of non-proliferative and total number of vessels.

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I.2.APLICAREA METODELOR HISTOCHIMICE ȘI IMUNOHISTOCHIMICE ÎN PROCEDURILE DIAGNOSTICE ȘI CU POTENȚIAL IMPACT TERAPEUTIC ÎN STUDII EXPERIMENTALE

1. Cimpean Anca Maria, Dusan Lalošević, Vesna Lalošević, Pavle Banović, Marius Raica, **Ovidiu Alexandru Mederle**- Disodium cromolyn and anti-podoplanin antibodies strongly inhibit growth of BHK 21/C13-derived fibrosarcoma in a chick embryo chorioallantoic membrane model- In VIVO, vol. 32: 791-798 (2018) ISSN: 0258-851X, IF=0,953.

2. Osakwe, H; Nicolescu, C; Nicolescu, L; Hoinoiu, B; **Mederle, O**; Mussuto, E; Popoiu, C; Boia, E -The impact of residual bowel after extended bowel resection on bacterial overgrowth and bacterial translocation, REV.CHIM, Volume: 69 Issue: 8 Pages: 2121-2128, 2018, ISSN: 0034-7752, IF=1,412

Pe modele experimentale, am studiat și am testat efectele anticorpilor anti-podoplaninei și cromolinei disodice asupra tumorilor derivate din linia celulară BHK21/C13-fibrosarcom dezvoltate pe membrana chorio-allantoidă a embrionului de pui (CAM). Imunofenotipul fibrosarcomului derivat din fibroblaste BHK-21 / C13 demonstrează că aceste fibroblaste reprezintă o linie celulară specială cu fenotipul vimentin + / CD34- / CD117 + / PROX1 + / podoplanin- / EGFR +, sugerând un comportament foarte agresiv bazat pe mai multe particularități moleculare, descrise anterior pentru această linie celulară. Răspunsul eterogen la cromolina disodică, bevacizumab și anti - podoplanina sprijină utilizarea acestor celule pentru evaluarea viitoare a altor terapii noi.

Scopul a fost să demonstrăm că, după rezecția intestinală extinsă, există o legătură directă între numărul bacteriilor intestinale (supra-aglomerarea bacteriană), modificările morfologice intestinale ale mucoaselor și a altor organe (splină și ficat). Am dorit, de asemenea, să comparăm rezultatele noastre cu studii similare la om. Prin compararea rezultatelor microbiologice și histopatologice ale șobolanilor cu procente diferite de rezecție intestinală, am reușit să concluzionăm că lungimea rezecției intestinale est invers proporțională cu sunt șansele de adaptare și supraviețuire intestinală și direct proporțională cu șansele bolii de a progresa la nivelul intestinului și la nivel hepatic prin colestază.

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Disodium Cromolyn and Anti-podoplanin Antibodies Strongly Inhibit Growth of BHK 21/C13-derived Fibrosarcoma in a Chick Embryo Chorioallantoic Membrane Model

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Abstract. *Aim: To characterize baby hamster kidney fibroblast (BHK 21/C13) cells and test the effects of antibodies against podoplanin and disodium cromolyn on BHK 21/C13 cell line-derived tumors grown on chick embryo chorioallantoic membrane (CAM). Material and Methods: BHK 21/C13 cell-derived fibrosarcomas developed in hamsters were implanted on CAM and treated with anti-podoplanin antibodies and disodium cromolyn. BHK 21/C13 cell immunophenotype was assessed. Results: Fibrosarcoma cells were positive for vimentin, CD117, smooth muscle actin, vascular endothelial growth factor epidermal growth factor receptor, homebox prospero gene 1 and negative for platelet-derived growth factor B, neuron-specific enolase, S100, CD34, Ewing sarcoma and podoplanin. CAM- grown fibrosarcomas were highly sensitive to disodium cromolyn and anti-podoplanin antibodies. Conclusion: Immunophenotyping BHK 21/C13 cells and their response to drugs represent the first step in revealing cell line utility and a reliable tool for experimental cancer research.*

Experimental models are still powerful tools for medical research. *In vitro* and *in vivo* models are designed to help researchers in their work for understanding disease mechanisms and for the discovery and testing of new therapeutics which by their future clinical application may improve the prognosis and survival of patients with various diseases (1- 3). Despite researchers' efforts to create a perfect experimental model for each disease, several issues remained unresolved (4). Ethics rules become more and more strict regarding the use of animals in experimental models (5, 6) and thus the development of alternative experimental models which lack the use of animals is a real challenge for the scientific world. Even though in the future microfluidic systems such as tumor/organ on a chip model (7, 8) or 3D-printed organs may be used to develop new experimental models (9, 10), cell lines will remain the main component of any experimental model. Countless options for the use of cell lines are available today. There are normal and tumor cell lines used for various purposes from vaccine production (11) to testing cell toxicity of different agents (12, 13).

Baby hamster kidney fibroblasts (BHK -21/C13) is a well-known cell line sensitive to various viruses such as herpesvirus (14), hepatic (15) or rabies virus (16), but not polyoma virus. These cells are able to undergo malignant transformation, with fast growing behavior due to rapid proliferation and invasion of adjacent tissues when they are subcutaneously injected into hamsters. A giant fibrosarcoma-like tumor without evidence of lymphatic or distant metastasis may develop at the site of inoculation. Few studies in the field of tumor experimental models used BHK-21/C13 cell line to obtain fibrosarcomas and even fewer to test the effects of different therapeutic agents.

The BHK-21/C13 cell line has not been characterized regarding their phenotype except for some old articles which reported the expression of fibroblast growth factor by BHK-21/C13 cells (17) and the effects of vascular permeability factor on their proliferation and migration (18, 19). Regarding BHK-21/C13 cell response to different therapeutic

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in vivo 32: 791-798 (2018)

agents, previous research was mainly focused on the inhibitory effects of potential or certified antiviral agents (20, 21). Other drugs with different properties such as antiallergic, antitumor or antiangiogenic actions, have not been tested yet on BHK-21/C13 cell-derived sarcomas as far as we are aware.

BHK-21/C13 cells have an unknown phenotype. Therefore, we proposed to immunohenotype tumor derived from BHK-21/C13 cells for markers with a potential prognostic role or which could be used as therapeutic target. Here we designed a combined experimental model [in hamsters and chick embryo chorioallantoic membrane (CAM)] which allowed us to study the ability of BHK-21/C13 cells to develop sarcomas and the reaction of chick embyo CAM -implanted sarcomas to bevacizumab, disodium cromolyn and anti-podoplanin antibodies, three therapeutic agents with controversial effects on tumor tissues.

Materials and Methods

Ethics and animal protection. All procedures involved animals were performed according to the present international guidelines recommended by European Union (Directive 2010/63/EU). The Ethics Commission of Victor Babes University of Medicine and Pharmacy approved all the laboratory and experimental procedures (No.5 /5821/26.05.2016.

Cell lines and culture procedure. BHK- 21/C13 cell line was provided by American Type Culture Collection (Manassas, VA, USA). The cells were cultured according to the manufacturer's protocol and following the method previously described by Lalosevic et al. (22).

Inoculation and tumor development in Syrian hamsters. Cultured BHK-21/C13 cells were subcutaneously inoculated into 10 Syrian hamsters (6 weeks old, weight of 250 g) . Two weeks later, a well-developed tumor mass was detected at the inoculation site. The tumor was characterized by fast growing behavior and invasiveness into the surrounding tissues without local or distant metastases.

Tissue processing and routine staining. Three weeks after initial inoculation, the hamsters were sacrificed and tumors were removed. Fresh tumor tissue of about 2 mm were collected for the future implant procedure on chick embryo CAM model. Remaining tumor was formalin fixed for about 24 hours and then paraffin embedded according to routine protocol. Three -micrometer sections were made and one of these was stained with hematoxylin and eosin method for morphological assessment. Based on evaluation of quality slide, specimens were selected for immunohistochemistry. The same tissue processing was also applied for the specimens collected from chick embryo CAM specimens (treated and untreated).

Chick embryo CAM model, and drug delivery template. Twenty-two fertilized chicken eggs were prepared for the development of the experimental model. Briefly, the eggs were incubated at 37°C for 72 hours in incubators with controlled temperature and humidity. On the fourth day of incubation, 2 ml of albumen was removed from each specimen and the CAM was made visible by making a window in the superior part of the egg shell. On the seventh day of incubation, the chick embryo CAM was ready for implantation of BHK-21/C13-derived fibrosarcoma tissue collected from the tumor previously obtained from the hamster model. Two -milimeter-thick tumor piece was implanted inside a silicon ring previously fixed on the chick embryo CAM. Eggs were organized into three groups for testing therapeutic agents (bevacizumab, disodium cromolyn and anti-podoplanin antibodies). A control group (four eggs) received saline solution, while fibrosarcomas implanted in another 18 eggs were treated with bevacizumab, disodium cromolyn or anti-podoplanin antibodies (2 μl each day for 5 days on six specimens each, at a concentration of 100 μg/ml). Treated and control specimens were observed daily under stereomicroscopy and, at the end of day 5 of the treatment, the chick embryo CAM was fixed *in ovo* with 10% buffered formalin for 1 hour. It was then parraffin- embedded and examined by immunohistochemistry following the protocol described below. A brief overview of the procedure from hamster inoculation to chick embryo CAM implantation is summarized in Figure 1.

Immunohistochemistry. Three-micrometer sections were loaded in a Bond Max Autostainer previously scheduled to perfom a simple immunostaining procedure with step by step program provided by the manufacturer (Leica Microsystems, Medist Life Sciences, Bucharest, Romania). Briefly, this program included a dewaxing step followed by antigen retrieval and incubation with primary antibodies (30 minutes at room temperature) selected for the present study and detailed in Table I. Visualization of the final product for each antibody was performed using Bond Refine Detection System Brown specific for Bond Max Autostainer. The workflow also included an automated mounting procedure using Leica Permanent Mounting (Leica Microsystems).

Image aquisition and data interpretation. An Axio Zoom A2 Research Microscope (Zeiss, Munchen, Germany) was used for the evaluation of routine and immunohistochemically stained slides. This system allowed us to capture and process microscopic images and to assess blood vessel changes around and inside the treated and untreated specimens.

Results

The tumor expanded rapidly into the tissue of the Syrian hamsters. Two weeks after initial inoculation, a macroscopically well- defined apparently encapsulated tumor mass was observed but when we tried to remove it, we found that it was highly and deeply invasive into surrounding tissues (Figure 1a and b). Microscopically, the specimens collected from hamster had a fibrosarcoma morphology, being composed of closely packed spindle - shaped highly mitotic cells, and scant cytoplasm, including elongated nuclei and variable nucleoli (Figure 1c). Specimens harvested from this tumor were implanted onto the surface of chick embryo CAM. Fibrosarcoma tumor volume increased rapidly and the tumor became highly vascularized (Figure 1d) with the acquisition of blood vessels from adjacent chick embryo CAM. Blood vessel acquisition was confirmed by stereomicroscopy (Figure 1e) and by the assessement of histological specimens (Figure 1f).

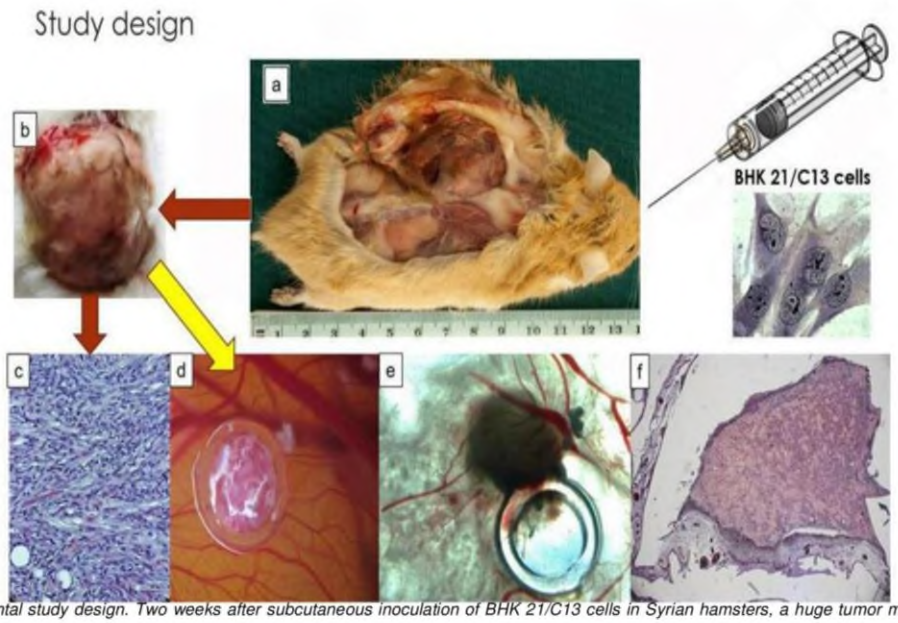

Figure 1. *Experimental study design. Two weeks after subcutaneous inoculation of BHK 21/C13 cells in Syrian hamsters, a huge tumor mass was clinically and macroscopically detected, with a well-vascularized surface and deeply infiltrative behavior (a). Small biopsies from the tumor mass (b) were collected, some of them being paraffin-embedded for histopathological evaluation (c). Fresh tissue from the tumor was implanted on chick embryo chorioallantoic membrane (CAM) (d, e) and treated with three different drugs. Tissues were processed by routine histopathological methods followed by staining with hematoxylin and eosin for preliminary evaluation of tumor biopsies from CAM (f), Original magnification, c: ×200, f: ×100).*

Table I. *Antibodies, dilutions and working system used for immunohistochemistry.*

Antibody	Manufacturer	Clone	Dilution	Working system
Vimentin	Novocastra	V9	Ready to use	Fully automated with Bond Autostainer, Bond Polymer Refine Detection System, diaminobenzidine (Leica Microsystems, UK)
CD34	Novocastra	QBEnd10	Ready to use	
Podoplanin	Dako Cytomation	D2-40	Ready to use	
S100 protein	Novocastra	Polyclonal	Ready to use	
CD117	Novocastra	C KIT	Ready to use	
SMA	Novocastra	1A4	Ready to use	
Ki67	Novocastra	MIB 1	Ready to use	
PROX1	Reliatech	Polyclonal	1:400	
VEGF	Dako Cytomation	VG 1	Ready to use	
EGFR	Dako Cytomation	Polyclonal	Ready to use	
PDGF BB	Reliatech	Polyclonal	1:200	

~~SMA: Smooth muscle actin, PROX1: homebox prospero gene 1, VEGF: vascular endothelial growth factor, EGFR: epidermal growth factor receptor, PDGF-BB platelet derived growth factor B.~~

Accurate immunophenotyping of the fibrosarcomas grown in hamsters was performed before implantation on chick CAM. As expected, all tumor cells were positive for vimentin. Vimentin expression was accompanied by a strong immunoreaction for CD117 in the tumor cells with a relatively high proliferation rate as evaluated by Ki67. CD34 expression was restricted to the endothelial level of tumor blood vessels, while tumor cells were totally negative. Smooth muscle actin (SMA)-positive tumor cells were present inside fibrosarcoma, isolated or in an island -like distribution around tumor blood vessels, giving the appearance that they emerged from perivascular cells. Two

in vivo 32: 791-798 (2018)

Figure 2. *Immunoprofile of BHK 21/C13 cell-derived fibrosarcoma. CD34: Transmembrane phosphoglycoprotein specific for endothelial cells; CD117: mast/stem cell growth factor receptor, proto-oncogene c-Kit or tyrosine- protein kinase KIT; PROX1: homebox prospero gene 1; VEGF: vascular endothelial growth factor; EGFR: epidermal growth factor receptor; PDGF BB: platelet-derived growth factor BB. Original magnification, ×200.*

lymphatic markers, podoplanin (D2-40) and homebox prospero gene 1 (PROX1) have significant value in the evaluation of any malignancy for the assessment of tumor lymphangiogenesis and of tumor cells expressing podoplanin or PROX1. BHK - 21/C13 cell- derived fibrosarcoma was characterized by a podoplanin-negative/PROX1-positive tumor cell immunophenotype. Podoplanin highlighted lymphatic vessels in the peritumoral tissue by labeling of lymphatic endothelial cells. No intratumoral lymphatic vessels were detected inside the tumor mass. PROX 1 immunoexpression was restricted to fibrosarcoma cells with nuclear and cytoplasmic pattern. Amongst three growth factors, vascular endothelial growth factor (VEGF), epidermal growth factor receptor (EGFR) and platelet derived growth factor BB (PDGF BB), EGFR had the highest expression with a consistent distribution inside the cytoplasm of fibrosarcoma tumor cells. VEGF highlighted tumor blood vessel endothelium with a high intensity and with a weak to moderate and inconstant expression inside tumor cells. Staining for PDGF BB was mostly negative, with scattered cells around tumor blood vessels showing a scant positive reaction.

During the next step of evaluation of BHK-21/C13 cell-derived fibrosarcoma on chick embryo CAM, the implanted tumors were treated with three different drugs: disodium cromolyn (a well- known mast cell stabilizer), bevacizumab (Avastin) and anti-podoplanin antibodies. We focused on their effects on tumor cells and vascular network. Fibrosarcoma cells reacted to each of these drugs in a specific and different manner. Disodium cromolyn, induced massive necrosis of the fibrosarcoma grown on chick CAM, but did not influence the blood vessels surrounding the tumor implant (Figure 3a and b). Despite the total necrosis observed for the initial implant, disodium cromolyn favored migration of tumor cells along the blood vessels of the CAM. This suggested an increase of tumor cell invasiveness

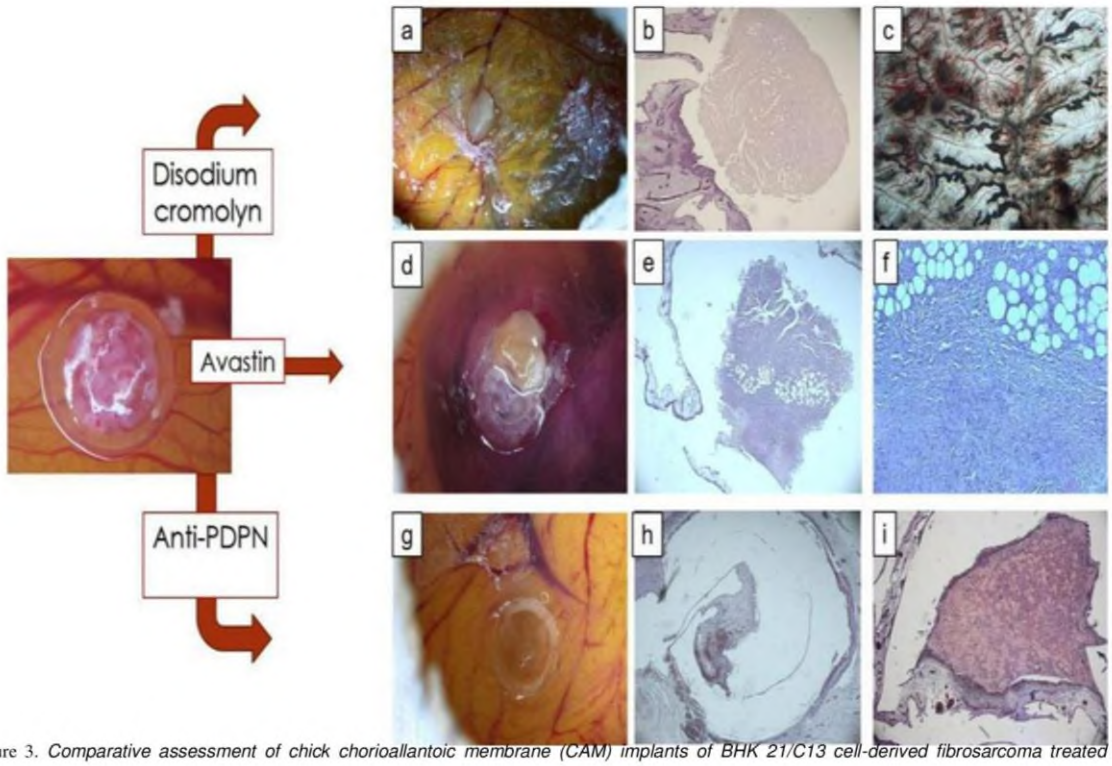

Figure 3. *Comparative assessment of chick chorioallantoic membrane (CAM) implants of BHK 21/C13 cell-derived fibrosarcoma treated with disodium cromolyn (a-c), Avastin (bevacizumab) (d -f) or anti-podoplanin (PDPN; g-i). Macroscopic view of the specimens revealed strong differences regarding tumor mass and vascularization (a, d, g). The strongest inhibition of tumor mass was reported for anti -PDPN antibodies (h), followed by disodium cromolyn (b) and Avastin (e). Despite the strong effect of disodium cromolyn on fibrosarcoma implanted on CAM, this drug was responsible for the appearance of distant metastases (c). A high inflammatory infiltrate was also noted for implants treated with anti-PDPN antibodies. Lipomatous differentiation around the implant (f) . Original magnification, a-e, g-i: ×100, f: ×200.*

and migration after disodium cromolyn treatment. In contrast, bevacizumab had no discernable effects on tumor cells, despite the previous observation of VEGF expression in fibrosarcoma tumor cells. Weak lipomatous changes inside the implanted tumor were observed (Figure 3d-f) . The highest regression of implanted fibrosarcoma was noted for the specimens treated with anti -podoplanin antibodies. The tumor volume was dramatically reduced macroscopically (Figure 3g) and microscopically (Figure 3h) after anti-podoplanin treatment. Compared with the other two drugs, anti-podoplanin treatment was followed by activation of the chick CAM immune system (not active under normal conditions) followed by a strong inflammatory response inside and around the implanted fibrosarcoma (Figure 3i).

Regarding the effects of the three drugs on the vascular network around the fibrosarcoma implants, it was noticed that disodium cromolyn stimulated the development of peritumoral blood vessels (accompanied by extensive migration of tumor cells along them), while bevacizumab partially induced a decrease of their density around the implant. Anti-podoplanin antibodies also reduced the number of peritumoral blood vessels, despite a high degree of inflammatory infiltrate noted around and inside the implant.

Inflammation was present for specimens treated with disodium cromolyn and anti -podoplanin antibodies but was lacking from the specimens treated with bevacizumab. The highest inflammatory infiltrate was noted for anti-podoplanin-treated specimens followed by those treated with disodium cromolyn, where scattered small patches of inflammatory cells were noted between peritumoral blood vessels of the CAM.

Discussion

BHK 21/C13 are versatile cells, subclone 13 being derived from the kidneys of five unsexed, 1- day-old hamsters. These cells have a high proliferative rate, as we also observed in

in vivo 32: 791-798 (2018)

our study during development of fibrosarcoma both in hamster and chick CAM model. They are highly adherent cells used in molecular biology and virus -related studies, especially to produce veterinary rabies vaccines (22). But BHK 21/C13 cell line is not exclusively used for vaccine production. In the early 1970s, BHK 21/C13 cell cultures were used for testing different therapeutic agents such as colchicine (23), bleomycin (24) and other chemotherapeutic drugs (25) . During these tests, it was observed that BHK 21/C13 cells are highly resistant, few cytostatic drugs being effective. This may suggest that more targeted therapies should be tested on such types of cell lines. We proposed and tested here for the first time the effects of bevacizumab, disodium cromolyn and anti-podoplanin antibodies on fibrosarcoma developed from BHK 21/C13 cells in chick embryo CAM model. Despite their therapeutic resistance to drugs, fibrosarcoma cells implanted on chick CAM showed a high sensitivity for two out of the three agents used in the present studies, rarely disodium cromolyn and anti-podoplanin. Disodium cromolyn has been tested on other aggressive malignant cells such as melanoma (26), exhibiting similar effects regarding tumor cell necrosis and stimulation of vascular network development. Compared with the effects on malignant melanoma cells, disodium cromolyn seems to favor the invasiveness and migration of fibrosarcoma tumor cells in close association with blood vessel development. No data have been reported to our knowledge regarding the potential mechanism of this migration of tumor cells along the tumor blood vessels mediated by the action of disodium cromolyn in fibrosarcoma cells, nor for other malignant cells.

Podoplanin-negative fibrosarcoma cells were highly sensitive to anti -podoplanin antibodies. This discrepancy may be due to high glycosylation of podoplanin in tumor cells previously reported in glioblastoma cell line LN229 (27). This aberrant glycosylation of podoplanin may give negative immunohistochemical findings despite podopanin presence inside the fibrosarcoma cells due to aberrant glycosylation not being recognized by usual anti- podoplanin clone D2-40. This may be an explanation for massive necrosis of tumor cells in specimens treated with anti-podoplanin.

The present study may be considered as the first attempt to characterize the immunophenotype of BHK -21/C13 cells. Of the 11 markers used in the present study to define the immunophenotype of this cell line, four of them had potential impact on the future use of these cells. Tyrosine kinase receptor CD117 (c-KIT), intensely expressed in BHK-21/C13 cell-derived fibrosarcoma, is a well -known target for imatinib mesylate and future studies may therefore be able to use these cells to test new drugs with a similar target. Currently the expression of this tyrosine kinase receptor in fibrosarcoma is questionable. The presence of CD117 in fibrosarcomas was rarely reported in the literature (29). In cats with fibrosarcomas, the presence of CD117 was not correlated with survival or histological grade (29). Indirectly evidence suggested that rat kidney fibroblasts expressed a glycosylated form of CD117, namely s-KIT (30) . Because of their early embryologic origin, it seems that BHK-21/C13 cells retain CD117 expression specifically for cells with pluripotency features. The persistence of CD117 in BHK-21/C13 fibroblasts supports the aggressiveness of these cells, this aspect being previously reported for CD117-positive fibroblasts-like stromal cells present in ovarian cancer stroma of patients with a unfavorable clinical outcome (31). Moreover, their ability to differentiate into smooth muscle actin- positive cells during fibrosarcoma development (reflected by the expression of SMA) supports the fact that these cells are not fully differentiated and still have an immature phenotype.

Aggressiveness and immaturity of BHK -21/C13 fibroblasts are also supported by the expression of PROX1 in tumor cells with both nuclear and cytoplasmic localization. During embryonic life, PROX1 is expressed in neural crests and is a marker of endodermal compartment. Renal development depends on PROX1 expression, especially at the level of Henle loop, most probably due the interaction between epithelial and stromal cells (32). In a zebrafish model, it seems that PROX1 has a dual role in renal development: after its initial roles in the specification of inter-renal primordium, it is critical for the maturation of the inter-renal organ (33) . Malignant transformation is followed by up- regulation of *PROX1* in tumor cells reported for several types of cancer, such as gastric (34, 35), pancreatic (36), vascular (37) and renal (38) cancer. For all these cancer types, PROX1 overexpression is related to high aggressiveness and poor prognosis (35). PROX1 distribution and expression in BHK- 21/C13 fibrosarcoma may be considered a promoter of aggressive behavior of these cells, clinically detected during the development of the tumor in both hamster and the chick embryo CAM model.

Weak and inconsistent expression of VEGF was related to lack of response to bevacizumab in the chick embryo CAM model. A previous report suggested that VEGF secreted by human fibrosarcoma cells promotes and sustains distant metastases after inhibition of primary tumor (39) . We observed a similar phenomenon of development and persistence of distant metastases in the specimens treated with disodium cromolyn inhibition of primary tumor but not in those treated with bevacizumab. High EGFR expression of BHK -21/C13 fibrosarcoma is in concordance with previous data showing EGFR expression and modulation in soft-tissue sarcomas cell lines *in vitro* and *in vivo* by a combination of gefitinib and doxorubicin (40). These findings support the use of BHK -21/C13 fibroblasts in future research of tumor cell behavior after anti-EGFR agents.

Conclusion

Here we established the immunophenotype of BHK-21/C13 fibroblast-derived fibrosarcoma demonstrating that these fibroblasts represent a particular cell line with vimentin+/ CD34−/CD117+/PROX1+/podoplanin−/EGFR+ phenotype, suggesting highly aggressive behavior based on several molecular peculiarities not previously described for this cell line. The heterogeneous response to disodium cromolyn, bevacizumab and anti - podoplanin support the use of these cells for the future evaluation of other new targeted therapies.

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Conflicts of Interest

The Authors have no conflict of interests to declare in regard to this study.

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The Impact of Residual Bowel After Extended Bowel Resection on Bacterial Overgrowth and Bacterial Translocation

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The intestinal mucosa is a major barrier in preventing bacteria invading the body but under certain circumstances mucosal gut barrier can be breached leading to the migration of bacteria to distant organs (mesenteric lymph nodes, spleen and liver). This process is termed bacterial translocation. The abnormal changes in gut ecosystem due to bowel resection led to bacterial overgrowth demonstrated postoperatively in male wistar rats duodenal and stool culture. We proved that after an extended bowel resection there is a direct relation between the number of the intestinal bacteria, the abnormal mucosal structural and functional changes and bacterial translocation to distant organs. A total of 22 male wistar rats divided in 4 groups were studied, short bowel syndrome was simulated in 16 by extended bowel resection (60%, 70% and 75%) while 6 rats had no surgery. Bacterial toxins induced local inflammation which altered neuromuscular tissue function, deteriorated further the gut barrier and increased bacterial overgrowth and bacterial translocation demonstrated by rat's intestinal biopsy results. The sequence of events leading to translocation remained unclear. The correlation between the percentage of the duodenal aspirate Enterococcus and cecal stool bacteria in rats was R= 0.81, while the correlation between duodenal aspirate Enterococcus and cecal stool bacteria of rats without ileocecal valve resection compared to rats with ileocecal valve resection was R= 0.57 being statistically relevant. Simulated short bowel syndrome by extended intestinal resection caused bacteria overgrowth in residual rat's bowel and this subsequently led to bacteria translocation to distant organs.

Keywords: Bacterial translocation, Bacterial overgrowth, Intestinal resection, Bowel adaptation, Duodenal aspirate.

The intestinal mucosa is a major barrier in prevention of bacteria invading the body, but under certain circumstances this mucosal gut barrier can be breached leading to the migration of gut bacteria to mesenteric lymph nodes (MLNs), spleen and liver [1]. This process is termed bacteria translocation. There are 3 known factors that facilitated this process: Increased number of gut bacteria, impaired immune function and increased mucosal barrier permeability (1). Duodenal culture postoperative demonstrated Bacterial overgrowth (BO) and subsequent bacteria translocation (BT) in male rats (wistar). This experiment validated the fact that there is a direct relation between the number of the intestinal bacteria, the abnormal mucosal structural and functional changes and the mesenteric lymph nodes bacterial translocation to distant organs after extended bowel resection (EBR) in rats. Bacterial toxins induced local inflammation which further deteriorated the gut barrier and increased BO demonstrated by rat's intestinal biopsy results. Abnormal changes in gut ecosystem due to bowel resection led to BO. Translocation of indigenous bacterial from rats gut to MLNs, spleen and liver appeared to be an important step in the pathogenesis of increasing intestinal permeability and by promoting the absorption of endotoxins or other enteric bacterial products [2]. Though we know that one of the most predictable risk factors for translocation is the small bowel bacteria overgrowth (SBBO), the sequence of events that led to translocation remained unclear. We proved that EBR in male wistar rat's triggered chain of reactions that led to the increased number of bacteria (bacterial overgrowth), the abnormal intestinal mucosal morphologic and functional changes, and the bacterial translocation to distant organs. Bacterial translocation in humans though not yet proven is still presumed to be involved in multi-organic dysfunction syndrome, systemic inflammatory response syndrome, acute pancreatitis, cirrhosis, burns, ischemia–reperfusion syndrome, and intestinal obstruction [3]. Study was designed to compare intestinal bacterial flora of cecal stools and duodenal fluid of rats with different degrees of bowel resections (60%, 70% and 75%) prior to and at the end of the experiment, while the rationale for this was to prove that the intestinal bacteria had an adverse effect on the intestinal mucosal architecture and cell kinetics seen on rats gut histopathology analysis.

Our aim was to prove that after extended bowel resection there is a direct relation between the numbers of the intestinal bacteria (bacterial overgrowth), the abnormal intestinal mucosal morphologic changes and the BT to distant organs (MLNs, spleen and liver). We were also able to compare our findings with similar studies in humans.

Experimental part

Materials and methods

A total of 22 rats male wistar rats aged between 4 to 6 months (equivalent to human age of 12-18 years), and weighing between 235 and 300g divided into 4 groups were

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studied, out of which SBS was simulated in 16 (n= 16) through extended bowel resection (60%, 70% and 75% respectively). However, 6 rats (n= 6) had no surgery (2 received proton pump inhibitor (pantoprazole 20mg) and histamine 2 blocker (zantac 75mg), 2 received antibiotics (Neomycin 125mg/5mL) and probiotics (linex 60mg), 2 received no medication). The rationale for dividing 6 non-surgical rats into subgroups was to demonstrate the harmful effect of antibiotics and antisecretory medications (in 4 rats) on intestinal mucosal architecture and cell kinetics seen on jbiopsies even in the absence of an EBR. Experiment was performed in a facility that housed research rodents. Animals were bred in our institution research facilities. Experiment was carried out to a high ethics standard and with the approval of the ethics committee at the University of Medicine and Pharmacy (Approval number: 19/17.04.2017). Animals were bred in different cages in our laboratory according to current regulations. The standard rat cage used by research institutions (including ours) requires approximately 903 cm^2 of floor space. Bedding floor was wire bottomed and with wood-shaving product which helped to keep animals warm as well. Animals were kept in individual cages with free access to food and water. Medications (antibiotics/ probiotics, antisecrectory and pain killers) were mixed with the animal's water. Animals were fed standard pellet rat chow prior to surgery and after surgery. Rats were disease free of parasites, viruses and bacteria. Room environment where rats were housed was controlled at a temperature of 20-23 ℃: humidity of 30% to 70%: 14:10-h light: dark cycle). Rats were housed in individually ventilated caging (model Allentown caging). Cages were changed at least every 14 days and more often as necessary, according to established standard operating procedures. Animal care staff wore dedicated footwear and personal protective equipment that consisted of a disposable gown and gloves when performing animal husbandry tasks. Animal rooms were swept and then mopped. Sanitation Strategies was on daily basis except on weekends and holidays. Rat's age varied between 4 to 6 months (equivalent to human age of 12-18 years). The rationale for using younger rats was to compare the results with the results of SBS pediatric patients.

Study design: Group A: 60% intestinal resection without an ileocecal valve resection (A1 = 4 rats) and 60% intestinal resection with an ileocecal valve resection (A2= 4 rats). Total number of rats: 8

Group B: 70% intestinal resection without an ileocecal valve resection (B1= 3 rats) and 70% intestinal resection with an ileocecal valve resection (B2= 3 rats). Total number of rats: 6

Group C: 75% intestinal resection with ileocecal valve resection (C= 2). Total number of rats: 2

Group D: Rats without intestinal resection but under daily proton pomp inhibitor treatment (D1= 2 rats), Rats without intestinal resection but under daily antibiotics and probiotics treatment (D2= 2 rats) and Control study rats without surgery (D3= 2 rats). Total number of rats: 6

Body weight, nutritional status and stool were frequently analyzed. Rats with 75% of bowel resection were sacrificed 2 weeks after surgery. Rats with 70% bowel resection were sacrificed 3 weeks after surgery, while rats with 60% bowel resection were sacrificed 4 weeks after surgery together with the other non-surgical rats. The rationale for sacrificing animals with more EBR earlier was based on the fact that they gradually exhibited signs of intestinal failure (bloating, diarrhea, poor appetite, malnutrition, foul smelling stool (steatorrhea), weakness and weight loss). MLNs, spleen, liver, duodenal fluid and cecal stool were obtained for bacterial detection, while samples of the harvested intestine were sent for histopathology analysis. At the time of harvest, a 50-mm segment of the mid-ileum and colon were removed and opened along the mesenteric border. Tissues were fixed in 4% paraformaldehyde and embedded in paraffin. Sections (5μm) were stained with hematoxylin and eosin and examined using light microscopy.

Operative procedures*:* Rats were in good health and were randomized by body weight and fasted overnight prior to surgery. The following day, rats underwent laparotomy (as described) between 9:00 AM and 15:00 PM. Animals were placed briefly on inhalation general anestesia (isoflurane 2-2.5%) and oxygen of 0.5-0.7L/min. The rationale for using isoflurane was because it offers many advantages over other inhalational anesthetics. Its faster induction and recovery, relative sparing effect on cardiovascular function and cerebral blood flow autoregulation, and negligible metabolism makes it particularly useful in anesthetic management of small animals. A ventral abdominal midline incision was performed. The locations for bowel transection and partial small bowel-colon resection were identified by using defined landmarks. The length of the residual intestine was measured in 10-cm segments from the ligament of Treitz advancing along the anti-mesenteric border of a slightly stretched intestine. Rats gut were removed (intestinal resection) at the rate of 60, 70 & 75% respectively (with or without the ileocecal valve), followed by intestinal end to end anastomosis. There was no anastomotic leak found at the time of animal organ harvesting. Pathology samples of intestine, MLNs, liver and spleen were obtained on initial resection for pathology comparison of specimens at end of the experiment when the remainder intestinal specimen of non-surgical rats was obtained. Duodenal fluid culture of rat's prior to surgery were obtained and were all negative for bacteria. Pathology samples of rat's intestine, MLNs, liver and spleen obtained at initial resection for comparison at the end of the experiment were all normal.Antibiotics (neomycin) were added to the rat's drinking water prior to and after surgery until tissue collection on days 14, 21 and 30 respectively.

Tissue collection*:* Rat's intestines were stripped of mesenteric and vascular connections and sequentially removed from the peritoneum. The lumen was flushed with saline solution to clear intestinal contents. Segments used for this study were collected sequentially at the equivalent site in each rat. MLNs were dissected from the mesentery and then parts of the spleen and liver were also harvested and placed in bottles containing formaldehyde for preservation. Blood was drawn by cardiac puncture. Serum was collected and stored at -80℃.

Bacterial culture and identification*:* MLN were homogenized with sterile glass tissue grinders, and then put on MacConkey agar plate to identify gram-negative enteric bacterial pathogens. Positive bacterial colonies were counted after incubation for 24 h at 37℃. Bacterial

translocation to MLN was considered present when a sample had more than 10 colony-forming units per gram tissue. Positive colonies were sub-cultured on blood agar for an additional 24 h at 37°C, and enterobacteriaceae species were identified in our clinical laboratory. Bacterial overgrowth was found in duodenal fluid of greater than 100,000 colony-forming per unit per mL. *Enterococcus* accounted for most of the bacterial translocation followed by Escherichia coli.

Euthanasia method**:** Animal euthanasia was performed in our research laboratory with trained personnel approved on the Animal Protocol. Inhalation anesthetics gas isoflurane was used to render the rodents unconscious. Animals were placed in sealed chambers where high level of anesthetic gas was introduced. Upon achieving unconsciousness death was caused by CO2 at a flow rate of 10-30% volume per minute, followed by continued exposure to CO2 for at least 15 min after respiratory arrest. Animals showed no sign of stress. Rat's organs (MLNs, intestine, spleen and liver) were harvested for histopathology and microbiology studies, After euthanasia death was verified prior to disposal, after which animal remains were cremated.

Results and discussions

Rats with 60% intestinal resection without ileocecal valve resection (table 1, [A1.1 & A1.2]): Duodenal aspirate and cecal stool cultures were positive for Enterococcus 30 days postoperatively.

Histopathology result showed an increase in villous height, crypt dept, the intestinal diameter thickness (mucosal hyperplasia and bowel dilatation), the pseudo-stratified immature enterocytes and rare calciform cells (fig 1).

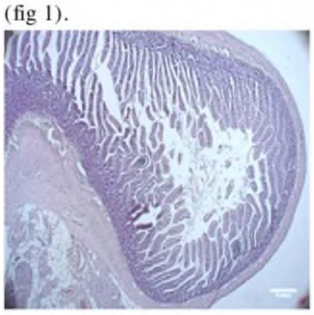

Fig. 1.

Rats with 60% intestinal resection with ileocecal valve resection (table 1, [A2.1 & A2.2]): Duodenal aspirate cultures were positive for Enterococcus, while cecal stool cultures were positive for Enterococcus and Klebsiella 30 days postoperatively. Histopathology result showed an increase in villous height, crypt dept, the intestinal length thickness (mucosal hyperplasia and bowel dilatation), the pseudo-stratified immature enterocytes with rare calciform cells and lymphoplasmocytic mucosal infiltration (fig 2).

Rats with 70% intestinal resection without ileocecal valve resection (table 1, B1): Duodenal aspirate and cecal stool cultures were positive for Enterococcus 21 days postoperatively. Histopathology result showed villous atrophy and no calciform and detrusor cells (fig 3).

Rats with 70% intestinal resection with ileocecal valve resection (table 1, B2): Duodenal aspirate cultures were positive for Enterococcus, while cecal stool cultures were positive for Enterococcus and E. coli 21 days postoperatively. Histopathology result showed increased villous atrophy with some calciform cells at the base of the intestinal villi (fig. 4)

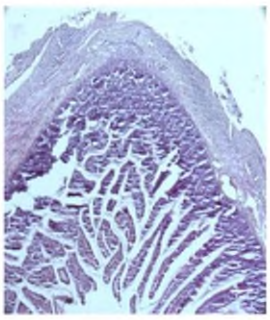

Fig. 2.

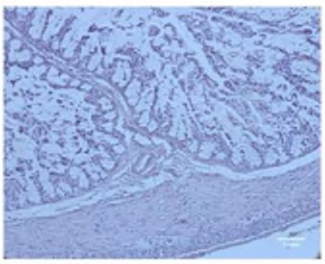

Fig. 3.

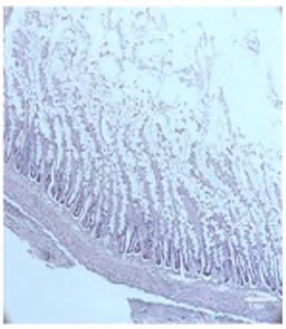

Fig. 4.

Rats with 75% intestinal resection with ileocecal valve resection (table 1, C): Duodenal aspirate cultures were positive for Enterococcus, while cecal stool cultures were positive for Enterococcus and Ecoli 14 days postoperatively. Histopathology result showed increased intestinal atrophy and proliferation of programmed death cells (apoptosis) within enterocytes, mucosal hyperplasia with immature enterocytes but no calciform cells. Epithelial desquamation mixed with mucosal hyperplasia and leucocytes infiltration, intestinal glands hypertrophy with lumenal detrusor cells associated with rounded hypertrophied calciform cells, moderate and numerous areas of leucocytes infiltration. Leucocytes infiltration was associated with numerous detrusor cells at the lamina propria (fig. 5a and 5b).

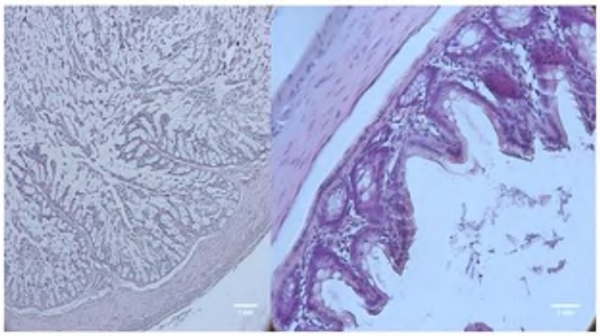

Fig. 5a Fig. 5b

Table 1
RATS DUODENAL FLUID CULTURE AND CECAL STOOL ANALYSIS BEFORE AND AFTER SURGERY

		Results
A	***A1***: 60% intestinal resection **without** an ileocecal valve resection	**A1.1:** -Duodenal aspirate cultures before surgery were negative, but were positive for enterococcus 30 days postoperatively; -Cecal stool cultures were positive for enterococcus 30 days postoperatively;
		A1.2:-Duodenal aspirate cultures before surgery were negative, but were positive for enterococcus 30 days postoperatively; -Cecal stool cultures after 30 days were positive for enterococcus 30 days postoperatively;
	A2: 60% intestinal resection **with** an ileocecal valve resection	**A2.1:**-Duodenal aspirate cultures before surgery were negative, but were positive for enterococcus 30 days postoperatively; -Cecal stool culture were positive for enterococcus + klebsiella 30 days postoperatively;
		A2.2:-Duodenal aspirate cultures before surgery were negative, but were positive for enterococcus 30 days postoperatively. -Cecal stool cultures were positive for E. coli + proteus 30 days postoperatively;
B	***B1***: 70% intestinal resection **without** an ileocecal valve resection	**B1.1:** -Duodenal aspirate cultures before surgery were negative, but were positive for enterococcus 30 days postoperatively. -Cecal stool cultures after 30 days were positive for enterococcus
	B2: 70% intestinal resection **with** an ileocecal valve resection	**B2.1:** -Duodenal aspirate cultures before surgery were negative, but were positive for enterococcus 21 days postoperatively. -Cecal stool cultures were positive for enterococcus + E. coli after 21 days.
C	75% intestinal resection with ileocecal valve resection	-Duodenal aspirate cultures before surgery were negative, but positive for enterococcus 14 days days postoperatively -Cecal stool cultures were positive for enterococcus + E coli 14 days postoperatively
D	***D1***: Rats without intestinal resection but on proton pomp inhibitor treatment	-Duodenal aspirate cultures before surgery were negative, but were for enterococcus 30 days postoperatively. -Cecal stool cultures after 30 days were negative for enterococcus, E. coli and salmonella
	D2: Rats without intestinal resection but on antibiotics and probiotics treatment	- Duodenal aspirate cultures before surgery were negative and still negative for enterococcus 30 days postoperatively. -Cecal stool cultures after 30 days were negative for enterococcus, E. coli and salmonella
	D3: Control study rats without surgery	-Duodenal aspirate cultures before surgery were negative and still negative for enterococcus 30 days postoperatively. -Cecal stool cultures were negative for enterococcus, E. coli and salmonella 30 days postoperatively.

Non surgical rats with daily histamine 2 blockers/proton pump inhibitors (table 1, D1) and daily antibiotics/probiotics (table 1, D2) medication: Duodenal aspirate cultures were positive for Enterococcus, while cecal stool cultures were negative for Enterococcus, E. coli and Salmonella. Histopathology result of rats treated with histamine 2 blockers/proton pump inhibitors showed lympho-plasmocytic mucosal inflammatory infiltration, reduced number of calciform cells and numerous enterocytes proliferation (fig 6).

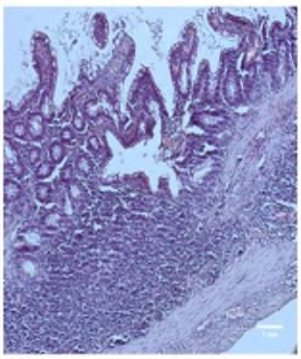

Fig. 6.

Histopathology result of rats that received probiotics/ antibiotics showed mild mucosal inflammatory infiltration, villous hypertrophy, glandula dilatation and enterocyte atrophy (fig 7).

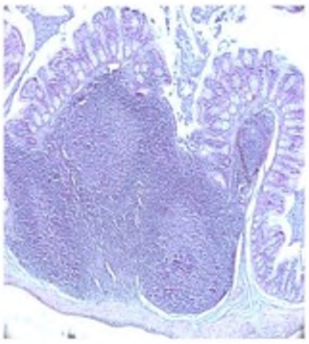

Fig. 7.

Control study rats (table 2, D3): Duodenal aspirate cultures were negative for bacteria, but histopathology showed intestinal villi without atrophy and no enterocyte hypertrophy (fig 8).

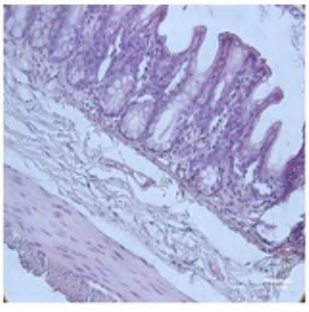

Fig.8.

Microbiology organ culture result of rats with 60% intestinal resection (without an ileocecal valve resection): MLNs cultures were positive for Enterococcus, while spleen and liver cultures were negative for bacteria. Rats with 60% intestinal resection (with ileocecal valve resection): MLNs, spleen and liver cultures were positive for Enterococcus and Klebsiella. Rats with 70% intestinal resection (without ileocecal valve resection): MLNs, spleen and liver cultures were positive for Enterococcus. Rats with 70% intestinal resection (with ileoocecal valve resection): MLNs, spleen and liver cultures were positive for E.coli and Proteus. Rats with 75% intestinal resection (with ileocecal valve resection): MLN, spleen and liver cultures were positive for Enterococcus and E coli. Rats with daily histamine 2 blockers/proton pump inhibitors treatment, rats under daily antibiotics/probiotics treatment and control study rats: MLN, spleen and liver cultures were negative for Enterococcus, E. coli, and Salmonella.

Microbiology culture (duodenal fluid, cecal stool, MLNs, spleen and liver), histopathology and laboratory tests showed a milder form of SBBO and BT in rats with lesser intestinal resection compared to rats with greater EBR. Bacterial concentration was higher in rats with ileocecal valve resection compared to rats without ileocecal valve resection. However, the non surgical rats that received just probiotics/antibiotics treatment had milder form of BO and no BT compared to non surgical rats on proton pump inhibitors/antihistamine 2 blockers because the chronic inhibition of gastric acid secretion by histamine 2 receptor blockers/proton pump inhibitors increased the number of gastric bacteria. Rats with intestinal resection gradually developed weight loss, significant bacterial overgrowth, malnutrition, malabsorption, maladaptation, diarrhea, self-filling loop, hepatomegaly, hepatic injury (bile duct proliferation), fibrosis, acute periportal and parenchymal inflammation at 2, 3 and 4 weeks postoperatively.

The percentage of the intestinal resection and the presence or the absence of the ileocecal valve determined the outcome of bowel adaptation. The correlation between the percentage of the duodenal aspirate Enterococcus and cecal stool bacteria in rats was R= 0.81. However, the correlation between duodenal aspirate Enterococcus and cecal stool bacteria of rats without ileocecal valve resection compared to rats with ileocecal valve resection was R= 0.57 being statistically significant. Correlations were analyzed with pearson's correlation test.

EBR were followed by a compensation response within the residual bowel termed adaptation, a process characterized by an early increase in blood flow to the intestinal remnants and by long term stimulation of intestinal growth, which enormously enlarged the absorptive surface area [4]. The latter led to an increase in villous height, crypt dept, intestinal length and thickness in diameter (mucosal hyperplasia and bowel dilatation) [4]. Resection induced adaptation provoked functional alterations that affected intestinal morphology and kinetics of cell turnover [5]. In terms of morphologic changes the bowel increased in its length and caliber as hyperplasia and hypertrophy of intestinal layers were noted. In addition to morphologic changes the rate of enterocytes turnover was enhanced, as demonstrated by increased proliferation and by the rate of programmed cell death (apoptosis) within enterocytes [5]. Mucosal hyperplasia and villous hypertrophy occurred in the early stage of the disease. SBBO caused various degrees of intestinal villous atrophy and mucosal damage due to the effect of the endotoxins [5]. Loss of intestinal barrier was associated with translocation of enteric bacteria in animal model, but comparative studies showed that BT in humans was not

Table 2

RAT LABORATORY BLOOD TESTS, LAST DAY OF EXPERIMENT

	Normal values	*A1.1*	*A2.1*	*B1.1*	*B2.1*	*C*	*D1*	*D2*	*D3*
Blood Volume	*5.6-7.1 ml/100g*								
Clotting time	*2-5 minutes*								
RBC	*6.76-9.75 x 10^6/mm³*	6.1	5.0	4.3	4.7	4.1	6.3	7.7	7.9
HCT	*36.1% to 44.3%*	30	31	25	27	24	37	39	41
WBC	*6.6-12.6 x 10^3/ mm³*	10.3	10.7	14.1	13.0	16.6	6.25	7.20	5.0
Hb	*11.5-16.1 g/dL*	11.1	11.2	10.8	10.9	10.7	11.9	13.9	14
N	*1.77-3.38 x 10^3/ mm³*	3.6	3.4	4.2	5.7	5.9	3.2	2.0	1.8
L	*4.78-9.12 x 10^3/ mm³*	11.9	12.7	13.0	12.8	14.3	4.8	4.0	3.2
E	*0.03-0.08 x 10^3/ mm³*	0.06	0.09	1.0	1.2	1.7	0.01	0.02	0.01
M	*0.01-0.04 x 10^3/ mm³*	0.01	0.02	0.02	0.04	0.05	0.01	0.02	0.01
B	*0.00-0.03 x 10^3/mm³*	0.01	0.01	0.02	0.02	0.03	0.01	0.01	0.00
Pl	*150-460 x 10^3/mL*	100	95	85	80	62	190	255	334
Tot PR	*5.6-7.6 g/dL*	5.3	5.5	5.2	5.0	4.8	6.3	6.8	5.6
Albumin	*3.5-5.5 g/l*	32	34	29	26	19	39	44	48
Glucose	*50-135 mg/dL*	35	18	147	149	158	63	77	98
BUN	*15-21 mg/dL*	36	56	64	66	71	22	16	18
Creatinine	*0.2-0.8 mg/dL*	0.9	1.0	1.2	1.1	1.4	0.4	0.6	0.4
Na	*143-156 mEq/L*	140	141	139	136	133	144	148	151
K	*5.4-7 mEq/L*	5.5	5.7	7.1	7.0	7.4	6.0	6.2	5.9
Cl	*100-110 mEq/L*	101	100	105	112	115	103	102	106
P	*3.11-11 mg/dL*	4.2	3.6	7.2	6.0	10	5.0	6.7	9.0
Ca	*5.3-13 mg/dL*	5.6	5.9	5.0	5.1	4.8	70	8.2	12.0
ALT	*17.5-30.2 U/L*	16	15	47	36	54	47	18	28
AST	*45.7-80.8 U/L*	250	274	301	292	588	190	95	72
ALK PH	*56.8-128 U/L*	309	289	324	333	401	227	64	44
Cholesterol	*40-130 mg/dL*	145	162	232	222	276	124	127	98
Tot Bilirubine	*5.1-17 mmol/L*	25	23	65	68	72	19	16	11
Amylase	*25-125 U/l*	220	243	203	302	420	169	135	85
Lipase	*0-160 U/L*	350	365	342	378	410	218	157	123
CRP	*0-10 mg/dL*	187	192	202	224	370	59	37	5
D Lactate	*0.0-0.25 mg/dL*	0.38	0.50	0.48	0.68	0.94	0.30	0.18	0.08
IgA	*70-312 mg/dL*	67	63	58	61	54	98	99	220
IgM	*56-350 mg/dL*	356	373	370	399	424	320	287	129
IgG	*640-1350 mg/dL*	550	528	503	487	420	489	695	840

A1.1: Rats with 60% intestinal resection but without ileocecal valve resection
A2.1: Rats with 60% intestinal resection including ileocecal valve resection
B1.1: Rats with 70% intestinal resection but without ileocecal valve resection
B2.1: Rats with 70% intestinal resection including ileocecal valve resection
C: Rats with 75% intestinal resection including ileocecal valve resection

linked to intestinal permeability increase or villous atrophy [6]. Extended bowel resection in rats led to delayed intestinal transit time and intestinal stasis, this predisposed host to SBBO [7]. Motility disorders that followed intestinal resection and/or gastric acid inhibition contributed to the development of SBBO [7]. Mucoasal damage caused by intestinal motility may also be related to the psychological stress of laparatomy and subsequent bacterial overgrowth. Obstruction of the intestinal content outflow at the anastomotic site due to adhesion, strictures and web after an EBR helped to promote SBBO [8]. The possibility of bacteria overpopulation in spleen and liver due to anastomotic leak was excluded postoperatively during organ harvest. Gastric acidity acted as the initial line of defense against ingested bacteria, even though the bactericidal effect of gastric hydrochloric acid may have neutralized excess gut bacteria, it still was unable to stop BO and subsequent BT [9]. Gastrointestinal and pancreatic secretions helped to dilute bacteria mass and were significant obstacles to bacterial overgrowth [10]. The malnutrition that followed EBR was another factor that further decreased gastric acidity and host immune function, and exposed host to SBBO [10]. Nitric oxide production by the intestinal microbiota played an important role in gut bacterial flora regulation [11]. The impediment of food passage, gastric, pancreatic and liver secretions from a small intestinal segment primarily reduced the number of cells per crypt, which led to a lower cell production, responsible for the reduction of the functional villous cell compartment [12]. Apoptosis (programmed cell death) also increased crypt and villous compartments after resection [12]. Intestinal adaptation occurred by increasing the size of the structures present in the residual intestine, not by the increased number of structural units. Intestinal resection resulted in an increase in the number of villous and crypt enterocytes [12]. This adaptive response occurred secondary to increased crypt cell proliferation early after resection [13]. Deficiency of host defense mechanism and particularly the absence of secretory immunoglobulin A (SIgA) promoted SBBO and BT. The adaptation changes that occurred after EBR depended on the resected length, the location of the residual intestine and the amount of energy animals derived from enteral feeding [13]. The absence of the ileocecal valve exposed host to excess bacteria due to lack of the ileocecal valve brake function, this resulted in reflux of colon content that led to excess bacterial overload [13]. Though, the ileocecal valve was strongly predictive of bowel adaptation it was not predictive of survival, but the amount of energy that animals derived from enteral nutrition was predictive of survival. Evidence from experimental animal studies (rats) indicated that probiotics exert barrier-enhancing, antibacterial, immune-modulating and anti-inflammatory effect [14]. The capacity of probiotics to maintain bowel bacteria flora equilibrium was very modest and thus failed to prevent BO. The use of oral antibiotics (neomycin) prior to and after surgery failed to stop BO and BT to MLNS compared to other studies where the use of oral antibiotic cocktail (metronidazole, neomycin, and polymyxin) was reported to have completely blocked bacterial translocation. Even though the use of antibiotics was presumed effective for gut bacteria decontamination, our study did not find any useful effect of gut bacterial antibiotic decontamination. Moreover, the balance between intestinal resection and antimicrobial effect of antibiotics (neomycin) failed to stop SBBO. The intestinal mucosa demonstrated the harmful effect of drugs which together with EBR predisposed host to BO. Excess bacteria in the intestinal lumen caused deconjugation of bile salt acids which led to steatorrhea, this can progress further to liver failure even with the best treatment [15]. Absolute small bowel length and cholestasis (conjugated bilirubin ≥2.5 mg/dL), were the strongest predictor of mortality [16]. Gut bacteria became potentially harmful when gut ecosystem underwent abnormal changes due to EBR. We detected BT to distant organs (spleen and liver) compared with other studies where the BT studied were limited to just the MLNs [17].

The lack of sufficient mucosal surface in operated rats and the long time intestinal adaptation process were crucial in determining bowel functional capacity. Strategies to increase adaptation remained elusive despite an abundance of experimental data.

Conclusions

SBS simulated in experimental animals (rats) by EBR caused BO in the residual bowel and this subsequently led to BT to MLNs, spleen and liver. SBBO and BT were the two most common complications in SBS with great impact on morbidity and mortality. These results demonstrated how the residual intestinal length influenced the evolution of SBS subjects postoperatively. However, by comparing the microbiology and histopathology results of rats with different percentages of intestinal resection, we were able to conclude that the greater the intestinal resection the lesser the chances of bowel adaptation and survival, also the greater the chances of the disease progressing to intestinal failure and cholestasic liver disease. We were able to sustain the fact that after an EBR there is a direct relation between the number of the intestinal bacteria (bacterial overgrowth), the abnormal intestinal mucosal morphologic and functional changes, and the bacterial translocation to distant organs. We also examined the evidence that after EBR adaptation occurred in rats just like in humans and focused on the factors that influenced adaptation and the strategies used to optimize this process. This studies permits better prediction of SBS outcome, which may help to direct future management of these life threatening and challenging cases.

List of abbreviations

MLNs: Mesenteric lymph nodes
BO: Bacterial overgrowth
BT: Bacterial translocation
SBS: Short bowel syndrome
SBBO: Short bowel bacterial overgrowth
EBR: Extended bowel resection

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I.3. APLICAREA METODELOR HISTOCHIMICE ȘI IMUNOHISTOCHIMICE ÎN PROCEDURILE DIAGNOSTICE ȘI CU POTENȚIAL IMPACT TERAPEUTIC ÎN BOLI CRONICE (OSTEOPOROZA, FIBROZA PULMONARĂ)

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Osteoporoza este cea mai comună boală metabolică a oaselor. Patogenia osteoporozei este complexă și multifactorială, caracterizată prin scăderea densității minerale osoase (procesul decalcificării osoase focale) și prin deteriorarea micro arhitecturii osoase (cavități de resorbție profundă, delimitate de lamelele osoase mai subțiri, cu zone cu rezistență redusă, micro fracturi osoase).

Osteoclastele se dezvoltă din precursorii liniei celulare monocitare-macrofage mononucleare după stimularea cu factorul de stimulare a coloniilor macrofage (M-CSF) și a receptorului factor nuclear activat pentru ligandul kappa (RANKL). Activatorul receptorului factorului nuclear-KB (RANK) este un membru al familiei factorului de necroză tumorală exprimat de osteoclaste și precursorii lor. Osteoprotegerina (OPG) aparține familiei receptorilor de factor de necroză tumorală (familia TNFR) și inhibă diferențierea și fuziunea celulelor precursoare osteoclastice și blochează activarea osteoclastelor mature. Studiul demostrează că RANKL este semnificativ crescut în cazul osteoporozei postmenopauzale și este un marker al creșterii resorbției osoase. OPG este moderat crescut în cazul osteoporozei și este un marker al formării osoase, stimulând turnover-ul osos. Densitatea osoasă (BMD) este încă standardul de aur pentru diagnosticul osteoporozei, dar markerii țesutului osos (BTM) pot furniza informații utile cu privire la procesul de remodelare a acestuia. Scopul acestui studiu a fost de a determina corelațiile dintre BMD și nivelurile serice ale BTM (fosfatază acidă tartrat-5b [TRAP-5b]), fosfatază alcalină specifică osului (BSAP), estradiol (E2) și magneziu [2+]) la femeile la postmenopauză cu osteoporoză. Studiul nostru a arătat că BMD se corelează negativ cu BTM și pozitiv cu nivelurile de estrogen (E2) și Mg (2+). Nivelurile TRAP-5b se corelează negativ cu E2, în timp ce BSAP se corelează pozitiv. Mai mult, nivelurile BSAP sau corelat negativ cu durata de deprivare a E2. TRAP-5b prezintă o bună specificitate în identificarea pacienților cu osteoporoză postmenopauză.

Aspectul fibrelor reticulare nu este luat în considerare în clasificările actuale ale fibrozei pulmonare. Scopul studiului a fost de a evalua distribuția și arhitectura fibrelor reticulare pentru utilizare potențială ca marker de țesut al severității fibrozei. Analiza cazurilor incluse în studiu a evidențiat mai multe aspecte: depleția fibrelor reticulare este în mod constant asociată cu stadiile avansate de fibroză; această schimbare majoră poate explica ireversibilitatea procesului fibrotic și absența eficacității tratamentului asupra recuperării funcției pulmonare.

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ORIGINAL PAPER

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The OPG/RANKL system and zinc ions are promoters of bone remodeling by osteoblast proliferation in postmenopausal osteoporosis

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Abstract

RANKL and its decoy receptor osteoprotegerin (OPG) is a mediator system involved in bone resorption and may be responsible for the homeostatic mechanism of normal bone remodeling. The serum levels of both OPG and soluble RANKL (sRANKL), the level of RANKL in primary cultures of osteoblasts, and the bone level of Zn^{2+} were measured in six women with postmenopausal osteoporosis and three women without osteoporosis (control group). As compared to control cases, patients with less than 15 years of estrogenic deprivation (cohort 1, n=3) presented increased levels of OPG (109.82%, $p<0.002$), sRANKL (229.13%, $p<0.001$) and $RANKL_{OBL}$ (272.35%, $p<0.001$), and decreased levels of Zn^{2+} (67.81%, $p<0.001$), whereas patients with more than 15 years of estrogenic deprivation (cohort 2, n=3) showed decreased levels of OPG (70.44%, $p<0.003$), and Zn^{2+} (61.41%, $p<0.001$), and increased levels of sRANKL (181.69%, $p<0.002$) and $RANKL_{OBL}$ (201.1%, $p<0.002$). The significantly increased levels of sRANKL and $RANKL_{OBL}$ in postmenopausal osteoporosis demonstrate osteoclastogenesis activation. According to the length of the estrogenic deprivation period, postmenopausal women with osteoporosis presented either increased (cohort 1) or decreased (cohort 2) OPG levels demonstrating osteoblast activation and osteoblast apoptosis stimulation, respectively. The bone levels of Zn^{2+} were significantly decreased showing limited proliferation and differentiation of the osteoblasts.

Keywords: bone Zn^{2+} ions, OPG/RANKL, osteoblasts, osteoporosis postmenopausal.

Introduction

Osteoporosis is the most commune bone metabolic disease. Osteoporosis pathogenesis is complex and multifactor, characterized by the decrease of the bone mineral density (a focal bone decalcification process) and by bone microarchitecture deterioration (deep resorption cavities, delimited by bone lamellas more thin, with low strength, bone microfractures areas) [1, 2].

The bone is permanent liable to a process of bony remodeling, which has cyclic character in which resorpted bone quantity (through osteoclasts action) is equal to the one which it is formed (through osteoblasts action), first stage being the resorption one, and than the formation stage [2, 3].

Osteoclasts develop from precursors of the mononuclear monocyte-macrophage cell line after stimulation by macrophage colony-stimulating factor (M-CSF) and receptor for activated nuclear-factor kappa ligand (RANKL). Receptor activator of nuclear factor-κB (RANK) is a member of the tumor necrosis factor family expressed by osteoclasts and their precursors [4, 5].

Osteoblasts, bone-forming cells are of mesenchymal origin and share a common precursor cell with adipocytes. During normal bone remodeling, marrow stromal cells and osteoblasts produce RANKL, which binds to the transmembrane receptor RANK on osteoclast precursors and induces differentiation and activation. Osteoprotegerin (OPG) is a soluble member of the tumor necrosis factor receptor family (TNFR family) and inhibits the differentiation and fusion of the osteoclastic precursor cells, and blocks the activation of mature osteoclasts. Osteoblasts also produce osteoprotegerin (OPG), a soluble "decoy receptor" that blocks RANKL and maintains control of the bone remodeling process [4, 6].

Osteoblasts differentiation from their mesenchymal precursors is a process dependent by the major transcription factor presence Cbfa-1/Runx-2 (core-binding factor-1/runt-related transcription factor-2), which regulates transcription at genomic level. Zinc ions (Zn^{2+}) are stimulating osteoblasts proliferation and differentiation, being promoters of the major transcription factor presence Cbfa-1/Runx-2. In osteoporosis, this mechanism is diminished as a result it is suppressed osteoblasts bone making activity, being favorable for microfractures/fractures bone appearance [4, 6, 7].

The discovery of the RANK/RANKL/OPG pathway and its implications in the pathogenesis of osteoporosis provides a molecular target for new therapies to improve bone health [4, 8].

Materials and Methods

Patients' selection

The study was made using two cohorts of patients

with postmenopausal osteoporosis, in comparison with a control group (patients without osteoporosis), which suffered a surgical procedure for femoral cervix fracture in the Orthopedic–Traumatology I Clinic of the County Hospital, "Victor Babeş" University of Medicine and Pharmacy, Timişoara, Romania, in the years 2007–2008:

- Cohort 1 (patients in menopause, with 15 years of estrogenic deprivation), includes three patients (n=3) having their age under 65 years and T-score ≤-2.5 DS at lumbar spine and femoral level.
- Cohort 2 (patients in menopause, with over 15 years of estrogenic deprivation) was formed from three patients (n=3), having the age over 65 years and T-score ≤-2.5 DS at lumbar spine and/or femoral level.
- Control group (patients without osteoporosis) was formed from three patients (n=3), age under 60 years and T-score ≥-2.5 DS at lumbar spine and/or femoral level.

Bone densitometry

Dual energy X-ray absorptiometry (DXA) scans measure the bone mineral density (BMD) at your spine and/or femur and assigns a T-score. The T-score is defined as the number of standard deviations above or below the mean BMD for normal young females. Bone mineral density of the femoral neck and lumbar spine (L1–L4) was measured using dual-energy X-ray absorptiometry (DXA-Hologic Inc. QDR–1000; Bedford, USA). The osteoporosis diagnosis was made according to the World Health Organization criteria, after DXA assessment (spine and/or neck of femur T-score, sT≤ -2.5 DS) [9].

Biochemical markers

OPG, RANKL levels (soluble and osteoblast culture) were measured by a sandwich ELISA (enzyme-linked immunosorbent assay) technique using the following tests:

The kit human RANKL (sRANKL and $RANKL_{OBL}$), from Biomedica Med. GmbH & Co KG, Vienna was also measured by a set of specific antibodies and standards of the same company.

The assay performance was characterized by a lower detection limit of (0 pmol/L + 3 SD): 0.08 pmol/L, conversion factor pg/mL to pmol/L its 1 pg/mL= 0.05 pmol/L and the mean of precision (intra-assay of variation was 3–5%, inter-assay of variation was 6 and 9% respectively) [10].

The kit human Osteoprotegerin ELISA, from BioVendor Laboratory Medicine, Inc., Czech Republic according to the manufacturer's protocol (the kit is a biotin labeled antibody based sandwich enzyme immunoassay for the quantitative measurement of human osteoprotegerin in serum, specificity – approx. 1%) cross-reactivity with recombinant mouse OPG, and the standard or sample is incubated with a mouse monoclonal anti-human osteoprotegerin antibody coated in microtiter wells [11].

Osteoblasts cultures supernatant was obtained through growth and differentiation of the bone cells, using specific Growth Medium (Osteoblast Growth Medium PromoCell), from PromoCell Gmb, Heidelberg. Osteoblast Growth Medium-Promo, which under osteo-inductive factors (ascorbic acid, β-glycerol phosphate, dexamethazone) suffers an osteoblast differentiation [12].

Zinc concentrations of bone were determined by flame atomic absorption spectrometry (Varian AA240FS Fast Sequential AAS, from Mecro System, USA), by direct aspiration methods with five standards levels (0.2, 0.5, 1.0, 1.5, and 2.0 µg/mL solution) and lower detection limit it 0.1 µg/mL solution [13].

Statistical analysis

All the values are reported as a mean ± SD (standard deviation); the statistical analysis was made using a Student's *t*-test for the paired data, and the Pearson's correlation. Were calculated coefficients values: $p<0.05$ has a significantly statistical value. Were calculated correlation coefficients, the values of correlation $r>0.5$ (positive and negative values) considered respectively.

Results

The bones were cut into small fragments that were dissociated to cell suspensions by enzymatic digestion with 10 ml 0.1% collagenase-I solution/100 mg bones, for 15 minutes at 37^0C.

The bone tissue were washed repeatedly in 20 mL DMEM (Dulbecco's modified Eagle medium), and supplemented the culture medium with 10% fetal bovine serum (FBS) were incubated in a humidified atmosphere with 5% CO_2 at 37^0C for 4–24 hours to hasten attachment. This was followed by the addition of normal culture medium, which was DMEM supplemented with 10% FBS and penicillin/streptomycin solution (1 mL 1% solution), to the dish after two days, cells were observed emerging from the cells, the confluent 70–80% after approximately seven days.

The cells were dishes by using 3 mL 0.05% trypsin-EDTA solution. The cells were placed into 24-well microplates at a density of 2×10^4 cells/cm^2, and continuously incubated in a humidified atmosphere with 5% CO_2 at 37^0C, utilization 20 mL Osteoblast Growth Medium-Promo Cell after 14 days allows us the obtaining of some, "clusters" of mesenchymal stem cells (MCS) with characteristic fusiform cells, having a "whirl" arrangement.

Also, the cultured incubated which under osteo-inductive factors (ascorbic acid 2×10^{-4}M, β-glycerol phosphate 7×10^{-1}M, dexamethazone 1×10^{-6}M; 10 µL solution/1 mL medium of cultures) suffers an osteoblast (OBL) differentiation for up to 28 days (Figure 1).

The obtained osteoblasts (OBL) have a characteristic morphology: big cells, "nest" disposal in "star arrangement", which forms bone trabecule, and after prolongations retreat delimitates bone canaliculus (Figure 2).

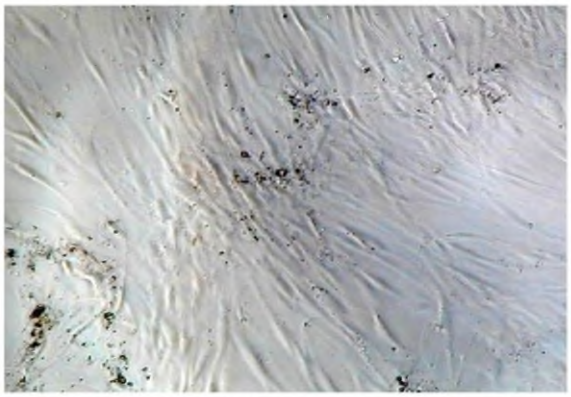

Figure 1 – *The "cluster" of mesenchymal stem cells (MCS). Phase-contrast micrographs, magnification at ×100: the "cluster" of mesenchymal stem cells with characteristic fusiform cells, having a "whirl" arrangement, after 7 days of culture.*

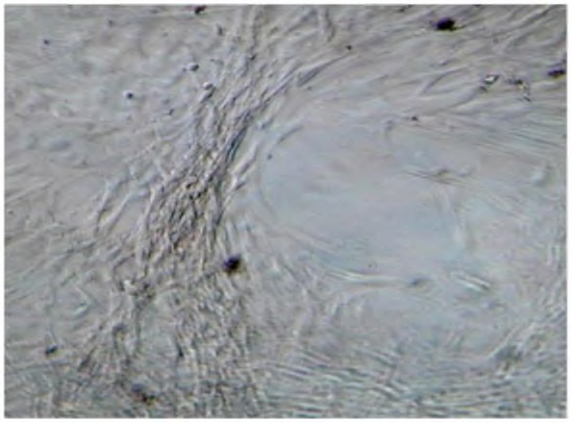

Figure 2 – *The culture of osteoblasts (OBL). Phase-contrast micrographs, magnification at ×200: the characteristic morphology in osteoblasts culture, after 28 days of culture.*

Analyzing the obtained results in the cells cultures realized in our study we can observe: a reduction of mesenchymal stem cells (MCS) to 68.46% of the cells density in cohort 1 (p<0.003), and a reduction to 48.42% of the cells density in cohort 2 (p<0.001), comparing the control group (Table 1).

Table 1 – *Mesenchymal stem cells and osteoblast number in case of postmenopausal women with osteoporosis as compared to control groups*

Characteristics (mean ± SD)	Cohort 1 (n=3)	Control groups (n=3)	Cohort 2 (n=3)
MSC ($\times10^4$ cells/cm^2)	0.65±0.15 p<0.003	1.41±0.1	0.16±0.07 p<0.001
OBL ($\times10^5$ cells/cm^2)	0.78±0.06 p<0.002	1.63±0.07	0.55±0.05 p<0.004

MSC – mesenchymal stem cells; OBL – osteoblasts; SD – standard deviation. The values reported as a mean ± SD; coefficients value p<0.05 were considered statistically significant, compared with control group.

There is a decrease of the differentiated osteoblasts (OBL): to 63.41% of the cells density in cohort 1 (p<0.002), and a decrease to 44.71% of the cells density in cohort 2 (p<0.004), comparing the control group (Table 1).

The obtained results of this study allow us to observe an increase of the serum levels of RANKL (sRANKL), determinate through ELISA method: in cohort 1, an increase of 229.13% of the average (p<0.001) and in cohort 2, an increase of 181.69% of the average (p<0.002), comparing the control group. In cohort 2, the sRANKL levels are more reduced in comparison with cohort 1 (Table 2, Figure 3).

Table 2 – *Bone markers levels in case of post-menopausal women with osteoporosis as compared to control group*

Characteristics (mean ± SD)	Cohort 1 (n=3)	Control groups (n=3)	Cohort 2 (n=3)
$RANKL_{OBL}$ [pg/mL]	79.31±5.92 p<0.001	29.12±1.0	58.56±3.08 p<0.002
$r_{RANKL\text{-}OBL/Zn(2+)}$	r=-0.949	r=-0.971	r=-0.898
sRANKL [pg/mL]	68.35±4.74 p<0.001	29.7±0.93	54.2±1.92 p<0.002
$r_{sRANKL/Zn(2+)}$	r=-0.970	r=-0.958	r=-0.985
$RANKL_{OBL}$/ sRANKL ratio	1.159±0.008 p<0.001 >1.0	0.983±0.012 <1.0	1.08±0.023 p<0.001 >1.0
OPG [pg/mL]	41.69±0.48 p<0.002	35.96±0.48	26.74±0.37 p<0.003
$r_{OPG/Zn(2+)}$	r=0.704	r=0.977	r=0.983
OPG/sRANKL ratio	0.611±0.047 p<0.001 <1.0	1.279±0.025 >1.0	0.492±0.027 p<0.001 <1.0
Zn^{2+} [µg/g bone]	7.31±0.43 p<0.001	10.78±0.25	6.62±0.11 p<0.001

The values reported as a mean ± SD; coefficient values p<0.05 were considered statistically significant, in comparing to control group, and coefficient of correlation r>0.5, values negative or positive, were considerate respectively.

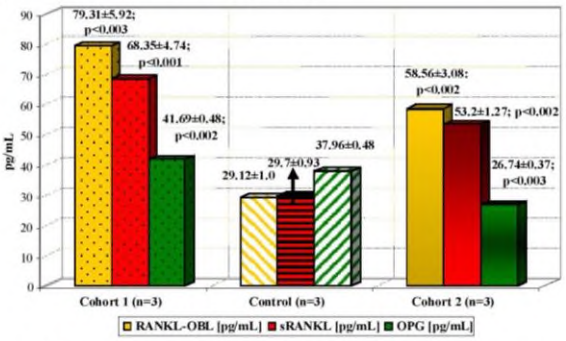

Figure 3 – *Bone markers levels at postmenopausal women with osteoporosis as compared to control group. The values reported as a mean ± SD; coefficient values p<0.05 were considered statistically significant in comparing to control group.*

The obtained results of this study allow us to observe an increase of the RANKL levels in osteoblast primary cultures supernatant ($RANKL_{OBL}$), determinate through ELISA method: in cohort 1, an increase of 272.35% of the average (p<0.001), and in cohort 2, an increase of 201.1% of the average (p<0.002), comparing the control groups. In cohort 2, the $RANKL_{OBL}$ levels are more reduced in comparison with cohort 1, phenomena specific to osteoporosis (Table 2, Figure 3).

Also, we can observe an increase of the $RANKL_{OBL}$/sRANKL ratio in cohort 1, an increase of 117.9% of the average ($RANKL_{OBL}$/sRANKL ratio >1.0; p<0.001) and in cohort 2 an increase of 109.86% of the average ($RANKL_{OBL}$/sRANKL ratio >1.0; p<0.001) characteristic to osteoporosis with bone fracture. In comparing, the control group in case of

which $RANKL_{OBL}$/sRANKL ratio had the average ($RANKL_{OBL}$/sRANKL ratio <1.0, characteristic to menopause without osteoporosis) (Table 2, Figure 3).

In cohort 1, an increase of OPG serum levels (increase of 109.82% of the average, $p<0.002$), comparing the control groups. In cohort 2, exists a decrease of the OPG serum levels (decrease to 73.15% of the average, $p<0.003$), comparing the control groups (Table 2, Figure 3).

In this study, we could demonstrate that OPG/ sRANKL ratio in cohort 1, a reduction to 47.77% of the average ($p<0.001$), comparing the control group. In cohort 2, a reduction to 38.54% of the average ($p<0.001$), comparing the control group. To both groups (with postmenopausal osteoporosis) OPG/sRANKL ratio is lower than 1.0 (OPG/sRANKL ratio <1.0), common cause osteoporosis disease. In case of the control group OPG/sRANKL ratio has the average, higher than 1.0 (OPG/sRANKL ratio >1.0), menopause characteristic, but without osteoporosis (Table 2, Figure 3).

Levels of ionic zinc (Zn^{2+}) from the bone tissue were determinate through atomic absorption spectrometry analyzed (FAAS, in acetylene flame). Analyzing the obtained results it is observed: in cohort 1 a decrease of Zn^{2+} bone levels to 67.81% of the average ($p<0.001$), and in cohort 2 a decrease of Zn^{2+} bone levels to 61.41% of the average ($p<0.001$), comparing the control group (Table 2).

In our study, we could demonstrate the existence of good positive correlation in cohort 1 between the low levels of the Zn^{2+} bone ions and high serum levels of OPG (r=0.704), some powerful negative correlations between the low levels of the Zn^{2+} bone ions or increased serum levels of sRANKL (r=-0.970), or increased of the $RANKL_{OBL}$ (r=-0.949) (Table 2, Figure 4).

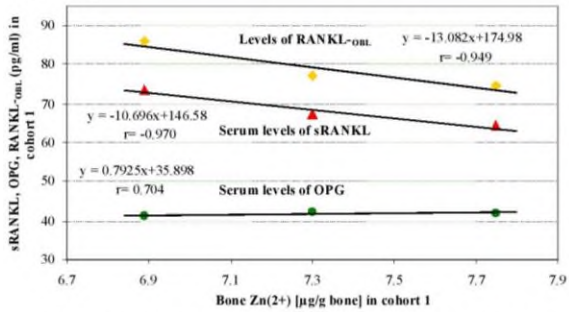

Figure 4 – ***Relationships between the bone levels of Zn^{2+} and the serum levels of bone markers in postmenopausal women with osteoporosis, in cohort 1 (n=3). The negative or positive values of the correlation coefficient (r>0.5) were considerate respectively.***

In our study, we could demonstrate the existence of a powerful positive correlation in cohort 2 between the low levels of the Zn^{2+} bone ions and the decreased serum levels of OPG (r=0.984), some powerful negative correlations between the low levels of the Zn^{2+} bone ions and increased serum levels of sRANKL (r=-0.985), or increased of the $RANKL_{OBL}$ (r=-0.898) (Table 2, Figure 5).

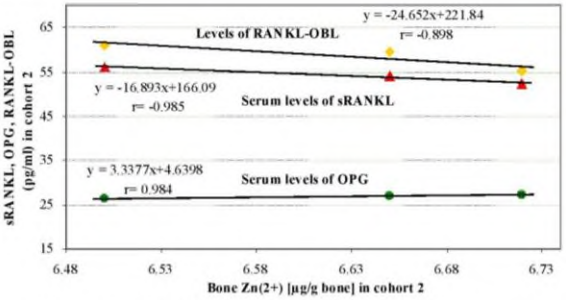

Figure 5 – ***Relationships between the bone levels of Zn^{2+} and the serum levels of bone markers in postmenopausal women with osteoporosis, in cohort 2 (n=3). The negative or positive values of the correlation coefficient (r>0.5) were considerate respectively.***

In control group exists a good positive correlation between OPG serum levels and bone Zn^{2+} levels (r=0.977), a powerful negative correlation between sRANKL levels (r=-0.958), and a powerful negative correlation between $RANKL_{OBL}$ levels and bone Zn^{2+} levels (r=-0.971), characteristics of postmenopausal women without osteoporosis (Figure 6).

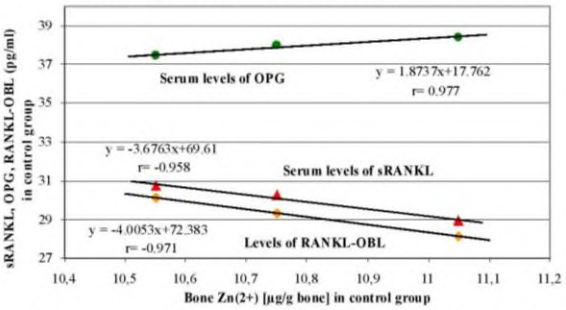

Figure 6 – ***Relationships between the bone levels of Zn^{2+} and the serum levels of bone markers in postmenopausal women with osteoporosis, in control group (n=3). The negative or positive values of the correlation coefficient (r>0.5) were considerate respectively.***

Discussion

The sRANKL molecule from the TNF family and its receptor, RANK, are the key regulators of bone remodeling and have a major role in the development and activation of osteoclasts. The sRANKL from the osteoblasts mediates osteoclastogenesis and, in contrast, OPG is a "decoy" receptor that acts by binding to neutralizing both the soluble RANKL. OPG is a soluble member of the tumor necrosis factor receptor family (TNFR family) and inhibits the differentiation and fusion of the osteoclastic precursor cells and blocks the activation of mature osteoclasts [8, 14, 15].

Osteoblasts–osteoclasts couple has a continuous interaction in the presence of molecular RANK/ RANKL/OPG system, with an important role in bone remodeling regulation. In postmenopausal osteoporosis, OPG/sRANKL ratio is lower than 1 (OPG/sRANKL ratio <1.0), comparing the one of the women which is in premenopause. Through a feedback-regulated mechanism, they will determine OPG secretion increase

at osteoblasts level, but manifesting a decrease of the "decoy" receptor for soluble OPG activity. OPG will not be connected with sRANKL, determining sRANKL connection with osteoclasts RANK having as a result RANK/ RANKL complex formation, which has a role in osteoclasts genesis stimulation and bone resorption stimulation being favorable to osteoporosis installation [3, 16, 17].

OPG is assuring "bone protection" through osteoblasts activation, turnover increase with bones formation stimulation. OPG is considered an important marker of bone synthesis. Postmenopausal, the OPG serum levels increase demonstrates osteoblasts activation and the decrease of OPG serum levels represents the consequence of age-related osteoblasts apoptosis (ARORC, age-related osteoblasts replicate capacity) [5, 18].

In osteoporosis, a high osteoblastic apoptosis leads to a decrease of multiplication capacity of the age-related osteoblasts (ARORC, age-related osteoblast respective capacity) specific to advanced age. This reduction leads to microfractures at bones level. Increased osteoblast irreparable apoptosis rate accelerates age-related osteoblast replication capacity decrease (Table 1).

In case of our study, the increased serum levels of OPG in cohort 1 (in postmenopausal osteoporosis in the initial phase) demonstrates osteoblast activation, and the decrease in cohort 2 (in postmenopausal osteoporosis in the belated phase) demonstrates stimulation of osteoblast apoptosis, associated with will increase significantly bone turnover; producing a decrease in bone formation and increasing bone resorption. The unbalance promotes appearance the osteoporosis. In postmenopausal osteoporosis, OPG serum level decrease can be an indicator of a higher risk of bones fracture (Table 2, Figure 1).

To both groups (with postmenopausal osteoporosis) OPG/sRANKL ratio is lower than 1.0 (OPG/sRANKL ratio <1.0), common cause of the osteoclast genesis through "decoy receptor" activity decrease of the OPG due to estrogens deficit postmenopausal installed. In case of the control group OPG/sRANKL ratio has higher than 1.0 (OPG/sRANKL ratio >1.0), menopause characteristic, but without osteoporosis. This unbalance represents an indicator of a higher risk of osteoporosis bones fracture (increased the risk factor). Thus, OPG/sRANKL ratio is an important determinant of bone mass and skeletal integrity (Table 2, Figure 3).

At bone's level the main RANKL actions are: mature osteoclasts activation for bone resorption which's stimulating "pool" increase of the active metabolic osteoclasts, assures osteoclasts survival through their apoptosis inhibition. RANKL (sRANKL and $RANKL_{OBL}$) it is considered an important marker of the bone resorption [4, 17, 19].

In case of our study, the serum levels of sRANKL and $RANKL_{OBL}$ are significantly *higher* (in cohort 1 and cohort 2), demonstrating osteoclast activation, associated witch will increase significantly the bone turnover; producing a decrease of bone formation and increasing bone resorption, the unbalance favorites the osteoporosis appearance (Table 2, Figure 3).

Also, we can observe an increase of the $RANKL_{OBL}$/sRANKL ratio in cohort 1 and in cohort 2 ($RANKL_{OBL}$/sRANKL ratio >1.0, which is characteristic to osteoporosis with bone fractures) comparing the control group in case of which $RANKL_{OBL}$/sRANKL ($RANKL_{OBL}$/sRANKL ratio <1.0), characteristic to menopause without osteoporosis (Table 2, Figure 3).

This results in increased osteoclast activation through a "switch-like" diversion of osteoprogenitor cell differentiation away from monocyte-macrophage cell development and toward osteoclastogenesis. Osteoblasts differentiation from their mesenchymal forerunners is a process dependent by the major transcription factor presence Cbfa-1/Runx-2 (core binding factor-1/runt-related transcription factor-2), which regulates transcription at genomic level. Zn^{2+} ions are stimulating osteoblasts proliferation and differentiation, being promoters of the major transcription factor presence Cbfa-1/Runx-2. Previous *in vitro* studies have suggested a direct effect of zinc on both the proliferation and differentiation of osteoblast-like cells [4, 7, 20]. Zinc inhibits the differentiation of osteoclasts and promotes osteoblast activity affecting the bone formation. The participation of trace elements in normal development and maintenance of the skeleton is related to their catalytic functions in organic bone matrix synthesis (Figure 7) [1, 3, 20, 21].

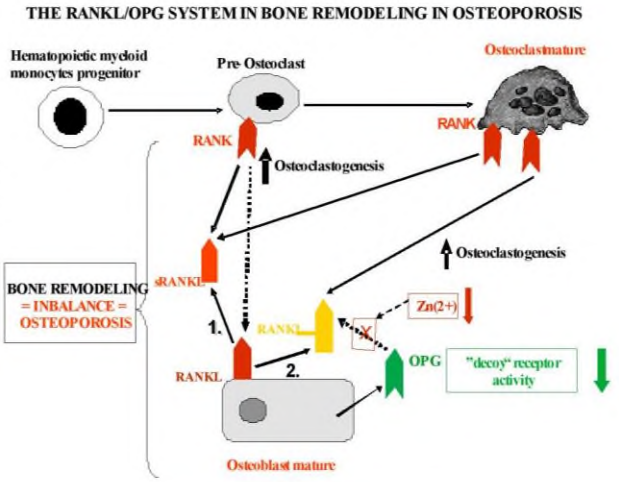

Figure 7 – ***The RANKL/OPG system in bone remodeling at post-menopausal osteoporosis. OPG – Osteoprotegerin; sRANKL – Soluble receptor activator of nuclear factor-κB ligand; RANKL – Receptor activator of nuclear factor-κB; $RANKL_{OBL}$ – Osteoblast receptor activator of nuclear factor-κB ligand; Zn^{2+} – Zinc ions; ↑ – Increased; ↓ – Decreased.***

In this study, analyzing the obtained results it is observed: in cohort 1 and cohort 2, a decrease of Zn^{2+} bone levels comparing the control group, as a result of bone turnover increase, but with a rate of the bone resorption superior to synthesis, determining in these conditions a negative bone balance and being favorable for microfractures and osteoporosis fractures apparition (Table 2).

Zinc is an essential trace element that increases osteoblast numbers and bone formation. Zn^{2+} is the most abundant trace element in bone, being present at a concentration of up to 300 µg/g bone, and it has been considered an important factor in bone metabolism. In osteoporosis, this mechanism is diminished, and as a result it is suppressed osteoblasts' activity. Zinc deficiency is associated with unbalance of the bone remodeling. Low zinc intake has been reported to be associated with low bone mass in women. It is defined as a disease characterized by low bone mass and microarchitectural deterioration of bone tissue leading to increased bone fragility and therefore to an increase of fracture risk [4, 19, 21, 22].

Conclusions

In conclusion, the sRANKL and $RANKL_{OBL}$ is significantly increased in postmenopausal osteoporosis and is a marker of increased of the bone resorption. The OPG is moderately increased in osteoporosis and is a marker of bone formation, stimulating bone turnover. The decreased of OPG serum levels represents the consequence of osteoblasts apoptosis. OPG/sRANKL ratio <1.0 and generates osteoclastogenesis through "decoy receptor" activity decrease of the OPG due to estrogens deficit postmenopausal installed.

In postmenopausal osteoporosis exists a decrease of the bone levels of Zn^{2+} because of activation and osteoblast differentiation reduction as bones mineralizing capacity decreases. In postmenopausal osteoporosis exists a powerful negative correlation between decreased levels of the bone Zn^{2+} and increased of the $RANKL_{OBL}$ or sRANKL because of osteoclast genesis activation and represents a major risk factor in the appearance of microfractures/fractures bone.

In postmenopausal osteoporosis in the initial phase exists a powerful positive correlation between decreased Zn^{2+} ions levels and increased OPG serum levels because of bone turnover stimulation, and in the belated phase exists a powerful positive correlation between decreased Zn^{2+} ions levels and decreased OPG serum levels because of osteoblast apoptosis increase.

The bone remodeling unbalance in postmenopausal women with osteoporosis is produced by decreased bone formation or increased bone resorption, and represents an indicator of a higher risk of osteoporosis bones fracture (increasing the risk factor).

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ORIGINAL RESEARCH

Correlations between bone turnover markers, serum magnesium and bone mass density in postmenopausal osteoporosis

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Introduction: Bone mass density (BMD) is still the gold standard for the diagnosis of osteoporosis, but bone turnover markers (BTMs) can provide helpful information regarding the bone remodeling process. The aim of this study was to determine the correlations between BMD and serum levels of BTMs (tartrate-resistant acid phosphatase-5b [TRAP-5b]), bone-specific alkaline phosphatase (BSAP), estradiol (E_2), and magnesium ($Mg^{[2+]}$) ion concentrations in postmenopausal osteoporotic women as compared to healthy postmenopausal subjects.

Materials and methods: The study included 132 women with postmenopausal osteoporosis and 81 healthy postmenopausal women without osteoporosis. Dual-energy X-ray absorptiometry scan assessed BMD at different skeleton sites. Serum levels of E_2, BSAP, and TRAP-5b were measured by enzyme linked immunosorbent assay. Serum levels of $Mg^{(2+)}$ were determined using the colorimetric spectrometry technique.

Results: Serum levels of BTMs were significantly higher in osteoporotic women than in controls. BSAP has a moderate sensitivity (76.5%) and specificity (84.3%) (cutoff point 21.27 U/L). At a cutoff point of 3.45 U/L, TRAP-5b presented a sensitivity of 86.3% and a higher specificity of 90.6%. Osteoporotic patients showed significantly lower concentrations of serum $Mg^{(2+)}$ than the control group. $Mg^{(2+)}$ levels correlated positively with BMD values (r=0.747, P<0.0001). Furthermore, $Mg^{(2+)}$ concentrations correlated positively with E_2 levels (r=0.684, P<0.0001). Spine BMD correlated negatively with BSAP levels (r=–0.36, P<0.0001).

Conclusion: Our study showed that BMD correlates negatively with BTMs and positively with E_2 and $Mg^{(2+)}$ levels. TRAP-5b presents a good specificity in identifying patients with postmenopausal osteoporosis.

Keywords: bone mass density, tartrate-resistant acid phosphatase-5b, bone-specific alkaline phosphatase

Introduction

Osteoporosis is defined by low bone mass and microarchitectural deterioration of bone tissue, with increased bone fragility and susceptibility to fractures. This deterioration of bone structure is caused by an imbalance in bone remodeling with increased osteoclasts' activity and decreased osteoblasts' activation.[1,2]

Estrogen deficiency represents a major risk factor for postmenopausal osteoporosis. It induces rapid bone loss in the early years after menopause and also slower bone loss associated with advancing age.[3,4] Estrogen deficiency contributes to the osteoporosis occurrence by skeletal and extraskeletal actions. Estrogen acts directly on bone cells through its receptors on osteoblasts and osteoclasts. It maintains a balance between bone formation (by enhancing osteoblasts' differentiation and inhibiting their apoptosis)

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and bone resorption (by impeding osteoclasts' differentiation and stimulating their apoptosis). Furthermore, estrogen receptors on different bone cells (stromal cells, immune cells, and so on) influence bone homeostasis.[5,6] Estrogen deficiency induces an upregulation of receptor activator of nuclear factor kappa-B ligand (RANKL) on bone marrow cells and also a decreased osteoprotegerin synthesis in osteoblasts, leading to an accelerated bone resorption.[4–6]

Bone mass density (BMD) (of the lumbar spine and hip), measured by dual-energy X-ray absorptiometry (DXA), is considered the gold standard for the diagnosis of osteoporosis.[7,8] The Fracture Risk Assessment (FRAX) tool, used for the prediction of major fracture probability >10 years, does not include all risk factors or bone turnover markers (BTMs).[8]

BTMs reflect the metabolic activity of osteoblasts and osteoclasts. In serial determinations (especially resorption markers), they could identify accelerated bone turnover, associated with an increased fracture risk. These patients (fast bone losers) would respond more promptly to antiresorptive medication.[9,10] Several studies confirmed that high levels of resorption markers could predict fractures, independent of BMD.[11–13] Also, BTMs proved to be useful in monitoring the efficacy and adherence to the antiosteoporotic treatment.[9] There are no published data regarding the sensitivity and specificity of BTMs in evaluating postmenopausal osteoporosis.

Every BTM determination presents some advantages and some limitations. Most of the bone resorption markers represent degradation products of bone collagen, with one exception, tartrate-resistant acid phosphatase (TRAP)-5b.[9]

TRAP presents the following two isoforms: TRAP-5a derived from macrophages and dendritic cells and TRAP-5b secreted by osteoclasts. Furthermore, serum levels of TRAP-5b reflect the number of osteoclasts and their activity.[14] The serum concentration is not influenced by food intake and liver or renal diseases. It provides good sensitivity and specificity, and it correlates well with other resorption markers.[15]

One of the most widely used markers of bone formation is serum bone-specific alkaline phosphatase (BSAP). BSAP is expressed on the cell surface of osteoblasts, and its synthesis correlates positively with bone formation rate.[10]

Many trace elements (magnesium [Mg], copper, manganese, zinc, selenium, and boron) contribute to the normal development and function of the skeleton, due to their catalytic activities during bone matrix formation.[16]

Mg represents an important cofactor for enzymes required for the normal synthesis of bone matrix. Hypomagnesemia can act directly on the bone cells, leading to abnormal apatite crystals, and indirectly by altering parathyroid gland secretion (associated with end-organ resistance to parathormone and low vitamin D) and inducing low grade inflammation (which accelerates bone loss).[17] The data regarding the correlation of fracture risk with hypomagnesemia in postmenopausal women are conflicting. Most of the authors agree that low serum $Mg^{(2+)}$ levels are associated with an increased risk for osteoporosis.[18–20]

The aim of this study was to determine the correlations between BMD and serum levels of bone resorption markers (TRAP-5b), bone formation markers (BSAP), estradiol (E_2), and $Mg^{(2+)}$ ion concentrations in postmenopausal osteoporotic women as compared to healthy postmenopausal subjects.

Materials and methods

The study included 132 women with postmenopausal osteoporosis (at least 1 year of amenorrhea) and 81 control subjects (healthy postmenopausal women without osteoporosis), evaluated in the Outpatient Department of Endocrinology of the County Hospital, Timisoara, from September 2016 to December 2017. The inclusion criteria in the osteoporotic group were women in the postmenopausal period, with lumbar or femoral neck BMD, expressed as *T*-score <2.5 standard deviation (SD) (World Health Organization criteria).[20] The control group included women in the postmenopausal period, with lumbar or femoral neck *T*-score >−2 SD.

Exclusion criteria were as follows: secondary causes of osteoporosis, other diseases that could influence the bone metabolism or electrolyte imbalance (especially Mg), fractures in the previous year, hormone replacement therapy, and any medication that could influence bone turnover.

BMD (g/cm^2) was assessed using the DXA scan (Hologic device; QDR Inc., Bedford, MA, USA), performed at lumbar spine and hip, determining the *T*-score.

Height and weight of the subjects were recorded, and body mass index (BMI) was calculated based on the formula weight (kg)/height^2 (m).

Each patient was informed about the study protocol and signed an informed consent. The study was approved by the Ethics Committee of Victor Babes University of Medicine and Pharmacy Timisoara, Romania.

For the serum markers, fasting venous blood samples were collected into preservative-free tubes and allowed to clot. After centrifugation at 2,000× *g*, serum samples were decanted and frozen in aliquots. The serum samples were stored frozen at −20 to −80°C (at −20°C for 5 days and at −80°C for 12 months).

E_2 concentration was determined by enzyme linked immunosorbent assay (ELISA) human kit, based on the principle of competitive binding. The microtiter wells were coated with an antibody directed toward a unique antigenic site on the E_2 molecule. The maximum inter- and intra-assay coefficient of variation (CV) were 6.8 and 7.25%, respectively, analytic sensitivity of 3–6 pg/mL (Human Gesellschaft für Biochemica und Diagnostica mbH, Wiesbaden, Germany).

Serum levels of BSAP were measured using the MicroVue BSAP human ELISA kit. BSAP is an immunoassay in a microtiter strip format, which uses a monoclonal anti-BSAP antibody coated on the strip to capture BSAP in the sample. The enzyme activity of the captured BSAP was detected with a *para*-nitrophenyl-phosphate (pNPP) substrate. The performance characteristics of this assay (of the Human ELISA kit utilizing) were minimum. Compared to the reference values, our detection limit 0.7 U/L, CV in circulation =5.0%–5.8%, corresponding for postmenopausal women CV =4.8%–5.2%, 14.2–42.7 U/L (Micro Vue™ BSAP; MDSS GmbH, Hannover, Germany).

TRAP-5b concentration was measured using a two-step MicroVue TRAP-5b assay. The reconstituted standards and controls were added to coated microwell plate wells along with sample diluents. Naturally occurring, inactive TRAP-5b fragments in the serum could interfere with the detection of TRAP-5b in physiological samples. This assay avoided the influence of the inactive fragments by using two different monoclonal antibodies. The assay performance was characterized by minimum detection limit 0.2 U/L, within-run CV =2.2%–3.6%, between-run CV =3.0%–4.6%, and reference values 4.3±1.5 U/L (MicroVue™ TRAP-5b; MDSS GmbH).

Serum levels of $Mg^{(2+)}$ ions were determined using the colorimetric spectrometry technique (VITROS Slides quantitative $Mg^{[2+]}$ concentration in serum with VITROS® 250 Chemistry System; Johnson & Johnson, Piscataway, NJ, USA). Minimum detection limit was 0.8 mg/dL (0.33 mmol/L), with a normal range of 1.6–2.4 mg/dL.

Statistical analysis

SPSS V17.0 for Windows (SPSS Inc., Chicago, IL, USA) was used for data entry and data analysis. The distribution of the quantitative data was studied using the Kolmogorov–Smirnov test, according to which parametric (Student's *t*-test) or nonparametric (Mann–Whitney *U* test) tests were applied. Continuous data were expressed as mean ± SD. Categorical data were expressed as frequency and percentage. For normally distributed data, Pearson's correlation coefficient was calculated, and for distribution-free data, Spearman's coefficient was calculated. We performed analysis on BSAP, TRAP-5b, and Mg in study groups. Sensitivity, specificity, and receiver-operating characteristic (ROC) curve were calculated to evaluate the diagnosis performance of BTMs in detecting women with osteoporosis. The level of statistical significance was established at $P<0.05$.

Results

Demographical data are shown in Table 1. The osteoporotic group subjects were significantly older than the control group. Although the menopause age was similar in both groups, the E_2 deprivation duration was longer in the osteoporosis group than in the control group (Table 1).

The serum concentrations of E_2 were significantly lower in postmenopausal women with osteoporosis than in the control group. In the study group, we found a strong, negative correlation between E_2 values and age (r=–0.389, r^2=0.151, 95% CI =–0.525 to –0.234, $P<0.001$). This correlation was not observed in the control group.

Table 1 Characteristics of the study subjects

Parameter (mean ± SD)	Study group with osteoporosis (n=132)	Control group (n=81)	*P*-value
Age (years)	65.5±1.8	56.5±2.4	<0.001
BMI (kg/m^2)	27.5±3.2	28.2±2.9	0.110
Menopause age (years)	46.5±1.5	47.5±0.5	0.215
E_2 deprivation duration (years)	17.5±1.5	9.5±0.5	<0.001
T-score			
Lumbar spine	–3.65±0.60	–1.75±0.11	<0.001
Femoral	–2.82±0.50	–1.30±0.31	<0.001
BMD (g/cm^2)			
Lumbar spine	0.62±0.04	0.77±0.05	<0.001
Femoral	0.69±0.05	0.82±0.12	<0.001

Note: Demographic characteristics in postmenopausal women with osteoporosis compared to control group: $P<0.05$, significantly different from control group.
Abbreviations: BMD, bone mass density; BMI, body mass index; E_2, estradiol; SD, standard deviation.

Table 2 Biochemical parameters in study groups

Parameter (mean ± SD)	Study group with osteoporosis (n=132)	Control group (n=81)	P-value
E_2 (pg/mL)	25.9±3.9	43.9±4.0	<0.0001
BSAP (U/L)	23.7±4.8	19.2±2.0	<0.0001
TRAP-5b (U/L)	4.8±1.3	2.9±0.3	<0.0001
$Mg^{(2+)}$ (mg/dL)	1.76±0.06	2.14±0.14	<0.0001
25(OH) vitamin D (ng/mL)	25.4±4.6	26.2±5.1	0.238

Note: Serum levels of bone markers in postmenopausal women with osteoporosis compared to control group: $P<0.05$, significantly different from control group.
Abbreviations: BSAP, bone-specific alkaline phosphatase; E_2, estradiol; Mg, magnesium; SD, standard deviation; TRAP-5b, tartrate-resistant acid phosphatase-5b.

BMD determined at different sites (lumbar spine and femoral neck) revealed significantly lower values in the osteoporosis group. Furthermore, vertebral BMD was lower than femoral values in both study groups (P=0.01). BMD values correlated negatively with age (r=–0.235, r^2=0.05, 95% CI =–0.390 to –0.067, P=0.006). In both groups, BMI values did not correlate significantly with BMD, or other parameters.

Biochemical test results are represented in Table 2. Serum levels of BSAP and TRAP-5b were significantly higher in the osteoporosis group than in the control group.

Age was significantly correlated with BSAP (r=0.408, 95% CI =0.280–0.521, P<0.0001) and TRAP-5b (r=0.654, 95% CI =0.563–0.729, P<0.0001).

Serum concentration of $Mg^{(2+)}$ ranged from 1.58 to 1.88 mg/dL in the osteoporosis group. Control serum levels of $Mg^{(2+)}$ ranged from 2.1 to 2.28 mg/dL, being significantly higher than in osteoporotic subjects (P<0.001). $Mg^{(2+)}$ levels were positively correlated with BMD values in all subjects (r=0.747, r^2=0.548, 95% CI =0.670–0.797, P<0.001) (Figure 1).

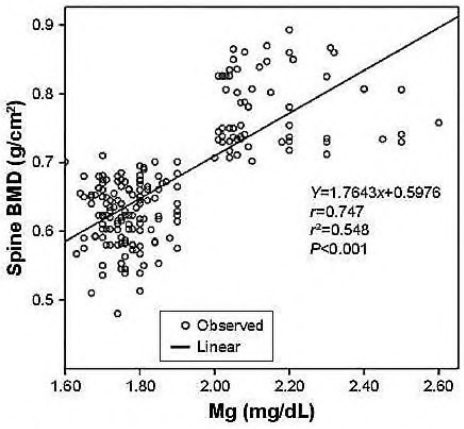

Figure 1 The correlation between BMD values (g/cm²) and $Mg^{(2+)}$ levels in the whole study group.
Abbreviation: BMD, bone mass density.

$Mg^{(2+)}$ concentrations correlated positively with E_2 levels (r=0.684, r^2=0.584, 95% CI =0.599–0.754, P<0.001). Furthermore, a negative correlation was found between E_2 deprivation duration and $Mg^{(2+)}$ levels (r=–0.804, r^2=0.646, 95% CI =–0.848 to –0.745, P<0.001). TRAP-5b did not correlate significantly with BSAP in either of the study groups.

Spine BMD correlated negatively with BSAP levels in all patients (r=–0.360, 95% CI =–0.510 to –0.266, P<0.001). Moreover, the correlation was strong and negative with TRAP-5b levels (r=–0.620, 95% CI =–0.701 to –0.522, P<0.001). Serum E_2 concentrations correlated positively with spine BMD measurements in both groups (r=0.757, 95% CI =0.690–0.811, P<0.001). Hip BMD did not correlate significantly with BTMs.

$Mg^{(2+)}$ levels did not correlate significantly with age in either group. $Mg^{(2+)}$ correlated negatively with BTMs, TRAP-5b levels (r=–0.610, 95% CI =–0.693 to –0.511, P<0.001), and BSAP levels (r=–0.417, 95% CI =–0.529 to –0.290, P<0.001). Vitamin D concentrations did not correlate significantly with BMD or BTMs' values.

TRAP-5b levels correlated negatively with serum E_2 concentrations in all patients (r=–0.629, 95% CI =–0.708 to –0.53, P<0.001). BSAP concentrations correlated positively with E_2 levels (r=0.419, 95% CI =0.293–0.5317, P<0.001). Moreover, BSAP levels correlated negatively with the duration of E_2 deprivation (r=–0.552, 95% CI =–0.645 to –0.443, P<0.0001).

ROC analysis showed that BSAP has a moderate sensitivity (76.5%) and specificity (84.3%) at a cutoff point of 21.27 U/L (area under the ROC curve 0.830, 95% CI =0.772–0.889, P<0.001) (Figure 2). At a cutoff point of 3.45 U/L, TRAP-5b presented a sensitivity of 86.3% and a higher specificity of 90.6% (area under the ROC curve 0.950, 95% CI =0.923–0.976, P<0.001) (Figure 3).

Discussion

Although DXA is considered the gold standard in the diagnosis of osteoporosis, it has some limitations in predicting

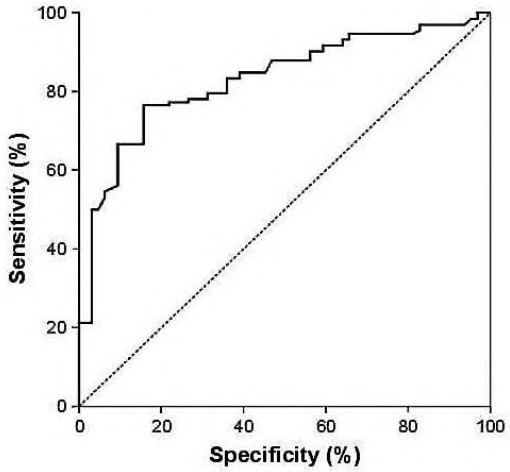

Figure 2 Receiver-operating characteristic curve of the BSAP, the difference between the patients with osteoporosis and healthy women.
Abbreviation: BSAP, bone-specific alkaline phosphatase.

the risk of fracture. Alteration in bone microarchitecture (affecting bone quality) increases the fracture risk, independent of low BMD. Bone dynamics and its assessment can be evaluated by BTM.[21]

BTMs are considered independent risk factors for osteoporotic fractures. A significant proportion of osteoporotic fractures occurred in patients with BMD above the diagnostic criteria (*T*-score >–2.5 SD). Thus, determining BMD alone could be an insufficient parameter for identifying subjects at the risk of fractures. BTMs may represent an independent diagnostic tool, with prognostic value, and also a complementary parameter to BMD for evaluating the risk of fracture.[22]

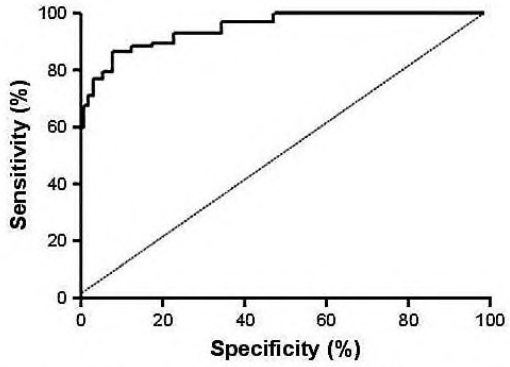

Figure 3 Receiver-operating characteristic curve of the TRAP-5b, the difference between the patients with osteoporosis and healthy women.
Abbreviation: TRAP-5b, tartrate-resistant acid phosphatase-5b.

Numerous studies confirmed the negative correlations between BMD values and BTMs.[22] Higher levels of pretreatment BTMs were associated with accelerated bone loss. For the same level of a certain BTM, there are significant interindividual variations regarding bone loss. Nonetheless, in a particular patient, BTMs cannot be used as predictors for rapid bone loss.[23,24] BTMs are also useful for the selection of patients who would respond better to antiosteoporotic treatment.[25] Previous data showed that the negative correlation between BTMs and BMD is stronger with aging, especially in patients older than 75 years (notably resorption markers).[26]

Other authors showed that the correlation between BTMs and BMD is stronger in early menopause. Both types of BTMs (resorption and formation) are more increased in early postmenopausal period, due to accelerated bone resorption.[24,27] The positive predictive value of BTMs for increased bone loss in elderly patients is rather low.[12]

In our study group, age correlated positively with BTMs, stronger with TRAP-5b as compared to BSAP, reflecting the bone dynamics. This is in accordance with other studies, which showed that in postmenopausal women, BTMs are increased, related to increased bone turnover.[28]

In elderly patients, BTMs are still increased, often explained by other mechanisms (vitamin D deficiency, intestinal malabsorption for calcium, and secondary hyperparathyroidism).[29] In our study group, we did not observe that the negative correlation of bone resorption marker and BMD became stronger with age, as our patients were younger than 75 years.

Which are the best BTMs for the assessment of bone resorption and formation is still a debate. We choose to evaluate TRAP-5b levels, due to the low diurnal variability, and serum concentrations being uninfluenced by food or liver and kidney dysfunctions. In contrast, as a bone formation marker, we determined serum BSAP, as it is not affected by renal function, has a long circulatory half-life of 1–2 days, and is affordable.

Several meta-analyses confirmed the negative, moderate correlation between BMD and BSAP.[23] In our patients, spine BMD correlated negatively with BSAP. In a previous study, we showed that BSAP levels were lower in osteoporotic patients with >15 years of estrogen deprivation.[30] In the present study, BSAP levels correlated negatively with E_2 deprivation period, demonstrating that the bone formation is decreasing as the E_2 deprivation period increases.

Some authors suggested that BSAP concentrations could discriminate between osteoporotic and healthy postmenopausal women.[31] We found a rather modest sensitivity

and specificity of BSAP, compared with other studies that informed the usefulness of BSAP as a useful tool in the first assessment of osteoporotic patients.

Several studies established age-specific reference intervals for specific BTMs.[32,33] Data regarding method-specific reference intervals for BSAP, or TRAP-5b, determined in large healthy pre- and postmenopausal population, are scarce in our country. This could influence the interpretation of the results.

Previous studies confirmed the negative correlation between TRAP-5b and BMD.[34] In our study, correlation of BMD was stronger for TRAP-5b, reflecting the imbalance of bone remodeling processes, predominantly bone resorption. The concentrations of serum TRAP-5b were significantly higher in the osteoporotic group, as compared with nonosteoporotic patients, thus reflecting the bone loss induced by menopause. TRAP-5b showed to be a slightly better BTM (90.6% specificity) in identifying osteoporotic women than BSAP (84.3% specificity). However, none of these markers can be used alone in diagnosing osteoporosis. They are rather useful in finding patients with high turnover, thus helping to select the optimal treatment and also to assess the response to the drugs.[35] In a large population-based study, which included women older than 75 years, followed-up for 9 years, high levels of TRAP-5b were associated with an increased risk of vertebral fractures.[36]

Some authors confirmed that TRAP-5b is independently correlated with BMD in women, proposing TRAP-5b as a screening marker for osteoporosis.[37]

The positive correlation of E_2 levels with BMD found in our study was confirmed by many studies, attesting that E_2 concentration is one of the most important factors determining BMD.[4]

Furthermore, in our study, increased age was associated with decreased E_2 and BMD, proving that low serum E_2 levels increase bone turnover, which represents a risk factor for fractures.

Odabasi et al demonstrated that BSAP and Mg play a role in bone turnover and osteoporotic aspects were associated with Mg deficiency.[38]

In our osteoporotic group, serum $Mg^{(2+)}$ concentrations were lower than in the control group. Several meta-analyses indicated similar results.[20]

Also, $Mg^{(2+)}$ levels correlated positively with E_2 levels and BMD values and negatively with BTMs. These findings are probably linked to a decreased osteoblastic multiplication capacity (correlated with advancing age) and to osteoblastic apoptosis. This demonstrates the impaired bone remodeling in osteoporosis, with reduced bone formation and increased bone resorption. Mg concentration in trabecular bone is significantly lower in osteoporotic women. The role of Mg in osteoporosis is also supported by studies, which demonstrated that Mg supplementation can increase bone density and stops bone loss in a significant proportion of subjects.[17]

The study presents some limitations: age difference between the two groups, which could influence the analyzed data; the limited number of BTMs analyzed; and no data on BTMs' reference values for our population.

Conclusion

Our study showed that BMD correlates negatively with BTMs and positively with E_2 and $Mg^{(2+)}$ levels. TRAP-5b levels correlate negatively with serum E_2, while BSAP correlates positively. Furthermore, BSAP levels correlated negatively with E_2 deprivation duration. TRAP-5b presents a good specificity in identifying patients with postmenopausal osteoporosis.

Disclosure

The authors report no conflicts of interest in this work.

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THE RETICULAR NETWORK CONTRIBUTES TO THE STAGING OF IDIOPATHIC LUNG FIBROSIS

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Abstract - The aspect of reticular fibers is not considered in the current classifications of lung fibrosis. The aim of our study was to evaluate the distribution and the architecture of the reticular fibers for potential use as a tissue marker of fibrosis severity. We included in our study 25 pulmonary samples obtained by video-assisted thoracoscopy surgery (VATS) from a number of 20 cases. The cases were subdivided according to four criteria into: degree II, III and IV. We noticed no significant changes in the reticular network from interalveolar septa to the cases scored with 0, an accumulation of reticular fibers in the interalveolar septa (stage II), the condensation and thick bundles with network disorganization in all areas affected by fibrosis (stage III), partial to full depletion of reticular fibers (stage IV). Depletion of reticular fibers was constantly associated with advanced fibrosis stages.

Key words: Reticular network, idiopathic lung fibrosis, staging

INTRODUCTION

Since 1944 when Hamman et al. (1944) described the pathological features of patients with unexplained interstitial pneumonia, idiopathic interstitial pneumonias have been extensively studied. Liebow (1969) presented a histopathological classification of the idiopathic interstitial pneumonias (IIPs) consisting of five patterns. The classification of the American Thoracic Society/European Respiratory Society (2002) comprises seven clinicopathological entities of IIPs. Idiopathic pulmonary fibrosis is one of the more frequent chronic interstitial lung diseases. Incidence was estimated at 10.7 and 7.4 per 100 000 per year for males and females, respectively. Despite numerous studies, many questions regarding the epidemiology, radiological features, clinical presentation, diagnosis, classification and therapy have remained unclear and should be elucidated.

Pulmonary fibrosis represents one of the particular responses to various lung parenchyma injuries. Idiopathic pulmonary fibrosis (IPF) is a disease characterized by lung parenchyma distortion by fibroblastic proliferation with extracellular matrix deposition and an inflammatory cell infiltration (Jones et al., 2011). Once established, lung fibrosis is a chronic, progressive, irreversible process with major impact on prognosis and increased mortality of diffuse interstitial pneumopathies.

The role of reticular fibers in the induction and progression of pulmonary fibrosis is relatively understudied and publications on this issue in the literature are rare. The changes in reticular fiber features may be hypothesized in lung fibrosis, based on their biochemical structure. Therefore, major changes of the reticular network are expected to occur in advanced stages of lung fibrosis. On the other hand, the aspect of reticular fibers is not included in the current classifications of lung fibrosis.

Quantification methods for fibrosis are not well standardized at the present time. Kumar (2005) described simple histological staining methods; morphometric, immunohistochemistry, *in situ* hybridization techniques used to quantify fibrosis in specific tissue compartments. Shahzeidi et al. (1993) showed that the activation of interstitial fibroblasts with enhanced type III collagen gene expression represent a part of the mechanism leading to increased collagen deposition in bleomycin-induced fibrosis. This phenomenon occurs before detection of fibrosis by conventional histological methods. Raghu et al. (1985) demonstrated that idiopathic pulmonary fibrosis and adult respiratory distress syndrome showed types I and III collagen accumulated in the interstitium. Type III collagen was initially predominant in the thickened alveolar septa and interstitium. None of these methods, taken alone or together can predict the behavior and prognosis of lung fibrosis.

The aim of our study was to evaluate the distribution and the architecture of the reticular fibers involved in the pathological diagnosis of pulmonary fibrosis for potential use of reticular fibers as tissue markers of fibrosis severity and the predictive value of the evolutionary potential of fibrotic process.

MATERIALS AND METHODS

We included in our study 25 pulmonary samples that were obtained by video-assisted thoracoscopy surgery (VATS) from 20 cases – 10 men and 10 women, with high-resolution computer tomography (HRCT) showing an interstitial pattern and for whom a specific etiology was not identified. The specimens were fixed in 10% buffered formalin for 48 h and paraffin embedded using the routine histological procedure. Five-micrometer-thick serial sections were performed from each paraffin block and sections were mounted on silanized slides. Sections from each case were stained with hematoxylin and eosin, Masson's trichrome staining method and Gordon and Sweet's silver staining (Sigma Reagents). Microscopic observation was performed by three independent observers using a Nikon Eclipse E600 (Nikon Corporation, Tokyo, Japan). Images were captured and processed with Lucia G software system.

As a first step, we established the morphological diagnosis and stage of lung fibrosis by using basic lesions according to current standard. The following parameters were evaluated: lung parenchyma: (score given for normal architecture – score 0; minor alterations – emphysema, collapse – 1; severe alterations – 2; major changes – 3; major changes, replacement – 4); chronic inflammatory infiltrate (absent – 0; isolated, rare, small groups – 1; focal, high density – 2; diffuse – 3; focal, even absent – 4); macrophages (rare – 0; small groups, focal distribution, intraalveolar – 1; diffuse, high density, heterogeneous – 2; diffuse, high density, homogeneous – 3; few macrophages, dust cells – 4); fibrosis (absent – 0; thin collagen fibers, not organized in bundles – 1; collagen bundles, heterogeneous pattern – 2; collagen bundles homogeneous pattern – 3; nodular, extensive fibrosis – 4). By summing up the values, the following stages of severity were given by the final score, as follows: I (1-3), II (4-6), III (7-9) and IV (10-12). In the second step, we analyzed the organization and density of reticular fibers in each section and compared with the different stages of fibrosis. The general score "0" corresponds to normal lung parenchyma, as was found in five cases included in the study as control. Biopsies from these cases were taken from patients operated on for other lesions of the lung, with the approval of the Committee of Ethics of the University.

RESULTS

All five cases diagnosed with normal parenchyma were scored with 0. From the 20 cases with pulmo-

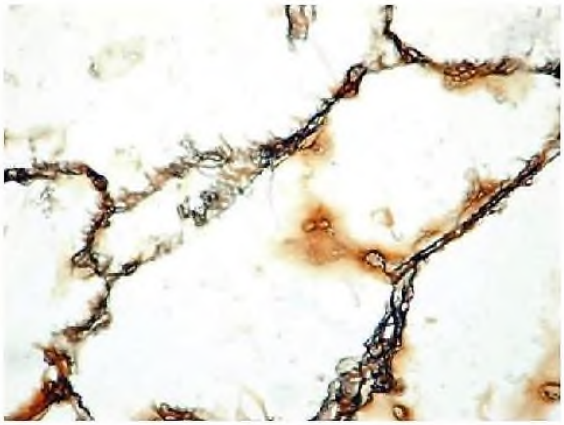

Fig. 1a. Normal distribution of reticular fibers, Gordon & Sweet's silver staining, X 100.

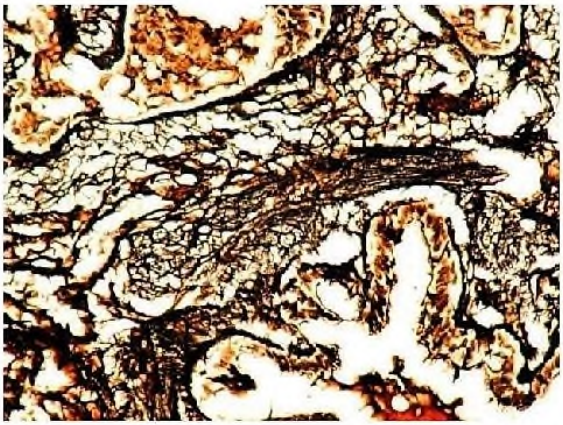

Fig. 1c. Massive accumulation of the reticular fibers, Gordon & Sweet's silver staining, X 100.

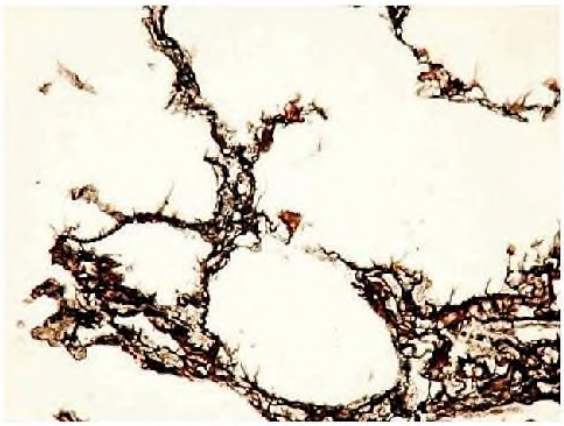

Fig. 1b. Accumulation of the reticular fibers in the interalveolar septa, Gordon & Sweet's silver staining, X100.

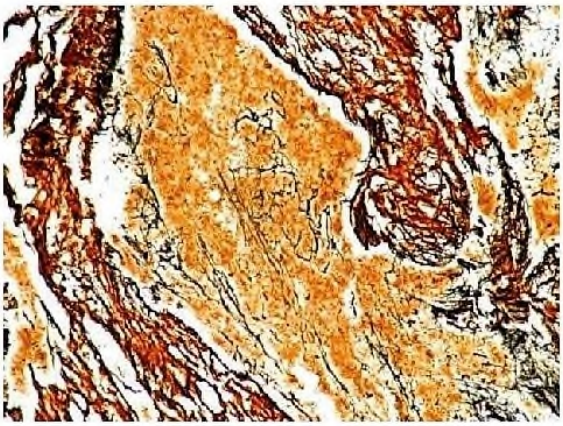

Fig. 1d. Disorganization of the reticular network, Gordon & Sweet's silver staining, X 100.

nary fibrosis included in the present study, none met the criteria for degree I (1 to 3 point final score). The cases were subdivided according to the four criteria into: degree II (4 cases, 20%), degree III (12 cases, 60%), and degree IV (4 cases, 20%). We noticed that the number and organization of reticular fibers were different depending on the morphologic stage of pulmonary fibrosis. We noticed no significant changes in the reticular network from interalveolar septa to the cases scored with 0. Reticular fibers appeared fine, filigreed, located in the network; this is considered to be the normal reticular network (Fig. 1a).

Along with progression of fibrosis and architectural changes of lung parenchyma, we found changes in the quantity, quality and organization of reticular network. At stage II of fibrosis, we noticed a high number of reticular fibers that accumulated in the interalveolar septa (Fig. 1b) causing significant thickening of these areas. Reticular fibers are still layed out in a partially organized network. Later, at stage III, the massive accumulation of reticular fibers leads to their condensation, and thick bundles with network disorganization in all areas affected by fibrosis were found (Figs. 1c, 1d).

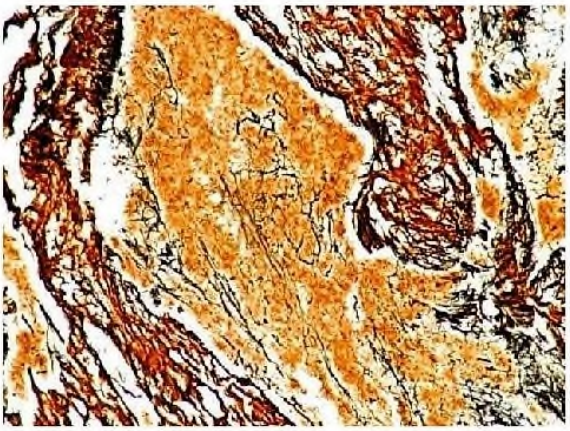

Fig. 1e. Partial depletion of the reticular network, Gordon & Sweet's silver staining, X 100.

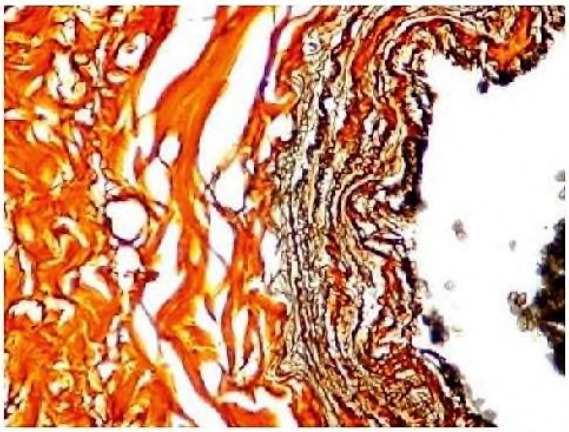

Fig. 1g. Persistence of the vessel wall reticular network, Gordon & Sweet's silver staining, X 200.

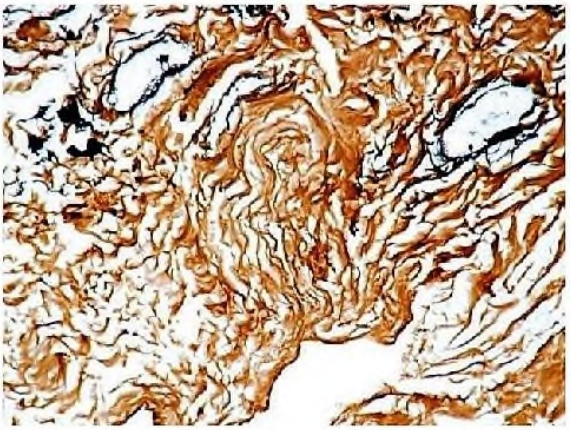

Fig. 1f. Complete depletion of the reticular network, Gordon & Sweet's silver staining, X 100.

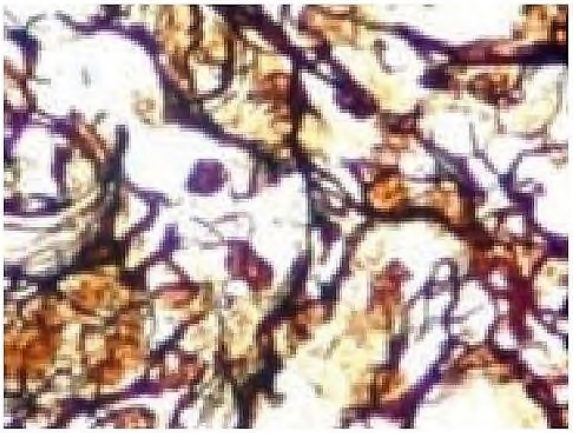

Fig. 1h. Disordered network of reticular fibers in the area of inflammatory infiltrate, Gordon & Sweet's silver staining, X200.

In the severe, advanced stages of extensive fibrosis (stage IV), thick bundles of collagen fibers partially or completely replaced the lung parenchyma with partial (Fig. 1e) to full depletion (Fig. 1f) of reticular fibers. The reticular network of the septa was noticed only focally in the immediate vicinity of the alveolar epithelium and occasionally in the septal blood vessel wall.

No significant changes were found in the blood vessels' walls in terms of the reticular component, even in cases with severe injuries (Fig 1g).

A well developed but irregular reticular fiber network was found in the areas with inflammatory infiltrate (Fig. 1h). In these areas, the aspect mimics the lymphoid tissue architecture and not that of normal lung parenchyma.

DISCUSSION

Several studies have described the reticular fiber network in the normal lung. Toshima et al. (2004) used electron microscopy scanning and showed that collagen fibers predominate in the alveoli and alveolar

septa, smaller fibers, forming basket-like networks, in the normal lung. Mercer et al. (1990) analyzed the distribution of collagen and elastic fibers in the lungs and noticed that connective tissue fibers were present in the alveolar duct wall in both human and rat species. Suki et al. (2005) showed that connective tissue of the lung is not a static structure, even in normal situations. It is a dynamic balance between continuous breakdown and remodeling modulated by mechanical forces, influenced by external or internal changes such as a disease or environmental stimuli.

The reticular fiber network changes, which are useful in diagnosis, were noticed in different situations: to differentiate between normal hypophysis and pituitary adenoma (Ceausu et al. 2010), well-differentiated hepatocellular carcinoma and benign hepatic nodules (Hong et al. 2011), in the diagnosis of superficial cervical endometriosis (Kim, 2001), and pathological changes of the lung after prolonged inhalation of high oxygen concentrations (Matsubara et al. 1986).

Regarding lung fibrosis, Takyia et al. (1983) showed that the mode of organization of the fibrotic lung connective matrix could be correlated with the evolution, stability, remodeling ability and reversibility. Our study shows that the identification of reticular fibers in the pulmonary fibrosis areas is an important factor in the evaluating, staging and prognostic assessment of fibrosis itself. Pulmonary fibrosis, for long considered as the final stage of evolution of diffuse interstitial pneumopathies, is itself a progressive, evolving, pathologic process, diagnosed in various stages of severity.

Bateman et al. (1981), using an immunofluorescence technique, demonstrated that type III collagen increases in early active fibrosis areas in the lung. We noticed an inverse relationship between the number and organization of reticular fibers and the severity of pulmonary fibrosis. The presence of reticular fibers is correlated with early and moderate forms of fibrosis, and their depletion with severe stages.

Idiopathic pulmonary fibrosis is a disease that is currently in the stage of hypothesis regarding its etiology, pathogenesis, mechanisms, experimental model, evolution and prognosis. Leslie (2012) proposed a pathogenic sequence of events in idiopathic pulmonary fibrosis as follows: stretch injury to epithelial-mesenchymal transition, formation of the fibroblastic reticulum, local alveolar collapse, collagen deposition, vascular ingrowths, simplification of lobules, and honeycomb lung. King et al. (2011) considered that the fibrotic response is due to abnormal activation of alveolar-epithelial cells. The production of mediators, proliferation of mesenchymal cells, formation of fibroblast and myofibroblast, attraction of circulating fibrocytes and epithelial to mesenchymal transition, are the next steps.

In these conditions, we estimate that in the early stages of fibrosis reticular fibers are formed in larger quantities than normal and are then replaced by the production of precursors of collagen fibers.

CONCLUSIONS

Analysis of the cases included in the study revealed several particular aspects. These indicated reticular fiber as a potentially useful tissue marker for the histopathological classification of fibrosis stage. Depletion of reticular fibers is constantly associated with advanced fibrosis stages. This major change may explain the irreversibility of the fibrotic process and the absence of efficacy of therapy on recovery of lung function.

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I.4. APLICAREA METODELOR HISTOCHIMICE ȘI IMUNOHISTOCHIMICE ÎN PROCEDURILE DIAGNOSTICE ȘI CU POTENȚIAL IMPACT TERAPEUTIC ÎN AFECȚIUNI PARTICULARE (ACTINOMICOZA, HIPERSENSIBILITATEA PULMONARĂ)

1. Gurban Camelia Vidita, Florina-Maria Andrica, Cosmin Citu, Elena Hogea, Iosif Marincu, Marioara Cornianu, **Ovidiu Alexandru Mederle**- Combined therapies in abdominal *actinomycosis*, REV.CHIM., 67, No.1, 2016, Pages: 171-173 ISSN: 0034-7752, IF=1,232

2. Prodea, M; Boia, ER; Ceausu, RA; Librimir, C; Iovanescu, G; **Mederle, OA**- Lung delayed hypersensitivity A case with particular features, REV. CHIM, Volume: 69, Issue: 8 Pages: 2071-2073, 2018, ISSN: 0034-7752, IF=1,412

Actinomicoza abdominală continuă să fie o tulburare greu diagnosticată datorită cursului insidios, a simptomelor nespecifice și a markerilor de laborator. Atât investigațiile moderne de imagistică (CT, RMN cu contrast), cât și examinarea histochimică a probelor de biopsie, ajută la stabilirea diagnosticului de actinomicoză abdominală. Terapia cu antibiotice combinată cu intervenția chirurgicală reprezintă o alternativă promițătoare pentru tratarea pacienților cu actinomicoză abdominală.

În plămân, pneumonia de hipersensibilitate (HP) este relativ rară și se caracterizează prin predominanța inflamației mononucleare a interstițiului pulmonar, a bronhiilor terminale și a alveolelor. Specimenele histopatologice prezintă inflamație interstițială însoțită de infiltrat celular gigant multinuclear tipic pneumoniei de hipersensibilitate. Cea mai importantă caracteristică clinică a HP este formarea granulomului, eventual progresând la fibroza pulmonară. Studiile imunofenotipice pentru limfomul non-Hodgkin pulmonar au utilizat anticorpi pentru Ki-67, CD3, CD5, CD10, CD19, CD20, CD22, CD23, CD45 și CD79a selectiv în funcție de subtipul suspectat. Cazul nostru a prezentat CD3 pozitiv în centrul germinal al foliculului limfoid, CD20 intens pozitiv în foliculul limfoid, CD5 rar și slab pozitiv în foliculul limfoid, CD15 negativ. Aceste aspecte au exclus diagnosticul limfomului non-Hodgkin. Există multe forme de hipersensibilitate întârziată la medicament. Dintre acestea, la plămâni au fost descrise două cazuri de reacții de hipersensibilitate respiratorie de tip IV datorate corticosteroizilor. Diagnosticul de hipersensibilitate de tip tardiv necesită analiza corelației dintre criteriile clinice, radiologice, fiziologice și morfopatologice.

Combined Therapies in Abdominal Actinomycosis

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This study is about a 43-year-old woman with anterior abdominal wall actinomycosis that appeared after abdominal trauma by dropping. The histopathological examination revealed an organized parietal hematoma with Actinomyces israelii colonies. The evolution was favorable under the treatment with Penicillin G. 5000000 International Units administered by intravenous infusion every 6 hours for 4 weeks and with orally administred Ospen for eight weeks more. In conclusion, the combined surgical drainage with antibiotic treatment proved to be an optimal therapy for patients with abdominal actinomycosis.

Keywords: Actinomyces israelii, abdominal traumatism, tumoral mass, penicillin

Actinomycosis is a rare, chronic debilitating disease, characterized by a granulomatous infection being caused by *Actinomyces species* [1, 2]. The most common etiologic agent of actinomycosis is *Actinomyces israelii*. Actinomyces species represent a group of anaerobic, Gram-positive, filamentous bacteria that normally colonize the respiratory, gastrointestinal and female genitourinary systems [3-5]. Actinomycosis have predilection for cervicofacial (50%), abdominal (20%) and thoracic (15%) regions. Moreover, it, occasionally, affects the anterior abdominal wall or viscera, being hardly diagnosed [2]. In the present paper, we report a case of anterior abdominal wall actinomycosis occurred after abdominal trauma by falling in the gutter line.

Experimental part

Materials and methods

The hemoleucogram parameters were assessed by using a hematology ADVIA®2120i analyzer. The laboratory tests were determined by enzymatic methods (quantitative technique) using a Vitros™750drySlides analyzer. They were correctly identified at the genus level with the Vitek 2 system ANC Card ATCC 12102 (colorimetric identification card, bioMérieux, Marcy-France).

Case report

This case report presents a 43-year-old woman from an urban area, who was hospitalized in the Surgery Department of Dr. Carol Davila Clinical Hospital, Bucharest, Romania on the 4th of November 2014. The patient visited the family physician one week before accusing abdominal pain in the lower quadrant and progressive abdominal discomfort. The patient has never been recorded with any history of chronic disease. The patient presented abdominal pain, discomfort and pressure in the left paraumbilical region with extension to the left abdominal flank, being suspected of having a tumor. On palpation, the doctor identified in the left subumbilical region, a tender tumoral mass of firm consistency, painful at deep pressure on the lower area of the abdomen, thereby the patient was asked to perform an abdominal magnetic resonance imaging (MRI). No signs of peritoneal irritation were noted and laboratory tests were normal.

Three months prior to the current admission, the patient suffered an abdominal trauma, from falling in an uncovered manhole of a gutter line on the street.

The MRI using contrast substance performed on the 26th of October 2014 showed an imprecise contoured image, 94.1/76.1/47.5mm in dimensions with hypo/hypersignal at the T1, T2 and TIRM (turbo inversion recovery magnitude) levels with interrupted continuity of muscular fibers in the left rectus abdominis muscle at 7 cm distance from the distal insertion. Diffuse interstitial edema at the level of the left side of rectus abdominis muscle and partially at the level of the right side of rectus abdominis muscle, having the aspect of hematic infiltration is presented in figure 1 and 2. According to the MRI using contrast agents, a non-homogenous appearance was observed. The lesion extended deeply percolating the abdominal wall, the omentum and the loco-regional of the intra-abdominal fat tissue.

Results and discussions

The patient has been hospitalized at the Surgery Clinics of Dr. Carol Davila Clinical Hospital in Bucharest on the 4th of November 2014. At the admission, the patient presented abdominal pain, discomfort and pressure in the left paraumbilical region with extension to the left abdominal

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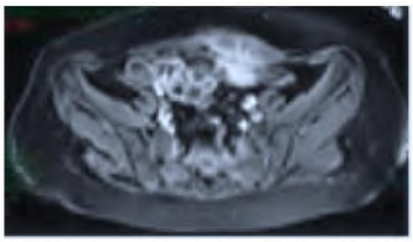

Fig. 1 MRI: an imprecise contoured image, 94.1/76.1/ 47.5 mm in dimensions with hipo/hipersignal at the T1, T2 and TIRM levels with muscular fibres continuity interruption on the left rectus abdominis muscle at 7 cm from the distal insertion. It was also found a diffuse interstitial oedema at the level of the left side of rectus abdominis muscle and partially at the level of the right side of rectus abdominis muscle, having the aspect of haematic infiltration

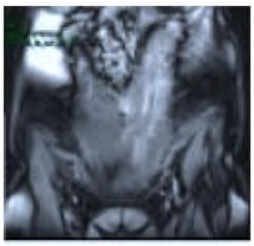

Fig. 2. MRI: By administering contrast agent intravenously an inhomogenous contrast was noticed.The lesion extended deeply percolating the abdominal wall, the omentum and the locoregional intra-abdominal fat tissue

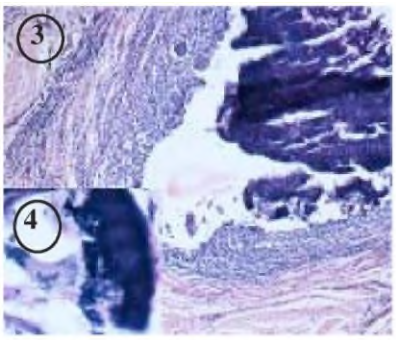

Fig. 3. Actinomicotic granuloma centered by Actinomyces israelii colony. Detail (HE x 200).

Fig. 4. Actinomicotic granuloma centered by Actinomyces israelii colony (HE x 100).

flank. The surgeon identified through palpation, a tender tumoral mass of 8/6 cm of firm consistency in the left subumbilical region, which was adherent to the surrounding areas. No signs of peritoneal irritation were noticed. The gastrointestinal transit was normal. The admission laboratory tests showed that the leukocytes, red cells, hemoglobin, platelets, blood sugar, urea, creatinine, alanine aminotransferase (ALT), aspartate aminotransferase (AST), Na, K, Ca, Cl, serum total protein, were normal. The adherences were removed by surgical intervention and a cross-cut subumbilical incision in the tumor was performed. It was observed an altered subcutaneous cell tissue having an inflammatory aspect which was removed. The left side of rectus abdominis muscle showed fibrous changes and was edematous. A muscular biopsy was taken and sent for a histopathologic examination, which revealed that the source of the examined tissue is from an organized parietal hematoma, surrounded by a xantogranulomatous inflammation extending to the adjacent adipose tissue wherehas been developed a process of septal panniculitis. There have also been found colonies of *Actinomyces* in the interior of the hematoma (fig. 3, fig. 4). The conclusion of the histopathologic examination revealed an organized parietal hematoma with colonies of *Actinomyces*.

The patient has received antibiotic treatment with Ceftriaxone (2x2g/day) for 10 days to prevent post-operative infections. Proper wound care was taken daily. The postoperative evolution was favorable. The biological examinations conducted on the 11th of November 2014 before the discharge of the patient were normal.

Hence, on 15th of November 2014 the patient was transferred to the Infectious Disease Department to start the therapy for actinomycosis. The postoperative wound was about to be scarred and the intestinal transit was normal. The laboratory tests conducted on the 17th of November 2014 showed leukocytes= 10700/µL (normal range 4000-10000/µL) with PMN 76.6% (normal range 50-55%) and ESR= 40mm at 1h (normal range 6-15 mm at 1h). We also found a hypercholesterolemia of 282 mg% (natural range 0-200 mg/dL). The rest of the laboratory tests were normal.

The following treatment was initiated: intravenous Penicillin G 5000000 IU every six hours for 28 days, a probiotic (Eubiotic, 2 capsules/day) and Metamizole. After 4 days of treatment the patient had no fever, the abdominal pain disappeared gradually and the laboratory tests were normal. The patient was discharged on 6 December 2014 with the recommendation to continue the antibiotic treatment with Ospen (penicillin 1 capsule= 1MIU), 4x1 capsule/day for 8 weeks together with Eubiotic (2x1 capsule/day). It was also recommended a low animal fat diet to control the hypercholesterolemia and also to avoid the intense physical activity.

The clinical re-evaluation and follow-up of the patient after one year after the surgery revealed no complications, or relapses seen, indicating that the patient was cured.

Actinomycosis, a pseudotumoral disease caused by an anaerobic gram positive bacteria, called *Actinomyces israelii* is usually rare in developed countries [5, 6]. Actinomycosis has a worldwide distribution and can affect any age group, regardless of race or geographical area, the reported annual incidence of actinomycosis being aproximately 1/300,000 [3, 9]. Preoperative diagnosis has been very rare reported, only in 10% of cases [3, 10]. According to the statistics, the mortality of patients with actinomycosis is between 0% to 28%, depending on the site of infection and also on the time of diagnosis, the highest mortality rate being observed in CNS involvement cases [5, 8, 13, 14].

After penetrating the mucosa, the bacteria induce an inflammatory process with pseudo-tumors or abscesses [6]. Most of the actinomycosis cases (50%) have affected the head and the neck and only 20% have involved the abdomen. The most commonly affected organs are the appendix, caecum, colon, stomach, liver, pancreas, anorectal region, pelvis and abdominal wall [6, 7].

Risk factors for abdominal actinomycosis are recent abdominal surgical interventions or trauma, invasive abdominal exploration procedures, endoscopic manipulation, intra-abdominal malignancy tumors, perforations organs, etc. Women with intrauterine contraceptive devices have also presented a higher risk [3, 8].

We report a case of woman with anterior abdominal wall actinomycosis occured after 3 months of abdominal trauma by falling in the gutter line. As we know, this is the first case of abdominal wall actinomycosis, appeared after a trauma, in western Romania. The clinical spectrum of actinomycosis is nonspecific, including pain, weight loss, anorexia, fever, chills, constipation, diarrhea, abdominal discomfort, palpable mass or fistulas în case of actinomycotic abscesses. Laboratory tests reveals leucocytosis, positive inflammation markers and, sometimes, anemia [2, 7, 9].

In our case we noticed that the patient had only

progressive abdominal pain associated with discomfort and pressure in the left inguinal flank. The patient presented abdominal pain, discomfort and pressure in the left paraumbilical region with extension to the left abdominal flank, being suspected of having a tumor. Because she was afraid of having a malignant tumor she visited her physician, which proceeded all the needed investigations and tests and eventually she decided to have the surgery. Laparoscopic surgery was performed, and an adherent parietal omentum block belonging to the left iliac fossa was found. The laboratory tests showed only a mild leukocytosis with neutrophilia, which was treated with Penicillin.

The conditions for isolation and culture *Actinomyces israelii* are difficult because it needs over a week only for growing. In addition, the culture is negative in 76% of cases. The histopathological examination of the biopsy fragments remains gold standard in diagnosing the abdominal actinomycosis [2, 6, 10]. Microscopic examination reveals the characteristic sulphur granules containing filaments in 50% of the cases. These granules are PAS and Grocott positive, but the von Kossa reaction is negative [2, 3, 6, 10, 11].

These findings are not pathognomonic, because other species such as Nocardia, Staphylococci and Streptomyces can also produce sulphur granules [3, 6, 12]. Ehrlich-Ziehl-Neelson staining are used for the differential diagnosis of Nocardia and Actinomyces (negative for actinomycosis).

Abdominal actinomycosis should be differentiated from Crohn's disease, ulcerative colitis, diverticulitis, neoplasm, intestinal tuberculosis, and appendicitis [4, 8, 13].

Modern imaging investigations such as CT and MRI using contrast agents have an important role to establish the diagnosis of actinomycosis. In our case, the patient examination using MRI contributed to the screening of a diffuse interstitial edema at the level of the left side of rectus abdominis muscle and partially at the level of the right side of rectus abdominis muscle, having the aspect of hematic infiltration.

Laboratory tests have shown that *Actinomyces israelii* is susceptible to penicillin G. Therefore, the specialists recommend intravenous treatment with penicillin G, 10-20 MIU/day for 4 to 6 weeks, followed by oral penicillin V or amoxicillin 2-4 g/day for a period of 2-12 months. The long-term therapy with higher doses of penicillin allows the drug to penetrate through the fibrotic wall of the abscess and to reach in the core of the sulfur granules of actinomyces organism [7, 11, 12]. The combined surgical drainage with antibiotics therapy has proved to be beneficial in the most of the cases [2, 6, 8, 12, 13].

For penicillin-allergic patients, therapists used doxycycline, minocycline, tetracycline, clindamycin, erythromycin, imipenem, cephalosporins and ciprofloxacin having good results [4, 5, 12, 13, 14].

In our opinion, we consider that this case was a success not only because of the therapeutic approaches, but also for the appropriate management concerning the positive and differential diagnosis. According to the other clinical studies, the prognosis was excellent when actinomycosis has been early diagnosed and when it was effectively treated [5, 8, 13, 14].

Conclusions

Abdominal actinomycosis continues to be a hardly diagnosed disorder because of its insidious course, non-specific symptoms and laboratory markers. Both the modern imaging investigations (CT, MRI with contrast) and the histological examination of biopsy specimens help to establish the diagnosis of abdominal actinomycosis. The antibiotic therapy combined with surgery represents a promising alternative to treat patients with abdominal actinomycosis.

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Lung Delayed Hypersensitivity

A case with particular features

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Delayed hypersensitivity reactions are inflammatory reactions initiated by mononuclear leukocytes. These reactions are mediated by T cells and monocytes/macrophages rather than by antibodies. We describe a case of 50 years old man with lung type IV hypersensitivity. The case of lung delayed hypersensitivity presented has some particular histopathological and immunohistochemical features. The diagnosis of lung delayed type hypersensitivity requires analysis of correlation between clinic, radiographic, physiologic and pathologic criteria

Keywords: hypersensitivity reactions, mononuclear leukocytes, histopathological features

The original debate about the role of cell mediated and humoral immunity began in the 19th century between the French cellularists, led by Elie Metchnikof and the German humoralists. The humoralists believed that immunity was due to serum factors (antibodies and complement) which directly destroyed bacteria [1]. The cellularists believed that phagocytes were the basis for immunity. The 1940's experiments confirmed that both theories were essentially correct. Immune function is chemical (antibodies, complement) and also cellular (T cells, B cells and macrophages). Robert Koch was the first who demonstrated a delayed type hypersensitivity reaction in 1882 [2]. Coombs and Gell classified delayed type hypersensitivity as type IV [3]. Delayed hypersensitivity reactions are inflammatory reactions initiated by mononuclear leukocytes. These reactions are mediated by T cells and monocytes/macrophages rather than by antibodies. Both the CD4+ and CD8+ fractions of T cells have been shown to modulate a response. Were demonstrated four types of delayed hypersensitivity reactions based on the T-cell subpopulation involved: IVa- type 1 helper T cells, IVb- type 2 helper Tcells, IVc- cytotoxic T cells, IVd- IL8 secreting T cells. Th 1 cells mediate delayed-type hypersensitivity (DTH) trough secretion of ITF Yand IL-2. Disorders involving type IV reactions include contact dermatitis (eg, poison ivy), hypersensitivity pneumonitis, allograft rejection, tuberculosis, and many forms of drug hypersensitivity [4].

Contemporary debate regarding the reaction is focused on the role of the Th1 and Th2 cells originally discovered by Mosmann [5].

In delayed-type hypersensitivity reactions sensitized T cells mediate a cascade of cellular interactions. Initiation of these responses depends of vasoactive mediators from mast cells that are activated by antigen-specific T-cell-derived factors. Askenase and Loveren [6] discussed how this event initiates a sequence of steps that lead to T-cell recruitment of effector cells; and how this event differs from activation of mast cells by IgE antibody. They suggested that the conventional time-based separation of immediate and delayed hypersensitivity should be replaced by a classification based on the type of antigen-specific lymphocyte - B or T-responsible for the effects of hypersensitivity.

Refer to delayed hypersensitivity expression in the lung it was described hypersensitivity pneumonitis (extrinsic allergic alveolitis), an allergic lung syndrome considered to be a mix of type III and type IV hypersensitivity responses. It is caused by inhalation exposure to a wide variety of organic dusts. These dusts contain antigenic substances, including fungal/bacterial components, serum proteins and some chemicals.

Experimental part

Case presentation

A 50-year-old white male with cough, fever, signs of mild respiratory failure. Computed tomography (CT) scan of the chest demonstrated the mediastinal lymph nodes with the following dimensions: 2cm in the right paratracheal area, 0.6 cm in the lung hila and 1.7 cm infracarinal. Disabling, fibrous lesion, antero- basal, left lower lobe. Subpleural, bilateral, pulmonary ground glass opacities were noticed. Trachea and main bronchus were permeable. Hearth presented normal aspect. Pulmonary artery, thoracic aorta have normal caliber. Adrenal glands with normal aspect were found.

An open lung biopsy of the lingula and basal pyramid (paracardiac segment) was performed. Haematoxylin eosin staining indicated a biopsy from peripheral parenchyma with multiple lymphoid follicles separated one from each other. Focally we noticed severe bronchioalveolar destruction. We found alveoli irregular distension, massive accumulation of macrophages in the alveolar lumen, focally associated with edema, a high number of intravascular and extravascular eosinophils without a significant fibrosis. Occasionally, the blue corpuscles were present. The dusty macrophages were rare, crowded in small groups, perivascular, predominantly. The silver staining revealed a normal reticulin network in perivascular area and lung septa but disorganized in massive inflammation areas. The reticulin network was presented around lymphoid follicles.

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Immunostaining for CD3 indicated a positive reaction with heterogeneous distribution in the lung parenchyma. It was noticed an accumulation in the lamina propria of bronchioles (fig. 1a) and an abnormal distribution in the lymphoid follicles (germinal center) (fig. 1b).

CD20 expression was intense in the lymphoid follicles (fig. 1c) compare with CD5 immunoreaction which showed rare and weakly positive cells in the same area (fig. 1d). The reaction for CD15 was negative.

Bcl 2 positive reaction was restricted to the periphery of lymphoid follicles, respecting the normal distribution of this marker (fig. 1e). Immunostaining for Ki67 revealed a positive reaction in the germinal center of lymphoid follicles (fig. 1f).

Expression of CD68 was intense in the cells with macrophages morphology. Immunostaining with anti-mast cell tryptase antibody showed a high number of mast cells around the lymphoid follicles, in the connective tissue. Many mast cells were founded, concentrated in the immediate vicinity of the respiratory epithelium, in the lymph nodes from this area. In the lymph nodes which are not surrounded by epithelium, mast cells were disposed to the periphery of these, in a smaller number compare with previously situation. Only a few, isolated follicles presented mast cells in the central areas. The most of the nodules were situated in the vicinity of blood vessels. In this area was found a high number of mast cells with an oval shape, most of them partially degranulated (fig. 1g). Free mast cells granules were noticed among the collagen fibers, around vessels from the vicinity of nodules. The numerical distribution of mast cells was different from one node to another. The dimensions were relatively small. An extremely high number of mast cells were found in the interalveolar septa (fig. 1h).

Results and discussions

The diagnosis of delayed- type hypersensitivity was sustained in our case by immunohistochemical profile: CD3 positive with heterogeneous distribution in the lung parenchyma, CD20 intense positive in the lymphoid follicle, Bcl2 positive, with normal distribution in the lymphoid follicle, CD15 negative, CD68 positive, mast cell tryptase positive heterogeneous. An accurate differential diagnosis represents an important step for patient's evolution and prognosis. The differential diagnosis with other diseases which have the delayed type hypersensitivity as mechanism is important to make.

Enander et al, 1988 [7] studied the appearance of mononuclear cells, mast cells and mucus-producing cells in the lung and their linkage to the development of delayed hypersensitivity (DH) reactions. After in vivo treatment with the monoclonal GK1.5 (anti-L3T4) antibody resulting an inhibition of the DH reaction and a decrease number of mononuclear cells and mucus-producing cells, but not mast cells in the lung of sensitized and challenged mice. In our case, many mast cells were concentrated in the immediate vicinity of the respiratory epithelium, in the follicle from this area. In the follicles which are not surrounded by epithelium, mast cells were disposed to the periphery of these, in a smaller number compare with previously situation.

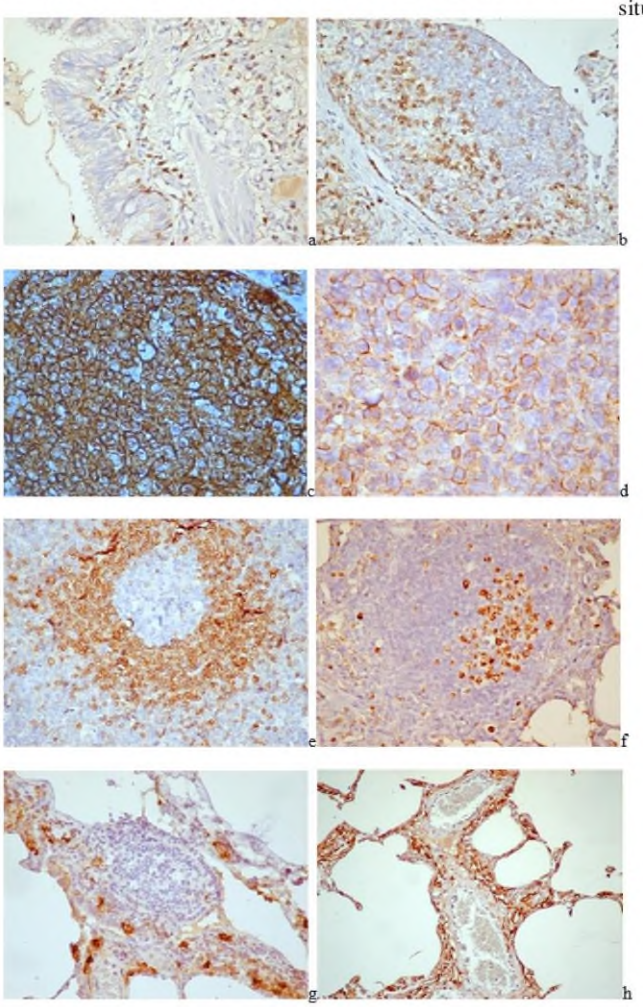

Fig.1a- Immunostaining for CD3. Heterogeneous distribution of CD3 in the lung parenchyma, with accumulation of positive cells in the lamina propria of bronchioles, X 100;
Fig.1b- Immunostaining for CD3. The abnormal distribution in the germinal center of the lymphoid follicles, X 100;
Fig.1c- Immunoexpression of CD20. An intense expression in the lymphoid follicles, X 400;
Fig.1d- CD5 immunoreaction. Rare and weakly positive cells in the lymphoid follicles, X 400;
Fig.1e- Immunoexpression of Bcl 2. The normal distribution, with reaction limited to the periphery of lymphoid follicles, X 100;
Fig.1f- Immunoexpression of Ki67. Positive reaction in the germinal center of lymphoid follicles, X 200;
Fig.1g- CD68 immunoreaction. Mast cells with an oval shape, most of them partially degranulated, X 200;
Fig.1h. CD68 immunoreaction. High number of mast cells situated in the interalveolar septa, X 100.

In the lung, hypersensitivity pneumonitis (HP) is a relatively rare and is characterized by the predominance of mononuclear inflammation of the lung interstitium, terminal bronchioles and alveoli. The histopathological specimens show interstitial inflammation accompanied by organizing pneumonia and multinucleate giant cell typical of hypersensitivity pneumonitis. The most significant clinical feature of HP is granuloma formation (absent to our patient), possibly progressing to lung fibrosis. Historically, HP has predominantly resulted from occupational exposures and therefore has a variety of names based on occupation or antigen association (farmer's lung) [8]. A combination of host and environmental factors should be considered as a requisite to developing this disease. Although the antigens differ widely, the clinical syndromes that results are very similar. HP occurs mainly in non-smokers, and clinically it may be in acute, subacute, or chronic forms.

It can be excluded another diseases with granuloma formation: TB (caseating granulomas, cavities with approximation of walls, granulation tissue, fibrosis and stelate scar), sarcoidosis (non-caseating epithelioid granulomas with tightly packed epithelioid cells, Langhans giant cells and lymphocytes (T cells), usually in interstitium adjacent to bronchioles and around and within vessel walls, pleura and connective tissue septa; necrotizing sarcoid granulomatosis: extensive, vascular, non-caseating sarcoid-like granulomas invading pulmonary arteries and veins with diffuse necrosis of lung parenchyma, Schaumann bodies, asteroid bodies).

Among the diseases that can be addressed in the differential diagnosis include chronic obstructive pulmonary disease (COPD). This is an inflammatory disease in which the cellular infiltrate is comprised primarily of CD81 /Tc1 and CD41 /Th1 lymphocytes and macrophages. This infiltrate, which persists long after cigarette smoking is ended, is diffusely distributed throughout the lung, including the small airways, submucosal glands, lung parenchyma, and pulmonary arteries. In addition, T cells, B cells, macrophages, and dendritic cells aggregate into organized lymphoid follicles in close proximity to the airways and within the lung parenchyma [9]. Compare with this, our case presented lymphoid follicles in the lung parenchyma and a high number of mast cells around blood vessels and in the interalveolar septa also.

Microscopic description of lymphoid interstitial pneumonia (LIP) included: lymphocytes with germinal centers, plasma cells, macrophages and epithelioid granulomas in lung interstitium, no effacement of alveolar architecture, no invasion of parietal pleura, although visceral pleura may have mild inflammation, late- diffuse interstitial fibrosis. Our case did not present ephiteloid granulomas, but its lung architecture showed alveoli irregular distension, massive accumulation of macrophages in the alveolar lumen, focally associated with edema. It was noticed a high number of intravascular and extravascular eosinophils without a significant fibrosis.

Immunophenotyping studies for lung non-Hodgkin lymphoma used antibodies to Ki-67, CD3, CD5, CD10, CD19, CD20, CD22, CD23, CD45 and CD79a selectively according to suspected subtype [10]. Our case presented CD3 positive in germinal centre of lymphoid follicle, CD20 intensely positive in the lymphoid follicle, CD 5 rare and weakly positive in lymphoid follicle, CD15 negative. These aspects excluded non-Hodgkin lymphoma diagnosis.

There are many forms of drug delayed hypersensitivity. From these, to the lungs have been described 2 cases of respiratory type-IV hypersensitivity reactions due to corticosteroids like a rare phenomenon. Constantinos, 2009 [11] presented a case of a patient who developed fever, arthralgias, myalgias, leukocytosis, and ARDS following a second infliximab infusion, after a 15-month drug holiday. Human antichimeric antibodies (HACA) were strongly positive, and no other etiology for acute respiratory distress syndrome (ARDS) was discovered. This may have represented an unusually severe delayed hypersensitivity reaction to infliximab. Our patient presented simphtomatology after professional dust exposure. In the literature, other cases with effects to the lungs were described by Riegert, 2002 [12]- a case of adult respiratory distress syndrome associated with infliximab therapy, Potenza, 2010 [13]- dysesthesia and laryngeal spasm developed 10 h after the sixth administration of oxaliplatin to a 46-year-old man with adenocarcinoma of the sigmoid colon.

Conclusions

Lung delayed type hypersensitivity diagnosis requires analysis of correlation between clinic, radiographic, physiologic and pathologic criteria.

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II. APLICAREA METODELOR HISTOCHIMICE ȘI IMUNOHISTOCHIMICE ÎN PROCEDURILE DIAGNOSTICE ȘI CU POTENȚIAL IMPACT TERAPEUTIC ÎN BOLI CU ETIOLOGIE PARAZITARĂ ȘI INFECȚIOASĂ

O direcție principală de activitate este concentrată în sfera acariozelor (demodicoza), a zoonozelor parazitare (criptosporidioză, toxoplasmoză, giardioză, tricofiție, cheiletieloză), microsporidiozelor (nosemoză) și a bacteriozelor (infecția cu *Lawsonia spp.*, *Salmonella spp*.).

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Ectoparazitoză ce afectează pielea omului și a animalelor și determină apariția leziunilor depilante, alopecice, eritematoase, care, deseori pot atrage complicații bacteriene sau micotice, demodicoza este produsă de specii ale genului *Demodex*, acarieni care parazitează în foliculii pilosebacei [Mederle Narcisa și col., 2010].

Demodicoza este considerată o boală multifactorială: sinergismul dintre factorii imunologici (imunosupresie primară, bazată pe o disfuncţie limfocitară sau pe un defect genetic al limfocitelor T sau, secundară, rezultat al corticoterapiei, stress, boli

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REVIEW ARTICLE

Diagnosis of canine demodicosis

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Abstract. This paper briefly reviews the dermatological diagnosis, the examination of skin scrapings, as well as the interpretations of some molecular methods. The aim of the paper is to assess the value of the diagnosis methods and to establish whether correlating the results may lead to a rigorous diagnosis in canine demodicosis.

Keywords: *Demodex canis*; demodicosis; diagnosis.

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Canine demodicosis is a skin disease of dogs, produced by mites of genus *Demodex*, parasitic in the pilosebaceous follicles. It frequently becomes pustular due to bacterial complications. The diagnosis of canine demodicosis is achieved with respect to the general principles of a diagnostic dermatology.

Anamnesis

This first step of the diagnosis should lead to the formulation of hypotheses before the clinical examination. A careful anamnesis should consider epidemiologic data (breed, sex, age), circumstance of disease installation, way of life and microclimate conditions. Information regarding previous conditions, treatments or presence of aggravating factors are also important.

Receptivity of dogs to demodicosis is influenced by numerous intrinsic factors immunological disorders, hormonal status, breed, hair length, age. Extrinsic predisposing factors include alimentation, fitness, presence stress factors, other diseases or pathogens etc. Their joint action of these factors increase the receptivity to demodicosis, as well as its outbreak and evolution (Deepa et al., 2005). Age is highly important in the epidemiology of the disease. Demodicosis typically occurs in young dogs, with the clinical onset, generally between three months and one year. In dogs older than one year, cases tend to become occasional. Most of the cases (64.97%) occur before the age of one year (Rabdea, 2005). However, demodicosis has been diagnosed in dogs as young as five weeks or old ones (14 years old) (Rabdea, 2005). Some data suggest correlations between the age of dogs and the clinical form of the disease (Scott et al., 1995).

Clinical examination

imunosupresive grave) și factorii de altă natură este determinant în ruperea echilibrului simbiotic dintre parazit şi gazdă, exacerbarea patogenităţii acarienilor şi declanşarea bolii. Acarienii sunt foarte numeroşi în piele şi sunt foarte bine apăraţi prin poziţia lor în foliculii piloşi şi în glandele sebacee, modul lor de hrănire cu sebum şi celule foliculare moarte îi fereşte de contactul şi de ingerarea unor cantităţi letale de acaricide, iar hipersecreţia de sebum pe care o provoacă măreşte şansele acestora de apărare pasivă şi le facilitează multiplicarea [Goethe, R., 1989, Kumari, P. și col., 2017, Kumari, P. și col., 2018, Radbea Narcisa, 2005].

Caracterul multifactorial al acariozei se regăsește și în versatilitatea manifestărilor clinice, mascarea leziunilor primare, expresiile clinice asemănătoare, dar și izbucnirea neașteptată a episoadelor clinice în funcție de intervenția factorilor individuali, aspecte simptomatice care ne obligă la o nouă abordare clinică, preclinică și de laborator a diagnosticului acariozei [Mederle Narcisa și col., 2010].

Demonstrarea transmiterii speciei *Demodex canis* la om, fenomen semnalat în prezent [Esenkaya Taşbent F. și Dik B., 2018], dar neconfirmat prin biologie moleculară, a reprezentat scopul studiilor preliminare realizate în 2007, respectiv, 2014: identificarea la om a speciei *Demodex folliculorum* în leziunile faciale exprimate prin eritem [Mederle Narcisa și col., 2007] și izolarea la o pacientă în vârstă de 60 de ani a două specii ale genului *Demodex: D. folliculorum și D. brevis*, în raclatele cutanate executate din leziunile faciale traduse prin prurit și mici colecții purulente, leziuni tratate fără succes cu antibiotic și steroizi. Leziunile faciale au fost însoțite de blefarită, probele recoltate din gene și sprâncene oferind, de asemenea, imaginea celor două specii de acarieni [Gartner Andreea și col., 2014].

ELSEVIER

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Demodex folliculorum and *D. brevis* – A cause of facial dermatitis and blepharitis

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Examenele complementare sunt orientative în diagnosticul demodicozei, dar asociate cu o examinare clinică și microscopică, acestea pot oferi o imagine complexă de diagnostic. Unele studii bibliografice subliniază importanța examinărilor paraclinice în diagnosticul și tratamentul demodicozei, în timp ce alți autori susțin irelevanța acestor teste la pacienții adulți, cu o ușoară reticență atunci când este vorba de vârsta tânără [Yarim, G. F. și col., 2015; Umeshdimri, A. și col., 2000; Tani, K. și col., 2002; Sushma, S.S. și col., 2000].

A fost realizat un studiu în care s-a urmărit evoluția valorilor serice ale proteinelor totale, albuminelor, globulinelor totale și imunoglobulinelor G, M, A, E, la pacienți canini diagnosticați cu demodicoză uscată (formele localizată și generalizată) și piodemodicoză (PD), prin tehnicile turbidimetrică și colorimetrică.

Evaluation of serum values of total protein (PT), albumin, total globulin and immunoglobulin (Ig) G, A, M, and E in canine demodicosis.

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Abstract : Demodicosis remains one of the main disease which affects the dog skin. Although in recent years there have been significant advances in the understanding of this important ectoparasitosis, more research in areas, such as pathogenesis and immunology is clearly needed. In this context, the aim of the present study is to determine the serum values of total proteins (PT), albumin, total globulin and immunoglobulins (Ig) G, A, M and E in the three clinical evolution forms of canine demodicosis. 67 dogs diagnosed with demodicosis were selected for study. The turbidimetric and colorimetric techniques revealed changes of PT and Ig serum values in dogs with demodicosis, as well as differences in serum Ig and PT concentrations between the three groups under study (LD, GD and PD).

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Studiul a relevat modificări ale valorilor parametrilor urmăriți față de limitele fiziologice (lotul martor), dar și modificări ale valorilor serice ale proteinelor totale, albuminelor, globulinelor totale și Ig în cele trei forme de evoluție clinică a bolii, aspect ce poate reprezenta un indicator în diagnosticul diferențial al demodicozei canine. În demodicoza uscată, albuminele (66.66%) și proteinele totale (64.44%) cresc, iar globulinele totale scad (71.11%). La câinii cu PD s-a remarcat scăderea concentrației proteinelor totale (36.36%) și a albuminelor (54.54%), acompaniată de creșterea concentrației globulinelor totale (54.54%). Creşterea concentraţiilor serice ale Ig şi ale globulinelor totale în PD conduc la scăderea accentuată a proteinelor totale în această formă de evoluţie clinică, responsabile de acest proces sunt bacteriile piodermice asociate lui *Demodex* care, vor determina, în cursul evoluţiei bolii o imunodepresie celulară. Concentrația de Ig G și Ig A este scăzută în DL și DG, cu insignifiante diferențe între cele două loturi și crește în PD: IgG (63.63%), respectiv, IgA (59.09%). Ig E se mențin la concentrații crescute față de limitele fiziologice, în toate cele trei forme de evoluție clinică a demodicozei, în timp ce, Ig M rămâne la valorile de referință, în 94% din câinii luați în studiu [Mederle Narcisa și Mederle O., 2017].

Acestui studiu i se alătură acela realizat la câini piodemodecici (PD) la care s-au urmărit fosfataza alcalină, aminotransferazele, urea, creatinina, proteinele totale, albuminele și globulinele. Rezultatele studiului au relevat modificări ale valorilor parametrilor urmăriți față de limitele fiziologice (lotul martor), dar și o creștere a fosfatazei alcaline și a aminotransferazelor asociată cu scăderea valorilor albuminelor și proteinelor totale, la lotul de câini tineri diagnosticați cu piodemodicoză [Negrescu Adina și col., 2017].

În concluzie, când scăderea imunoglobulinelor serice este determinată de acţiunea diferiţilor factori favorizanţi şi/sau imunodepresivi, sau când scăderea Ig serice este acompaniată de o imunodeficienţă mediată celular (deficit al limfocitelor T) în prezenţa unei stimulări antigenice permanente (*Demodex canis*), atunci apariţia bolii localizate este iminentă. Prelungirea acestei situaţii conduce la generalizarea bolii şi la menţinerea Ig la valori scăzute comparativ cu valorile Ig la câinii sănătoşi. Când se suprapun infecţiile bacteriene, iar boala capătă un caracter piogen, atunci valorile serice ale Ig se inversează prin intervenţia noilor factori imunosupresivi; în această situaţie, creşterea valorilor imunoglobulinelor serice este menţinută de parazitul însuşi. Creşterea constantă a IgE în toate cele trei forme de evoluţie clinică poate evoca implicarea IgE într-un fenomen de hipersensibilitate suprapus unei imunodeficienţe umorale sau celulare.

Studiul prezent pledează, prin rezultatele obținute, pentru importanța examenelor complementare care pot fi un indicator paraclinic important în diagnosticul piodemodicozei câinilor tineri. Rezultatele studiului au fost în acord cu rezultatele relatate de alți autori [Ravera, I. și col., 2015; Gasparetto, ND. și col., 2018].

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Abstract Letter

THE DIAGNOSTIC VALUE OF HEMATOLOGY AND BLOOD BIOCHEMISTRY IN PIODEMODICOSIS

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Demodicosis is a serious ectoparasitosis produced by Demodex sp. which is localized in hair follicles in animals and humans, also. Complementary examinations are indicative in the diagnosis of parasitosis, but associated with a clinical and microscopic examination, they have a value useful the clinician [4]. There are bibliographic studies [2,3] which emphasize some importance in the diagnosis and treatment of demodicosis, paraclinical examinations. They are also authors [1] who support the irrelevance of these tests in adult dogs, with a slight reluctance when it comes to young dogs.

Based on these considerations, the aim of the present study was to determine the values of certain blood parameters in dogs diagnosed with piodemodicosis and to appreciate the importance of these exams in the diagnosis of acariosis.

The investigations were performed on a group of 37 dogs diagnosed with piodemodicosis (PD), aged 2-3 years and on a control group of 20 dogs, aged 2-3 years. Blood samples were processed and blood counts were determined: blood count, alkaline phosphatase (ALP), alanine aminotransferases (ALT), aspartate aminotransferases (AST), serum urea, serum creatinine, total proteins, albumin and globulin. The results were statistically interpreted by the Microsoft Excel program.

Haematological investigations results in studied group (PD) reveal values of erythrocytes, hemoglobin, hematocrit and platelets at the lower limit of the normal values; decreased red blood cell count and decreased hematocrit concentration. Leukocytosis is found: lymphocytes and granulocytes increase and monocytes decrease. This aspect reveals the response of the host to the parasite, but also to the bacterial suprainfection. Blood biochemical parameters ranged, as average values, within the normal reference range, with mild serum increasing on ALP, ALT, AST associated with decreased albumin and total serum protein. These values highlight either a non-parasitic insufficiency or are the result of the toxic action of Demodex mite.

We did not record changes in kidney parameters (creatinine and serum urea). In the control group, the results of haematological and biochemical investigations were within the reference limits.The results of haematological and blood biochemical investigations reveal changes from the normal values of the monitored parameters. In young dogs diagnosed with piodemodicosis, haematological investigations and blood biochemistry are important in the diagnosis of acariosis.

Prelevarea prin puncție/biopsie a pielii parazitate cu acarianul *Demodex* și realizarea de preparate histologice și imunohistochimice au oferit, pe de o parte, o confirmare a diagnosticului clinic și microscopic și, pe de altă parte, o imagine a reacțiilor tisulare și imunohistochimice, determinate în pielea câinilor, de prezența acarianului [Radbea Narcisa și col., 2005].

Scientia Parasitologica, 2005, 1-2, 100-103

Aspecte histopatologice în demodicoza generalizată la câine

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REZUMAT. Imaginile histopatologice realizate în demodicoza canină, forma generalizată, au relevat prezenţa acarianului *Demodex canis* în foliculul pilos şi absenţa acestuia în glanda sebacee. În această formă clinică reacţiile celulare sunt intense, procesul de hipercheratoză este accentuat, apar foliculita murală, perifoliculita şi melanoza perifoliculară; infiltraţiile dermice sunt constituite din limfocite T, macrofage, monocite, histiocite şi eozinofile.

Examenul histopatologic al biopsiilor cutanate prelevate din leziunile de demodicoză însoţeşte şi completează rezultatele examenelor clinic şi microscopic. Examenul histopatologic poate confirma diagnosticul microscopic al raclatelor cutanate, dar constituie o probă insuficientă în stabilirea unui diagnostic diferenţial între demodicoza generalizată (DG) şi demodicoza localizată (DL) precum şi în anticiparea unei eventuale vindecări spontane. Examenul histopatologic poate însă diagnostica demodicoza atunci când apare suspiciunea unui examen microscopic negativ.

Luând în considerare aceste aspecte, **scopul** acestui studiu a fost acela de a urmări valoarea de diagnostic a examenului histopatologic în demodicoza canină generalizată.

Material şi metode

Pentru realizarea preparatelor histopatologice am constituit două loturi de câini de rase, sexe şi vârste diferite: lotul 1, constituit din 10 câini clinic sănătoşi (lotul martor) şi lotul 2, format din 10 câini cu demodicoză generalizată (DG) .

Biopsiile au fost recoltate din leziunile alopecice, din zonele care prezentau descuamări, hiperpigmentaţii şi eritem. Am evitat leziunile hemoragice, purulente şi necrotice pentru a nu deruta interpretarea rezultatelor. De la lotul martor au fost recoltate biopsii din pielea aparent

Biopsiile cutanate au fost recoltate sub anestezie generală, utilizând premedicaţie cu atropină sulfurică (0,2 mg/kg), urmată de administrarea intramusculară a xilazinei (1,5 mg/kg) şi ketaminei (10 mg/kg). Probele cutanate au fost introduse în formol 10%; preparatele histologice au fost efectuate la disciplina de Histologie - Citologie a Universităţii de Medicină şi Farmacie „Victor Babeş" Timişoara.

Tehnica histologică presupune derularea etapelor cunoscute: fixarea în formol, spălarea, incluzionarea în parafină, secţionarea, deparafinarea şi hidratarea secţiunilor şi colorarea. S-au folosit colorările: tricrom-Masson , colorarea cu hematoxilină-eozină şi colorarea PAS.

Rezultate şi discuţii

Preparatele histopatologice realizate din leziunile demodecice au relevat un generos tablou epidermo - dermic.

În leziunile de **demodicoza generalizată** (DG) (figura1) s-au remarcat următoarele leziuni:

Epiderm: hipercheratoză accentuată, ulceraţii la nivelul stratului bazal, hiperplazia celulelor stratului Malpighi cu apariţia spaţiilor intercelulare (acantoză şi spongioză), proliferări epiteliale.

Au fost realizate și interpretate preparate HP și IHC înainte și după aplicarea topică a kit-ului Demosimcan. S-au utilizat tehnicile de colorare tricrom-Masson, HE și reacția PAS și s-a apelat la kit-urile *BOND TM Polymer Refine detection CD 8, CD 20 și CD100 (Medist Life Science)* pentru a evidenția markerii imunohistochimici caracteristici în demodicoză.

Este primul studiu IHC realizat în demodicoza canină care utilizează kit-urile *BOND TM Polymer Refine detection CD 8, CD 20 și CD100.*

Imaginile histologice au relevat prezența acarianului în foliculii piloși (fig.1, 2), leziunile identificate crescând gradual din demodicoza localizată în piodemodicoză: foliculită murală, infiltrații limfoplasmocitare și granulare, hipercheratoză. Acarianul *Demodex canis* a fost absent în glanda sebacee, aspect ce asigură menţinerea integrităţii glandei, dar, infiltratul inflamator dermic determină compresiuni asupra glandei, finalizate cu atrofii. Aceste atrofii conduc la o creştere a activităţii glandei sebacee și ca urmare, apare hiperseboreea.

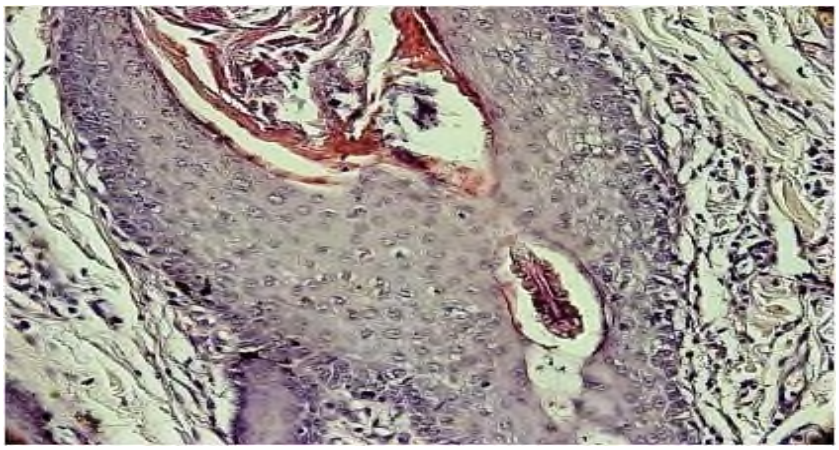

Fig. 1. Piele demodecică (col. HE)

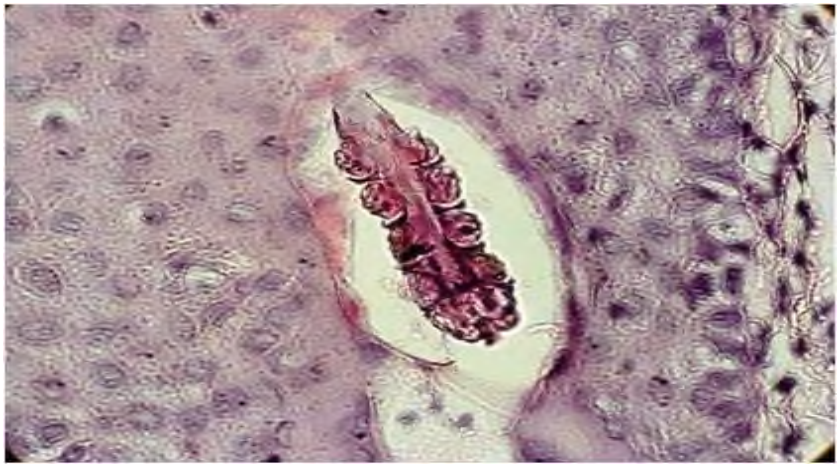

Fig. 2. Prezența acarianului *Demodex canis* în piele (detaliu) col HE

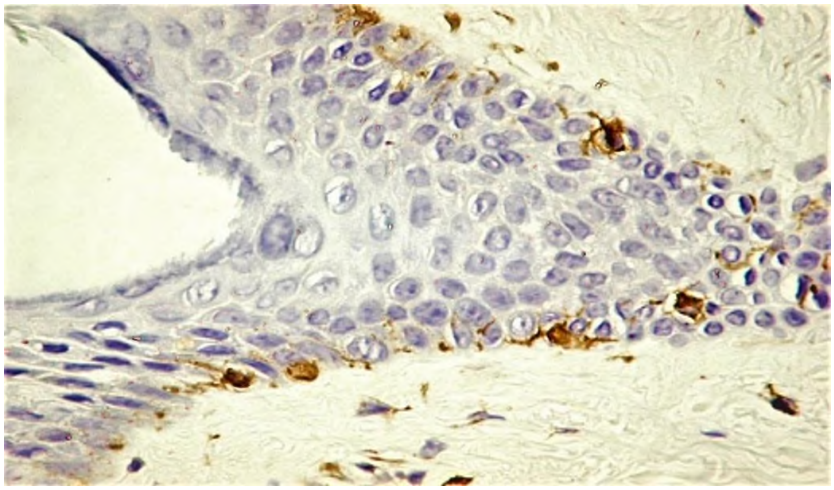

Fig. 3. Celule Langerhans evidențiate cu S100 in stratul bazal al epidermului

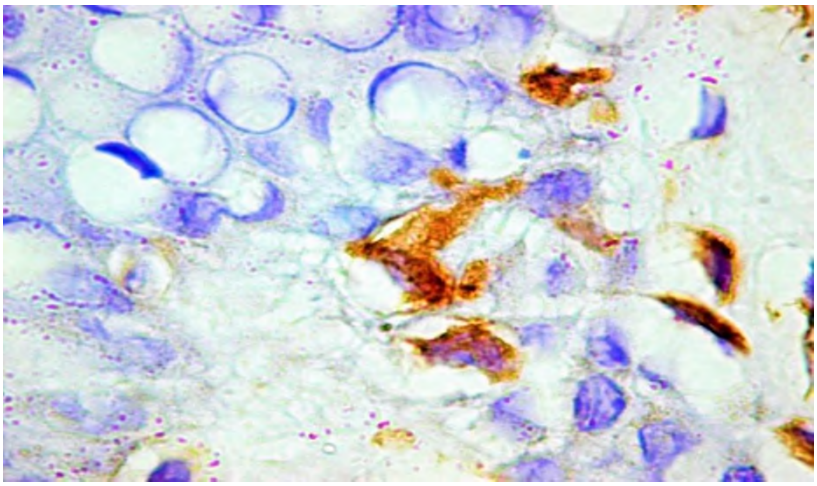

Fig. 4. Celule Langerhans evidențiate cu S100 in stratul spinos al epidermului

Preparatele IHC au relevat răspunsul sistemului imunitar cutanat la prezența acarianului: hiperplazii ale celulelor Langerhans, lezarea keratinocitelor, infiltrații limfocitare, migrarea neutrofilelor și a eozinofilelor, hiperplazia melanocitelor și a macrofagelor; au fost identificate limfocitele B și T cu markerii imunohistochimici caracteristici CD 8 și CD 20 și celule Langerhans, cu markerii S100 (fig.3, 4).

La o perioadă de trei luni de la administrarea kit-ului, acarianul a fost absent în preparatele HP, imaginile lezionale scad în intensitate; examenele IHC evidențiază o revenire la limitele fiziologice a reacțiilor cutanate celulare (keratinocitele, celulele Langerhans, limfocitele T și B, neutrofilele, eozinofilele, macrofagele) și o diminuare semnificativă a infiltratelor inflamatorii limfoplasmocitare (CD 8 și CD 20).

Au fost descrise tablourile histopatologice în demodicoza generalizată și în piodemodicoză, preparatele histologice relevând prezența acarianului în foliculul pilos și absența acestuia în glanda sebacee. O creștere a intensității reacțiilor cutanate celulare a fost

remarcată în forma generalizată (hipercheratoză, foliculita murală, infiltrații dermice) spre piodemodicoză (granuloame perifoliculare, melanoza perifoliculară) [Mederle Narcisa și col., 2010; Radbea Narcisa și col., 2006].

Pentru realizarea preparatelor IHC în piodemodicoză, s-a utilizat sistemul LSAB2. Imunofenotipizarea celulelor Langerhans și a limfocitelor a fost posibilă cu ajutorul proteinei S100. Keratinocitele și melanocitele stratului bazal au fost vizualizate prin metoda DAB, folosind citokeratina CK20 și pancitokeratina. Vimentina și citockeratina CK20 au permis identificarea fibroblastelor și a celulelor bazale. S-a remarcat un proces intens de migrare a neutrofilelor și eozinofilelor, hiperplazia melanocitelor și macrofagelor, scăderea numărului de celule Langerhans și keratinocite și creșterea numărului de limfocite B [Radbea Narcisa și col., 2006].

Universitatea de Ştiinţe Agricole şi Medicină Veterinară Iaşi

IMUNOHISTOCHEMICAL RESULTS IN CANINE PIODEMODICOSIS

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[2]U.M.F. Timişoara

We study 20 dogs, 10 infected with Demodex canis mite (piodemodicosis) and 10 control group. We performed immunohistochemmically method using LSAB2 system with following antibody: Langerhanh cells, Lymphocyte and citokeratine, visualization with DAB. Immunohistochemical staining reveals an intense process of neutrophils and eosinophils migration as well as a hiperplasia of melanocytes and macrophages. In piodemodicosis, there is a certain modification of the skin: the number of the keratinocytes and Langerhans cells decreases and that of the B cells increases as a consequence of the umoral immune response.

INTRODUCERE

The Immunohistochemical diagnosis reveals only immune response in skin canine demodicosis. There for he must associated with histopathological and cytopathological exams to complete diagnosis.

MATERIALS AND METHODS

Skin biopsies from each dog of the two series (one of 10 dogs infected with *Demodex Canis* and one of 10 healthy dogs used as control) were harvested and immunohistochemmically stained using the LSAB2 system. Langerhans cells and lymphocytes were primary immunophenotypised by the S100 protein. Citokeratin CK and pan – citokeratin with DAB visualization were used to mark the presence of keratinocytes and melanocytes of the basal layer. Fibroblasts and basal cells were identified by vimentin and, respectively, CK 20.

RESULTS AND DISCUSSION

Immunohistochemical staining revealed interesting aspects concerning the presence of immunocompetent cells (Langerhans cells, keratinocytes), and the reaction of dermis and epidermis of those cells as a response to he „foreign body": *Demodex Canis*.

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Rezultatele studiilor prezentate sunt în concordanță cu studiile altor autori care fac o corelație între reacțiile cutanate care apar în preparatele histopatologice cu leziunile clinice, macroscopice, subliniind, astfel, importanța examenului histopatologic în demodicoza canină: epidermul suferă un proces de hipercheratoză, tradus clinic prin descuamări şi cruste. Dilataţia foliculară accentuată de sebum, cheratină, debriuri si demodecşi conduce la ruperea foliculilor piloşi urmată de eliminarea conţinutului în derm şi apariţia, în final, a granuloamelor perifoliculare. Melanoza perifoliculară este asociată clinic cu leziuni de hiperpigmentare care însoţesc alopecia şi hiperseboreea, mascând la unele cazuri, eritemul preexistent [Sood, N. K. și col., 2012; Caswell, J., 1993; Mizutani, N. și col., 2016; Singh, S. K. și col., 2011; Singh, S. K. și col., 2014; Olivera, C.D. și col., 2015].

În situația în care, acarienii sunt numeroşi, răspunsul celular este minim sau absent, eozinofilele sunt absente, este prezentă furunculoza si limfadenita suntem îndreptăţiţi să considerăm că avem de a face cu o severă imunosupresie [Mizutani, N. și col., 2016].

În situațiile în care există prezumția de diagnostic, examenul microscopic al raclatului cutanat este negativ, aspectele clinice nasc suspiciunea de demodicoză, iar preparatele histopatologice evidenţiază acarianul în foliculii piloşi, prezenţa acestuia fiind însoţită de leziunile caracteristice, valoarea de diagnostic a examenului histopatologic și imunohistochimic în demodicoza canină este evidentă și necesară.

Studiile HP și IHC realizate în demodicoza canină contribuie la evidențierea imunohistochimică a infiltratelor inflamatorii limfo-plasmocitare (CD 8 și CD 20), precum și la descrierea fenomenului de migrare a celulelor Langerhans din stratul bazal spre dermul superficial.

OBȚINEREA PRIN TEHNOLOGII EMERGENTE ȘI PROCEDEE ASISTATE DE MICROUNDE ȘI ULTRASUNETE A GELULUI DEMOSIMCAN

– premieră națională –

Demodicoza este o dermatoză gravă, greu de tratat, deseori recidivantă, iar biologia şi patogenia agentului etiologic împiedică acţiunea medicamentelor [Scott, D.W. și col., 1995; Radbea Narcisa și col., 2005; Mueller, R. și col., 2012; Sarkar, P. și col., 2004].

Părerile referitoare la terapia demodicozei sunt împărțite între autorii care susțin tratamentul acaricid, fie el topic sau sistemic și, cercetătorii care pledează pentru administrarea produselor naturiste, neiritante şi neinvazive pentru piele si organism. Părerile devin unanime atunci când vorbim despre importanța cunoașterii biologiei parazitului și, mai mult, despre reacția pielii parazitate [Beyazit, A. și col., 2010; Lockwood, S. L. și col., 2017; Martinez-Subiela, S. și col., 2014]. Informațiile bibliografice actuale privind terapia demodicozei pun în lumină avermectinele, milbemicinele şi formamidinele, substanţele de referinţă în tratamentul specific al demodicozei canine [Paterson, T. E. și col., 2014; Cordero, A..M. și col., 2018; Hutt, J. H. și col., 2015]

Eficacitatea antiparazitară a acestor molecule de sinteză este recunoscută, însă, efectele secundare iritante, invazive pentru piele, ale unora dintre aceste substanțe, sunt deseori raportate [Six, R.H. și col., 2016; Snyder, DE. și col., 2017; Morita, T. și col., 2018; Duangkaew, L. și col., 2018; Fourie, J. J. și col., 2012; Becskei, C. și col., 2018; Beugnet, F. și col., 2016].

Recunoscute ca avantajoase din perspectiva eficacității și a costului, produsele naturiste, extractele din plante, aplicate topic și/sau administrate sistemic sunt, în prezent, candidate în terapia demodicozei canine: Graphitis 200, Maggacite (extract de Mallotus philippinensis, ulei aromat şi sulfură), AV/EPP/14 (extracte vegetale din *Acorus calanus*, *Azadirachta indica*, *Pongamia glabra*, *Cedrus deodora*, *Eucalyptus globulus*), Gliricidia (decoct din *Gliricidia sepium*) [Samal, P. și col., 2017; Tarpataki, N. și col., 2004; Ranjan, R. și col., 2014]. [Arsenovic, M. și col., 2015; Singh, S. și col., 2011].

Pielea, care este o structură destul de fragilă la contactul cu substanţele terapeutice, atunci când este parazitată, are o reactivitate mult modificată faţă de situația în care este „sănătoasă”. Înțelegerea biologiei parazitului, cunoașterea condițiilor de microclimat cutanat pe care acarianul le preferă sunt factori influenți în abordarea bolii și în alegerea protocolului terapeutic potrivit. În acest context, am încercat să obținem un produs natural care să fie eficace contra demodecşilor prin intervenția în habitatul lor cutanat, prin acțiunea în verigile slabe ale acarianului, dar, în acelaşi timp, inofensiv pentru pacient.

Această direcție de cercetare s-a concretizat în obținerea unui gel pentru tratamentul leziunilor uscate din demodicoză, gel care, un an mai târziu, va intra în componența kit-ului Demosimcan - șampon și gel.

Obținerea *Gelului pentru tratamentul leziunilor uscate din demodicoza canină*

Investigațiile întreprinse în această direcție au presupus efectuarea de studii multicentrice, cu implicarea Universității de Medicină și Farmacie „Victor Babeș” Timișoara, a disciplinei de Parazitologie a FMV Timișoara, SC PRIMOSAL București, Institutul de Cercetare - INCDT, București.

S-au urmărit realizarea, formularea, caracterizarea și testarea pe animalele parazitate a unui gel constituit din extracte naturale, cu absorbție cutanată rapidă, eficient în remisia leziunilor demodecice și fără efect iritant asupra pielii parazitate.

Pentru obținerea gelului s-a realizat selectarea principiilor active din plante, a urmat încapsularea acestora în hidrolizatul de colagen și selectarea variantei optime în urma studiilor efectuate (studii epidemiologice, paraclinice, clinice, histologice, imunohistochimice).

Prin aplicarea tehnologiilor emergente s-au obținut extractele hidro-glicero-alcoolice din plante care s-au stabilizat prin procedee asistate de microunde și ultrasunete.

Extracția asistată cu microunde este un instrument specific pentru extragerea compușilor naturali din plante pentru obținerea unui randament maxim de extracție fără a afecta structura chimică a compușilor naturali valoroși, pentru obținerea de „compuși țintă” și a compușilor termolabili [Călinescu, I. și col., 2017; Singleton, V. L. și col., 1974]. Extractele obținute prin această procedură prezintă un conținut ridicat de polifenoli cu o capacitate antioxidantă mai mare decât cele obținute prin metode convenționale efectuate la același profil de temperatură și în aceeași celulă de extracție [Teo, C. C. și col., 2008; Arimboor, R. și col., 2009; Brand-Williams, W. și col., 1995].

Colagenul, proteina de bază din organism, se găsește în derma pielii în proporție de 90%. Extras în diferite forme (hidrolizat, gelatină, gel) și procesat sub formă de biomateriale

(hidrogeluri, membrane, matrici, pulberi, tuburi), colagenul este recunoscut de organism ca și constituent propriu, nu ca material străin. De aceea, integrarea principiilor active din plante în hidrolizate colagenice (extracte proteice de origine animală) influențează în mod semnificativ calitatea produselor obținute, deoarece se fixează doar acei constituenți eficienți din punct de vedere terapeutic, evitându-se, astfel, reacțiile adverse asupra pielii câinilor. Spre deosebire de calea parenterală, calea cutanată asigură o bună complianță datorită uşurinţei la administrare, riscului minim de traumă, infecție sau altă traumă a țesuturilor. Dintre formele farmaceutice semisolide, se acordă un interes deosebit folosirii hidrogelurilor, ca suporturi pentru preparatele destinate aplicării cutanate, deoarece conținutul mare în apă reduce iritabilitatea; de asemenea, hidrogelurile prezintă o bună complianță pentru animale, caracteristici de eliberare a principiilor active încorporate și proprietăți reologice adecvate, o toleranță cutanată crescută, compatibilitate cu majoritatea excipienților, uşurinţă la aplicare şi respectiv îndepărtare de pe piele [Albu Mădălina și col., 2011].

Obținerea extractelor vegetale prin noi tehnici moderne de microunde și ultrasunete și încapsularea lor în colagen a avut ca rezultat un **complex biologic activ de noutate absolută pe plan național.** Complexul biologic este componenta de bază a *kit-ului Demosimcan.*

Colaborarea dintre colectivele de cercetare de la Universitatea de Medicină și Farmacie „Victor Babeș" Timișoara, USAMVB Timișoara, INCDT București – Departamentul Colagen și SC PRIMOSAL București s-a concretizat în publicarea unei lucrări ISI, în calitate de coautor, scopul acestor lucrări fiind acela de a evidenția rolul și implicarea colagenului, proteină de bază în organism, acceptat de acesta ca pe un component propriu, nu ca pe un „non-self", în vindecarea rănilor și în regenerarea osoasă [Mederle Narcisa și col., 2016].

Hindawi

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Research Article

Innovative Biomaterials Based on Collagen-Hydroxyapatite and Doxycycline for Bone Regeneration

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Abstract

Bone regeneration is a serious challenge in orthopedic applications because of bone infections increase, tumor developing, and bone loss due to trauma. In this context, the aim of our study was to develop innovative biomaterials based on collagen and hydroxyapatite (25, 50, and 75%) which mimic bone composition and prevent or treat infections due to doxycycline content. The biomaterials were obtained by freeze-drying in spongious forms and were characterized by water uptake capacity and microscopy. The *in vitro* release of doxycycline was also determined and established by non-Fickian drug transport mechanism. Among the studied biomaterials, the most suitable one to easily deliver the drug and mimic bone structure, having compact structure and lower capacity to uptake water, was the one with 75% hydroxyapatite and being cross-

Gelul pentru tratamentul leziunilor uscate din demodicoza canină prezintă următoarele avantaje: se realizează un tratament local (topic) sub o formă de administrare facilă; absorbţia produsului in piele este rapidă; se evită administrarea sistemică ce presupune riscul efectelor secundare; are în componenţă doar principii naturale (miere, propolis, oțet de mere şi extracte vegetale hidro-glicero-alcoolice) cu proprietăți hiposeboreică, stimulatoare a creșterii firului de păr, antialergică, detoxificantă a pielii, antiinflamatoare, antifungică, antipruritică, imunostimulatoare; nu are efect iritant asupra pielii parazitate.

Rezultatul cercetărilor este înregistrat la OSIM ca cerere de brevet – A 00075 din 1.02.2016 (Patent no 131619-A0).

II.2. CONTRIBUȚII LA STUDIILE ETIOLOGICE, CLINICE, MICROSCOPICE ȘI TERAPEUTICE ÎN MICROSPORIDIOZĂ (NOSEMOZĂ)

Studiile epidemiologice, clinice, terapeutice și de biologie moleculară în nosemoza albinelor susțin o altă direcție de cercetare postdoctorală.

1) Mederle Narcisa, Dărăbuș Gh., Morariu S., **Mederle O.,** Herman V., Oprescu I., Ilie M., Imre Mirela, Motoc Marilena, Chiș Codruța, **2018**, Therapeutic efficacy testing of plant dietary supplement used for prevention and control of nosemosis in bees, Conference of Life Sciences, USABMV Timișoara, Epub Ahead of Print.

2) Mederle Narcisa, Balint, A., Morariu, S., Hora, F.Ş., **Mederle, O**., Marincu, I., Dărăbuș, Gh., **2015**, Research on the prevalence of honey bees nosemosis in Arad County, J. of Biotechnology, 208, S5-S 120.

3) Mederle Narcisa, Maria Luisa Lobo, Sorin Morariu, Florica Morariu, Gheorghe Dărăbuș, **Ovidiu Mederle**, Olga Matos - Microscopic and Molecular Detection of Nosema ceranae in Honeybee Apis mellifera L. from Romania Status on pathogen worldwide distribution, REV.CHIM.(Bucharest), 69, No.12, 2018, ISSN: 0034-7752, IF=1,412

Importanța și implicarea albinelor în ecosistemele naturale și, implicit, în sănătatea omului, sunt cunoscute din vechi timpuri, în întreaga lume. Parazitozele albinelor și răspândirea acestora în toată lumea reprezintă una din cele mai serioase probleme care afectează apicultura modernă din zilele noastre. Lupta împotriva bolilor parazitare ale albinelor necesită folosirea unor tehnici de diagnostic și aplicarea unor tratamente, care trebuie să fie în concordanță cu informațiile despre biologia parazitului și a gazdei [Mederle Narcisa şi col., 2017].

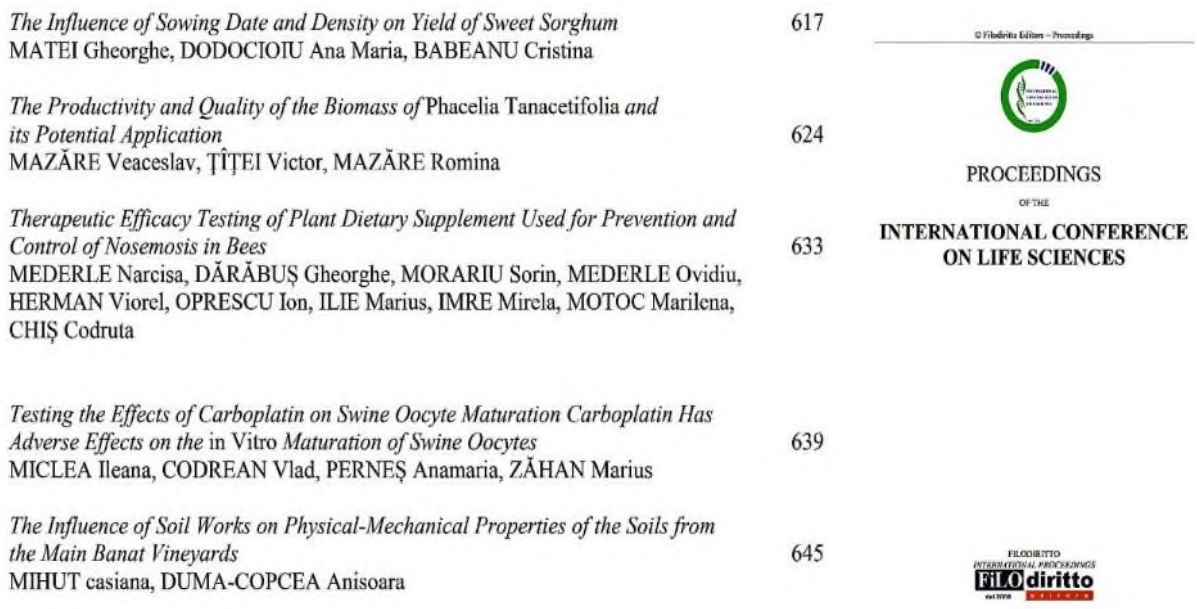
Una dintre bolile parazitare importante ale albinelor este nosemoza, o parazitoză produsă de *Nosema apis* și *Nosema ceranae,* care afectează albinele lucrătoare; determină diaree și mortalitate, aspecte ce conduc la apariția unui declin impresionant și progresiv în familiile afectate, remarcabile fiind atât scăderea numărului de albine, cât și diminuarea severă a producțiilor. În țara noastră, creșterea prevalenței nosemozei, identificarea parazitului și la albinele asimptomatice, implicarea speciei *N. ceranae* în episoadele diareice sau de depopulare a coloniilor impun o atenție deosebită a cercetătorilor în prevenirea și controlul acestei parazitoze, cu sublinierea unui aspect important: atenție la reziduurile din produsele apicole,

cu repercusiuni în sănătatea omului [Mederle Narcisa și col., 2017; Mederle Narcisa și col., 2015; Mederle Narcisa și col., 2018].

Dintre compușii de sinteză testați în prevenirea și controlul nosemozei, fumagilina are rezultate terapeutice încurajatoare, dezavantajul major al acestui produs fiind remanența în produsele apicole, dar și neafectarea sporilor parazitului, care se depozitează în fecale constituind surse de infecție pentru albine, în primăvară [Botias, C. și col., 2013; Mendoza, Y. și col., 2017; Nanetti, A. și col., 2015; Roussel, M. și col., 2015].

În acord cu cerințele UE, tendința actuală este obținerea de produse apicole „curate" (miere, lăptișor de matcă etc). Renunțarea la terapiile alopate ce lasă reziduuri în aceste produse și găsirea unor remedii naturale, care să fie eficiente, dar care, în aceeași măsură, să nu dăuneze stupului este eminentă și obligatorie [Bravo, J. și col., 2017; Maggi, M. și col., 2010]. În acest context, s-a născut ideea realizării unui produs natural care să nu dăuneze stupului și să nu prezinte reziduuri în produsele apicole, atât de apreciate și folosite pentru hrana și sănătatea omului: *Supliment alimentar din plante destinat prevenirii și combaterii nosemozei albinelor* - cerere de brevet de invenție înregistrat la OSIM cu nr. A 00075/14.02.2014 (Patent No 130489).

Produsul se încadrează în deziderateleeuropene, dovedindu-se eficient în reducerea infestației cu *Nosema spp.*, fără a avea efecte secundare și fără reziduuri în produsele stupului; produsul se bazează pe utilizarea principiilor activi din diferite plante medicinale [Mederle Narcisa și col., 2018].



Infecția cu *N. apis* rareori duce la moartea coloniei de albine, evoluând subclinic, de cele mai multe ori; *N. ceranae* este foarte patogenă pentru *A. mellifera*, fiind asociată cu sindromul depopulării graduale, cu mortalitățile din toamnă sau din iarnă și cu producții slabe de miere.

Combinația de microscopie convențională și secvențierea PCR/ADN a demonstrat că este o metodă eficientă pentru diagnosticarea microsporidiei *Nosema ceranae* și pentru evaluarea prevalenței acestui patogen la albinele melifere din trei județe din sud-vestul României. Prezența exclusivă a *N. ceranae* în toate stupinele monitorizate sugerează dominația acestei specii și capacitatea sa de a o înlocui pe *N. apis*, de-a lungul timpului, în această regiune a țării.

Prezentul studiu intenționează să contribuie la evidențierea importanței implementării măsurilor de prevenire, tratament și control al nosemozei albinelor în România [Mederle Narcisa și col., 2018].

ELSEVIER

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Research on the prevalence of honey bees' nosemosis in Arad County

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Microscopic and Molecular Detection of *Nosema ceranae* in Honeybee *Apis mellifera* L. from Romania

Status on pathogen worldwide distribution

NARCISA MEDERLE[1], MARIA LUISA LOBO[2], SORIN MORARIU[3], FLORICA MORARIU[3], GHEORGHE DARABUS[1], OVIDIU MEDERLE[4]*, OLGA MATOS[2]

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Until now, in Romania, a country in southeastern Europe located on the Balkans Peninsula, information regarding the status on A. mellifera honey bee infection by Nosema spp. has not been published. The aims of the present study were to survey the occurrence and identification of Nosema spp. in A. mellifera honeybee colonies from 37 apiaries located in Arad, Caras-Severin and Timis Counties, Romania. Also, based on published literature an update on the distribution of N. ceranae infection among A. mellifera colonies worldwide was performed. Overall, a frequency of 55.1% (389/690) of Nosema infection was observed in the analyzed regions (ranging from 52% to 66%), by light microscopy. By PCR and DNA sequencing, N. ceranae was the only microsporidia identified. Nosema ceranae is clearly a novel, emergent pathogen of A.mellifera with potentially very serious effects on the individual and honeybee's colonies in Romania. Data obtained provide new and important information on N. ceranae geographic prevalence and distribution, and on its impact at colony level and/or its role in colony losses. The present study intends to contribute to highlight the importance of implementing prevention, treatment and control measures of honeybee nosemosis, in Romania.

Keywords: Apis mellifera honeybee; Microsporidia; Nosemosis; Nosema ceranae; Molecular characterization

For centuries, the importance of honeybees to honey and wax production and to the pollination of most crops is well recognized. Honey remains an important international good with global production estimated at 1,07 million metric ton in 2007 (FAO, 2009), and almost fifty percent of leading global food commodities depend on honey bee pollination for either fruit or seed set (Klein *et al.*, 2007). This insect is the most efficient pollinator for most crop monocultures around the world (McGregor et al,1976; Delaney *et al.*, 2009). The western honeybee, *Apis mellifera*L., one of the most economically important species of the genus *Apis*, has been transported worldwide for beekeeping purposes from its native range in Europe, Africa and the Near East (Ruttner, 1988).

During the last decade multiple factors like pathogens, pesticides, and abiotic stressors have been identified associated with unusually high and inexplicable honey bee colony losses (Genersch *et al.*, 2010; Ratnieks and Carreck, 2010; Cornman *et al.*, 2012; Pettis *et al.*, 2013). Among the pathogens characterized and discussed in this context are two microsporidian species from genus *Nosema*, (Cox-Foster *et al.*, 2007; Higes *et al.*, 2008; Genersch, 2010) which infect adult honeybees (Bailey L, 1955), *Nosema apis* and *Nosema ceranae*. Both species are intracellular pathogens that are thought to represent very primitive, but highly specialized spore-forming fungi. Initially, nosemosis in *A.mellifera* was thought to be caused by a single species, *Nosema apis* Zander. However, in 1994, a microsporidium like *N. apis*, called *N. ceranae* was described in Eastern honey bee (*Apis cerana* Fabricius) from China (Fries *et al.*, 1996). Transmission of *Nosema* in honeybee colonies is mainly via the fecal-oral route. The infection process starts with the ingestion of infective spores by adult honeybees when they are eating contaminated food or when they are cleaning up fecal material from infected bees. The spores germinate in the insect midgut by extruding the polar tube and releasing their sporoplasm into midgut epithelial cells where they generate more spores and leave the body of infected host by defecation. Once in the gut, they invade the ventricular cells causing disease, but the clinical and epidemiological characteristics of the parasitization by either species are different; the infection by *N.apis* (type A nosemosis) does not usually cause the death of the colonies and is characterized by dysentery, general weakening of the adults, locomotion impairment and crawling (OIE, 2014). These symptoms are not present in *N. ceranae* infections (type C nosemosis) (Higes *et al.*, 2010), which produce alterations in the temporal polyethism, foraging activity and life span of infected bees (Goblirsch *et al.*, 2013; Dussaubat *et al.*, 2013; Alaux *et al.*, 2014). A large-scale depopulation phenomenon, named colony collapse disorder (CCD), has been reported in the United States of America (USA) (Chen *et al.*, 2008) and Europe (Topolska, Gajda & Hartwig, 2008). *Nosema ceranae* was suspected to be one of the contributors to this illness, particularly winter colony loses (Klee *et al.*, 2007). However, although some studies implicated

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II.3. CONTRIBUȚII LA STUDIILE ETIOLOGICE, CLINICE, MICROSCOPICE ȘI TERAPEUTICE ÎN ZOONOZELE PARAZITARE

Importanța socială a protozoarelor (*Criptosporidium spp., Giardia intestinalis, Toxoplasma gondi*), a dermatofiților (*Tricophyton spp*.) și a unor acarieni (*Cheyletiella spp*.) rezidă din caracterul zoonotic al acestor endo și ectoparaziți, expresiile clinice și lezionale la om având un mare impact în viața acestora.

Contribuții la stabilirea diagnosticului și instituirea unui protocol terapeutic integrat planului de control parazitologic au fost remarcate pe o perioadă de zece ani și s-au materializat în diferite publicații științifice indexate ISI și BDI.

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Sci Parasitol 16(1-2):63-66, June 2015
ISSN 1582-1366

SHORT RESEARCH NOTE

Study on cryptosporidiosis prevalence in children from Timiş County – Recent Updates

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Abstract. Cryptosporidiosis as a parasitic disease found all over the world is a major health issue. According to some researchers, *Cryptosporidium* sp. is among the first three or four enteric pathogenes in humans. The most serious problems occur in paedriatics, mainly in the countries where hygienic conditions are poor. The aim of the study was to collect information related to epidemiology from patients in hospitals, clinics and foster institutions for children and to examine faeces samples using ELISA method. Following the examination of 212 coprological samples taken from children with diarrhea symptoms, tuberculosis, AIDS, dystrophy or infested with *Giardia* spp. the results show a cryoptosporidiosis prevalence of 7.54% (16/2120).

Keywords: Cryptosporidiosis; Children; Prevalence.

Received 24/02/2015. Accepted 17/03/2015.

Introduction

The importance of criptosporidiosis has enormously increased in the last decade due to the antropozoonotic and zoopaediatric characters, the negative impact on livestock productions and the interference or perturbation of human health (Caccio and Pozio, 2006).

The human infection with *Cryptosporidium* was described all over the world, across the six contents, both in developed and developing countries, in adults and children from urban and rural areas alike (Fayer et al., 2000).

The morphological, biological and genetic features which differentiate a species of *Cryptosporidium* from other species is a major problem in understanding the way of transmission of the infection with cryptosporidiosis for the veterinarians and epidemiologists who are directly involved in the parasitological control of this morbid entity (Cama et al., 2008; Dărăbuş and Imre, 2010).

Materials and methods

The study was conducted in the January – September 2014 period. 212 faecal samples were taken from children in hospitals, clinics and foster institutions. The patiens were aged like this:

Scientific works, C series LVI(1), 2010
ISSN 1222-5304

MOLECULAR CHARACTERIZATION OF HUMAN *CRYPTOSPORIDIUM* ISOLATES IN BANAT REGION, ROMANIA

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Key words: *Cryptosporidium*, PCR-RFLP, human, Banat region.

SUMMARY

The aim of the present study was the genetic characterization of *Cryptosporidium* isolates, from humans with diarrhea attending to different hospitals located in Banat region. A total of 78 fecal samples were examined by modified Ziehl-Neelsen staining method. Five microscopically positive samples were investigated by PCR-RFLP of the SSU rRNA gene. The species and/or genotypes were determined using restriction endonuclease enzyme digestion with S*sp*I and V*sp*I. The results indicated the presence of *Cryptosporidium parvum* in three samples and *Cryptosporidium* cervine genotype in another two samples. These data suggest the animal origin of this zoonotic species and genotype.

This is the first study of molecular epidemiology in human cryptosporidiosis that has been made in Romania.

Members of the genus *Cryptosporidium* are parasites with large host specificity, infecting many vertebrate species (mammals, reptiles, birds and fishes), including humans.

Infections with *Cryptosporidium* species has been documented in both immunocompromised and immunocompetent patients, worldwide.

Epidemiological studies from humans, based on molecular genetics tools, have reported the identification of eight species (*C. parvum*, *C. hominis*, *C. meleagridis*, *C. felis*, *C. canis*, *C. muris*, *C. suis*, *C. andersoni*) and three genotypes (cervine genotype, chipmunk genotype, monkey genotipe) (Ong et *al*., 2002; Blackburn et *al*., 2006; Feltus et *al*., 2006; Leoni et *al*., 2006; Soba et *al*., 2006; Trotz-Williams et *al*., 2006; Xiao and Ryan, 2008; Imre, 2010; Dărăbuş and Imre, 2010). Usually, the first two enumerated species of *Cryptosporidium* are the most common species infecting humans.

Worldwide, the most frequently used gene, for *Cryptosporidium* species and genotype identification, has been the SSU-rRNA (18S) gene.

In Romania, the reports regarding the molecular analysis of *Cryptosporidium* isolated from humans are absent.

The primary objective of the present study was to determine the *Cryptosporidium* species/genotypes involved in human diarrhea cases and their epidemiological significance for humans in Banat region, Romania.

1. MATERIAL AND METHODS

Fecal samples from 78 patients (52 children's and 26 adults) with diarrhea were collected from different hospitals (Table 1), located in Banat region. The samples were stored in sterile plastic bottles at 4°C in 2.5% potassium dichromate until further processed.

All the stool samples were previously processed for the *Cryptosporidium* oocysts identification. The samples were microscopically examined with Ziehl-Neelsen modified (mZN) staining method. The microscopically positive samples were selected for PCR-RFLP analyses of the small subunit rRNA gene (18S) (Alves et al. 2001).

DNA was extracted from fecal samples using the QIAamp® DNA Stool Mini Kit (Qiagen GmbH, Hilden, Germany) according to the manufacturer's instructions.

The nested polymerase chain reaction protocol was performed in two steps (Xiao et al. 2001) and the reactions were prepared in 25 µl volumes. For the primary PCR reaction (about ~1325 bp), the PCR master mix was prepared by mixing: 10X *Taq* Buffer $(NH_4)_2SO_4$, dNTP (2.5 mM), $MgCl_2$ (25 mM), *Taq* DNA polymerase (5U/µl), genomic DNA (7-95 ng/µl), forward and reverse primers, and nuclease free water. After initial complete denaturation at 94°C for 3 min the reaction was performed for 35 cycles (denaturation at 94°C for 45", annealing at 55°C for 45" and strand extension at 72°C for 1 min) followed by final extension at 72°C for 7 min (Xiao and Ryan, 2008). The conditions for the secondary/nested PCR reaction (expected amplicon size ~ 840 bp) were the same as for the primary PCR, except the use of a different pair of primers and the annealing at 58°C (Xiao and Ryan, 2008). The specific primers used were SSU-F2 forward and SSU-R2 reverse for primary PCR and SSU-F3 forward and SSU-R4 reverse for secondary PCR (Xiao and Ryan, 2008, Xiao et *al*. 1999).

Amplification products were separated using horizontal gel electrophoresis on a 1.5% agarose multi-porpose gel containing ethidium bromide and using the 100-bp ladder as the control. Gel images were visualized under UV light and were captured using a gel documentation system.

Restriction Fragment Length Polymorphism Analyses (RFLP) of SSU rRNA (18S) gene PCR products was performed according to Xiao et al. (1999).

For detection and differentiation of *Cryptosporidium* species and genotypes 5 µl of the secondary nested PCR product was subjected to restriction digestion with V*sp*I and S*sp*I enzymes. The reaction mixture contained: amplicon DNA, nuclease free water, VspI/SspI enzyme (12U/µl) and their respective buffers. All restriction digestions were carried out 37°C for 2 h in a humid chamber.

RFLP products were analyzed on 1.5% agarose gel and visualized after ethidium bromide staining.

2. RESULTS AND DISCUSSIONS

Of the 78 human origin fecal samples, five (6,41%) were positive for *Cryptosporidium* oocysts. With modified Ziehl-Neelsen staining (mZN), the oocysts are clearly marked ovoid or round shaped, colored in red on a green ground.

Based on analysis of the SSU rRNA gene the nested PCR products were observed at 850 bp region. This specific amplification confirms the presence of *Cryptosporidium* spp. infection (fig. 1).

The RFLP analysis with S*sp*I revealed bands at 454 and 274 bp for three samples, and 465 and 395 bp for two samples (fig. 2).

The RFLP analysis with V*sp*I, for specie diagnosis, revealed bands at 624 and 111 bp for three samples which clearly indicated that the isolates were *C. parvum* (*C. hominis* would have given bands at 561 and 115 bp). For two samples, processed RFLP with V*sp*I enzyme, the revealed band was at 460 and 172 bp. This fragment sizes confirm the presence of *Cryptosporidium* cervine genotype (fig. 3).

More human cases have been associated with the *Cryptosporidium* cervine genotype, which has been reported in one patient in Slovenia, three patients in the United States, ten patients in Canada, and one in England (Ong et *al*., 2002; Blackburn et *al*., 2006; Feltus et *al*., 2006; Leoni et *al*., 2006; Soba et *al*., 2006; Trotz-Williams et *al*., 2006; Xiao and Ryan, 2008).

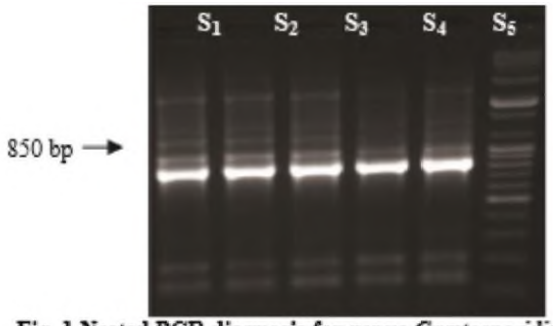

Fig. 1 Nested PCR diagnosis for genus *Cryptosporidium* (850 bp); S_{1-5}- processed samples; M –molecular marker

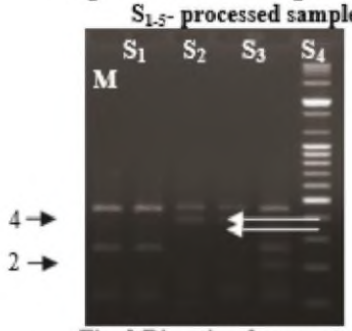

Fig. 2 Digestion fragments from SSU-rRNA (18S) gene with S*sp*I enzime; M - molecular marker; $S_{1, 2, 5}$ – characteristic migration bands for *C. parvum* $S_{3, 4}$ - characteristic migration bands for *C.* cervine genotype

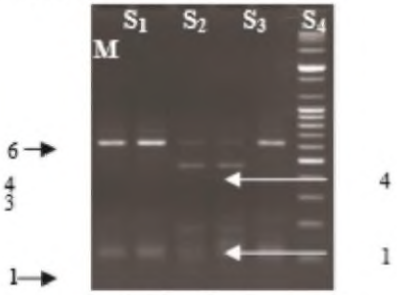

Fig. 3 Digestion fragments from SSU-rRNA (18S) gene with V*sp*I enzyme; $S_{1, 2, 5}$ – characteristic migration bands for *C. parvum*; $S_{3, 4}$ - characteristic migration bands for *C.* cervine genotype

Table 1

***Cryptosporidium* isolates and PCR-RFLP analysis at the SSU rRNA (18S) locus from humans in Banat region**

Sample origin from humans	Total no. of isolates studied	No. of isolates positive for:		Negatives
		Cryptosporidium parvum	*Cryptosporidium* cervine genotype	
"Louis Țurcanu" Children Hospital	19	1	2	16
"Victor Babeș" Hospital, from Timișoara	28	2	-	26
City Hospital from Reșița	25	-	-	25

Most species and genotypes of *Cryptosporidium* infect a limited range of animals, and when the host range or infectivity includes humans, the parasite acquires public health significance.

94

The predominance of *C. parvum* in humans may be due to high prevalence of bovine cryptosporidiosis in this zone (Imre, 2010; Dărăbuş and Imre, 2010).

C. parvum, the most spread specie, was reported in many countries worldwide (Xiao and Ryan, 2008; Xiao, 2010).

The increasing member of humans infected with the cervine genotype might be related to its wide range of mammalian hosts.

This is the first study of molecular epidemiology in human cryptosporidiosis that has been made in Romania.

3. CONCLUSIONS

3.1. Using molecular analysis of SSU-rRNA (18S) gene for *Cryptosporidium* oochist isolates from five human patients from Banat area, a parasitism with *Cryptosporidium parvum* was identified in three persons and *Cryptosporidium* cervine genotype was identified in two persons.

3.2. Parasitism with *C. parvum*, in humans, demonstrates the presence of a zoonotic specie, having as possible source of infection the ruminants.

3.3. The presence of *Cryptosporidium* cervine genotype was reported for the first time in Romania.

4. ACKNOWLEDGMENTS

The current research was based on grant 51-034/2007 PN II - Parteneriate) obtained by Prof. Dărăbuş from CNMP. Acknowledgments to Cristina Popescu, Molecular Genetics Laboratory CEEX II USAMVB Timişoara, for help in the interpretations of results.

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Immunofluorescence and immunohistochemistry methods in diagnose of human cryptosporidiosis

Mederle Geanina Narcisa [1], Mederle Alexandru Ovidiu [2] ✉, Darabus Gheorghe [1], Gartner Andreea [1], Ioanoviciu Sorin [3]

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Morphological and immunohistochemical features of *cryptosporidium parvum* in cattle

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"Banat" University of Agricultural Sciences and Veterinary Medicine, Timisoara, Romania**

INTRODUCTION

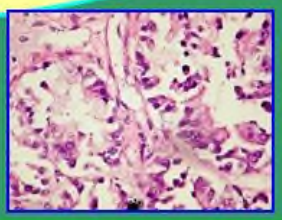
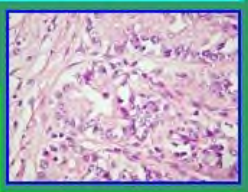
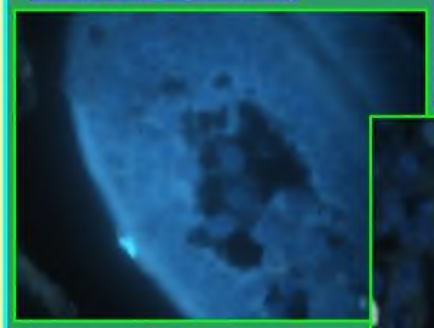
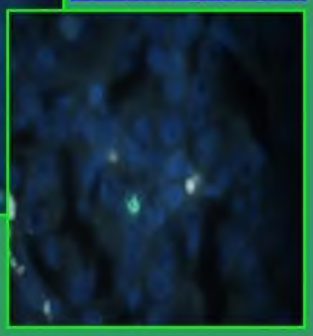
- *Cryptosporidium parvum* is primarily an intestinal parasite that infects both humans and young cattle
- Cryptosporidium parvum complete their life cycles in a single host, and their oocysts are highly infectious
- Oocysts range from 4.0µm to 5.0µm in diameter

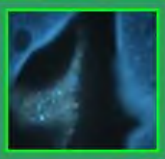
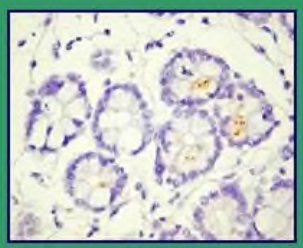
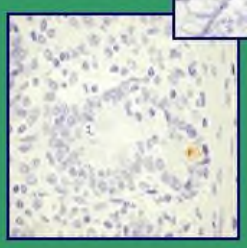
MATERIAL AND METHODS

- 11 cattle were investigated
- Specimens were fixed in bufferd formalin and paraffin embedded
- 5 µm thick sections were stained with haematoxylin-eosin method, and additional sections were stained with monoclonal antibodies against *cryptosporidium parvum* oocyst (Immunocruz Staining System). Nuclei were stained with Lillie's haematoxylin.

RESULTS

- Cryptosporidia were found in the brush borders of villous enterocytes throughout the entire small intestine except for the most proximal site
- Infection was most extensive in the ileum. The large intestine was not infected
- Changes included areas of stunted villi, particularly those overlying Peyer' s patch areas of the ileum
- The intestinal epithelium present focal area of necrosis. The lamina propria of the distal small intestine contained substantial infiltrates of neutrophils and a few macrophages, and some crypts contained dead leucocytes
- The caecal and colonic mucosa was infiltrated by a few neutrophils but was otherwise unaffected

CONCLUSION

- Our findings suggest that immunofluorescence and immunohistochemistry are recommended and useful methods to identify cryptosporidium parvum

Observations and immunohistochemical detection of spontaneous *cryptosporidium parvum* infection in lambs

Narcisa Mederle **, Ovidiu Mederle*, Raluca Ceausu* , Anca Maria Cimpean* , Marius Raica*, Gheorghe Darabus**
"Victor Babes" University of Medicine and Pharmacy, Department of Histology, Timisoara, Romania*
"Banat" University of Agricultural Sciences and Veterinary Medicine, Timisoara, Romania**

INTRODUCTION

- Criptosporidiosis, an infection of the intestinal mucosa by protozoan of enteric coccidian group- recognized in animals since the beginning of the 20-th century
- However, an association of *Cryptosporidium* - circumstantial until recently when its potential as an enteropathogen was recognized

AIM

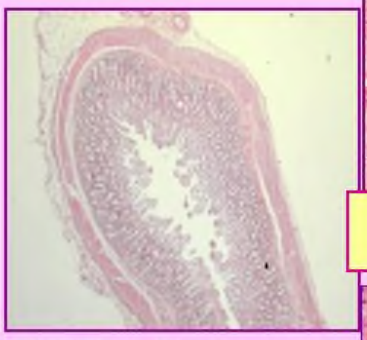
- The aim of this work was to describe by microscopic observations the different expression, distribution of cryptosporidium parvum oocyst and associate local efects in both hematoxilyne and immunohistochemical stains

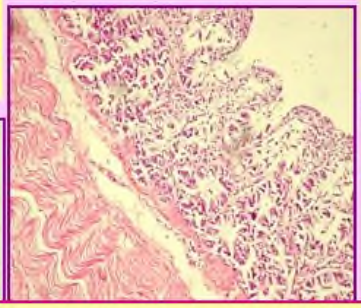
MATERIAL, METHODS

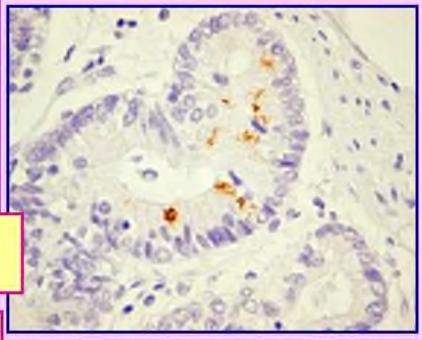
- Our study included 12 lambs biopsy
- Resected small intestine specimens were fixed in 10 % bufferred formalin for 48 hours and parrafin embeeded
- 5 µm thick sections were stained with haematoxylin-eosin method
- Immunohistochemical study included antibodies against cryptosporidium parvum oocyst (Santa Cruz Biotechnology,Inc.). We used ImmunoCruz as working system, DAB as chromogen, Lillie's hematoxyline for counterstain. Microscopic observation was performed using Nikon Eclipse E600. The images were captured and processed with Lucia G software system

RESULTS

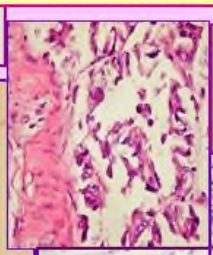
- *Cryptosporidium parvum* invaded the microvillous surface of enterocytes primarily in the distal small intestine
- Following the development of the parasitophorous vacuole just under enterocyte membrane, *cryptosporidium* infections result in atrophy and fusion of affected intestinal microvilli
- There was a marked infiltrate of lymphocytes, plasma cells, macrophages, and polymorphonuclear leukocytes
- The intestinal epithelium was focally eroded and contained a moderate number of necrotic cells



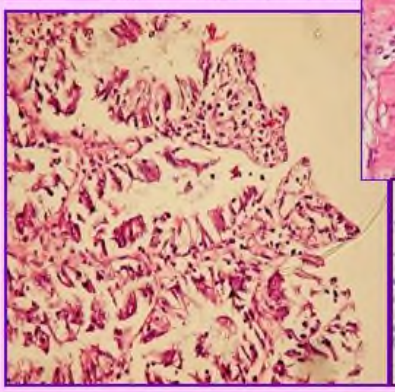




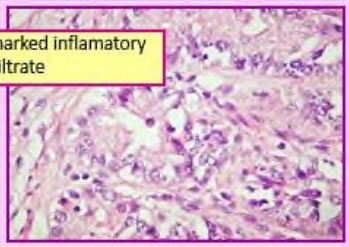
Cryptosporidium parvum invaded the microvillus surface of enterocytes

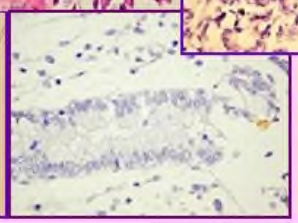


Focally eroded and presence of necrotic areas of intestinal epithelium



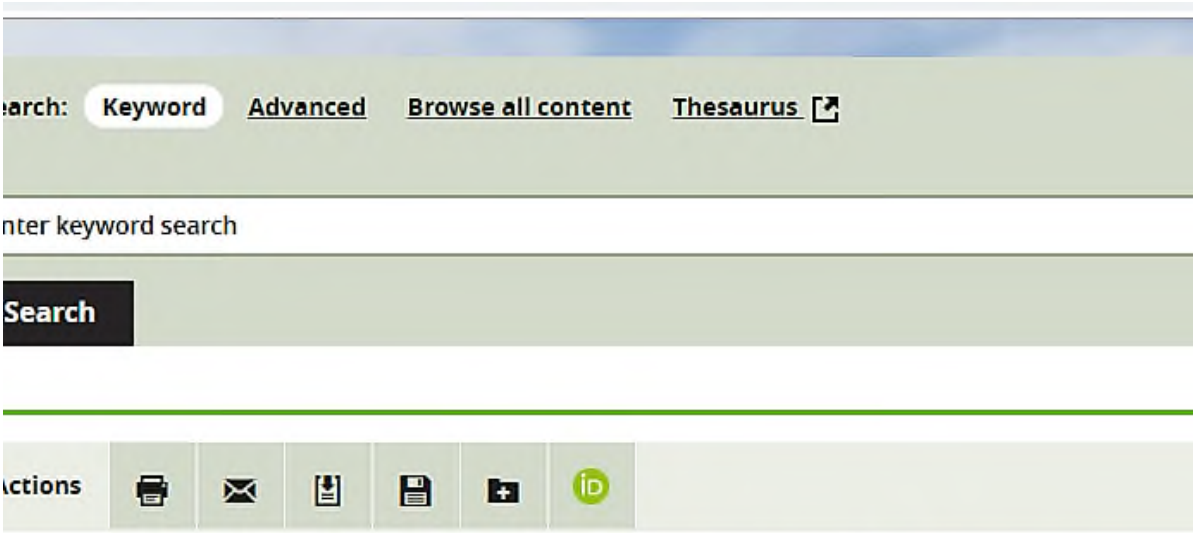
A marked inflamatory infiltrate





CONCLUSION

- Our findings suggest that the immunohistochemical methods is a reliable tool to identify cryptosporidium parvum infection in intestinal mucosa of lambs.



The study of some biochemical parameters in cryptosporidium experimental infection in broiler chickens.

Author(s) : Dărăbuș, G. ; Mederle, O. ; Mederle, N. ; Olariu, T. R. ; Oprescu, I. ; Morariu, S. ; Imre, K. ; Ilie, M. ; Hotea, I.
Author Affiliation : Facultatea de Medicina Veterinara Timisoara, Timisoara, Romania.
Journal article : Lucrari Stiintifice - Universitatea de Stiinte Agricole a Banatului Timisoara, Medicina Veterinara 2008 Vol.41 pp.344-347 ref.8

Abstract : The study of some biochemical parameters in experimental infection with *Cryptosporidia* was performed on three groups of 20 3-days broiler chicken each. The first group was infected with 2×10^5 oocysts of *C. meleagridis*, the second group with 2×10^5 oocysts of *C. parvum* and the third group was the control. At 6 days after the infection, 24 hours faeces were chemically analyzed. At 9 days after the infection some biochemical parameters in the blood of the chicken were assessed. Cryptosporidial infection in chicken reduces the digestion of proteins, cellulose and lipids. The growth of ALAT enzyme and the decrease of the cholesterol and triglycerides may signal a liver malfunction. The decrease of creatinin and uric acid may be due to the decreased absorption of non-protein nitrogen.

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Research note OPEN ACCESS

Molecular characterisation of *Cryptosporidium* (Apicomplexa) in children and cattle in Romania

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Abstract: To investigate the transmission of species of *Cryptosporidium* Tyzzer, 1907 in Timis County, Romania, 48 isolates of *Cryptosporidium* coccidia from 11 children, 29 calves and eight pigs were characterised by molecular analysis of two loci (SSU rRNA and 60-kDa glycoprotein gene). Overall, 22 isolates were amplified and sequence analyses revealed that all isolates were *Cryptosporidium parvum* Tyzzer, 1912. Two subtype families were identified, IIa and IId. Subtype IIdA22G1 (n = 4) was the single *C. parvum* subtype found in children. Subtypes found in calves included IIdA27G1 (n = 8), a novel subtype, IIdA25G1 (n = 5), IIdA22G1 (n = 2), IIdA21G1a (n = 1), and IIaA16G1R1 (n = 1). Subtype IIdA26G1 was found in a pig. These results were significantly different from previous Romanian reports, as the five subtypes of family IId identified in this study were never identified previously in this country. Thus, cattle may be a source of *Cryptosporidium* infections for humans and the transmission dynamics of *C. parvum* in Romania is more complex than previously believed.

Keywords: *Cryptosporidium parvum*, man, calves, molecular epidemiology, GP60 variability, eastern Europe

Protists of the genus *Cryptosporidium* Tyzzer, 1907 are important etiological agents of gastrointestinal disease of a wide range of vertebrate animals, including humans (Xiao et al. 2004, Fayer 2010). *Cryptosporidium* infection may be acquired by direct contact with infected persons or animals, and indirectly by ingestion of contaminated water and food (Fayer 2010, Xiao 2010). *Cryptosporidium* includes over 20 species (Xiao et al. 2004, Fayer 2010), with *Cryptosporidium hominis* Morgan-Ryan, Fall, Ward, Hijjawi, Sulaiman et al., 2002, which has anthroponotic transmission and *Cryptosporidium parvum* Tyzzer, 1912 with zoonotic or anthroponotic transmission as the two most common species infecting humans (Xiao 2010). *Cryptosporidium parvum*, *Cryptosporidium bovis* Fayer, Santín et Xiao, 2005, *Cryptosporidium ryanae* Fayer, Santín et Trout, 2008 and *Cryptosporidium andersoni* Lindsay, Upton, Owens, Morgan, Mead et al., 2000 were described from cattle, with an age-related distribution (Santín et al. 2004, Fayer et al. 2007). *Cryptosporidium parvum* is the major zoonotic species and is the dominant one in pre-weaned calves until two months of age (Santín et al. 2004). Natural infections in pigs by *Cryptosporidium* spp. have also been reported and are commonly attributable to two species (Xiao 2010), *Cryptosporidium suis* Ryan, Monis, Enemark, Sulaiman, Samarasinghe et al., 2004 and *Cryptosporidium scrofarum* Kváč, Kestránová, Pinková, Květoňová, Kalinová et al., 2013 (Fayer 2010, Kváč et al. 2013).

The present study was undertaken to genetically characterise *Cryptosporidium* spp. from humans and animals (Timis County, Romania), map the diversity of *Cryptosporidium* species in both populations and identify potential source(s) of human infection.

In this study we used 48 faecal samples from 48 individuals diagnosed as positive for *Cryptosporidium* spp. by a modified Ziehl-Neelsen staining. All faecal samples were collected in Timis County, Romania (2008), including 11 specimens from children under 12 years of age with diarrhea, 29 specimens from calves up to two months old and eight from nine-week-old pigs.

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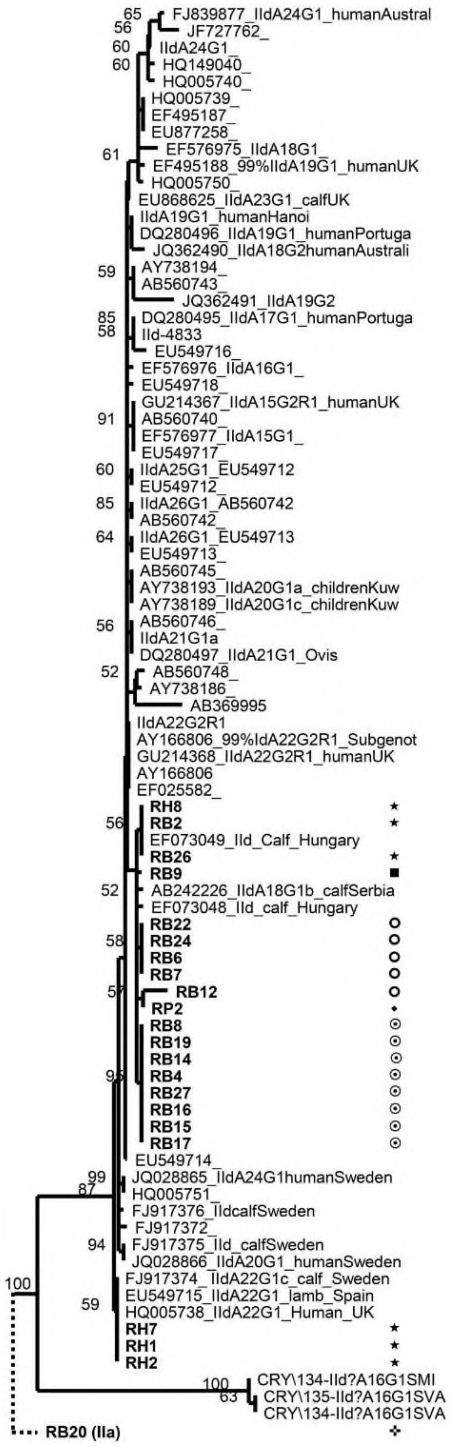

Fig. 1. Phylogenetic relationship between *Cryptosporidium parvum* subtypes characterised in this study and other sequences in GenBank, inferred by a neighbour-joining analysis of GP60 sequences, based on genetic distance calculated by Kimura two-parameter model. Numbers above branches indicate the confidence values obtained by bootstrap analysis with 1000 replicates. *Abbreviations*: RB – bovine faecal sample; RH – human faecal sample; RP – pig faecal sample; ★ – IIdA22G1; ■ – IIdA21G1a; ○ – IIdA25G1; ⬩ – IIdA26G1, ⊙ – IIdA27G1, ✣ – IIaA16G1R1.

Stool samples from the animals were collected directly from the rectum. Samples were stored in 2.5% potassium dichromate at 4 °C. The oocysts from samples were concentrated by a modified water-ether sedimentation method. The DNA was extracted by a MiniBeadBeater/silica method (Alves et al. 2001, 2003).

The identification of *Cryptosporidium* species and genotypes was performed by nested-PCR analysis of an 830 bp fragment of the SSU rRNA gene (Xiao and Ryan 2008). Subtyping analysis was done by nested-PCR analysis of a 400 bp fragment of the GP60 gene (Alves et al. 2003). The secondary PCR products were separated on 1.5% agarose gel and visualised by ethidium bromide staining under UV light.

All secondary SSU rRNA and GP60 products were purified with Jetquick Gel Extraction Spin Kit® (Genomed, Löhne, Germany) and sequenced in both directions on an ABI 3730XL automated sequencer (Applied Biosystems, Foster City, CA, USA). The sequences obtained in this study were aligned with reference sequences of *Cryptosporidium* spp. from the GenBank database using the MultAlin (http://multalin.toulouse.inra.fr/multalin/) software for species/genotypes and subtype identification. The *C. parvum* subtypes were named according to the number of TCA and TCG repeats in the trinucleotide repeat region and the mutations in the nonrepeating regions (Xiao 2010). To evaluate phylogenetic relationship among various *C. parvum* subtypes characterised in this and other studies, a neighbour-joining tree was constructed from the aligned sequences using the Treecom W program, based on genetic distance calculated by Kimura two-parameter model. The reability of branches was assessed by bootstrap analysis with 1000 replicates. The partial GP60 sequences generated in this study were deposited in the GenBank database (Acc. Nos. KF500407 and KC469687–KC469694).

Amplification of *Cryptosporidium* DNA was successfully achieved for 14 (two specimens from children and 12 from calves) of the 48 specimens diagnosed as positive for *Cryptosporidium* spp. by microscopy. The subsequent sequencing and alignment of all our nucleotide sequences with others of known identity deposited in GenBank database showed that all sequenced isolates belonged to *C. parvum*. Of the 14 isolates, six had 100% homology with the *C. parvum* nucleotide sequence DQ656355, six had 100% similarity with the *C. parvum* nucleotide sequence AB513881 and two had 100% homology with the *C. parvum* nucleotide sequence JN247404.

PCR products of the GP60 gene were obtained for 22 (four specimens from children, 17 from calves and one from a pig) of the 48 *C. parvum* isolates. The alignment of the sequences with reference sequences from GenBank confirmed that *C. parvum* was the only species found in the samples. The GP60 sequences of the 22 isolates confirmed six subtypes belonging to two zoonotic subtype families, IIa and IId. Four of the IId subtypes, IIdA21G1a, IIdA22G1, IIdA25G1 and IIdA26G1, are known subtypes, with GenBank Acc. Nos. AB560746, EU549715, EU549712 and EU549713. A fifth subtype, IIdA27G1, was a novel subtype identified in this study. The IIa subtype characterised in this study (IIaA16G1R1) had 100% homology to sequence AY149615 deposited in the GenBank database.

The remaining sequences (subtypes of subtype family IId) had only 99% homology to the sequences in GenBank with at least one polymorphism in the non-repeating region. The sequences from RB4, RB8, RB14, RB15, RB16, RB17, RB19 and RB27,

Table 1. Distribution of *Cryptosporidium parvum* GP60 subtypes in humans and animals in Timis County, Romania.

Subtypes	Host		
	Man ($n = 4$)	Calf ($n = 17$)	Pig ($n = 1$)
IIaA16G1R1	0	1	0
IIdA21G1a	0	1	0
IIdA22G1	4	2	0
IIdA25G1	0	5	0
IIdA26G1	0	0	1
IIdA27G1	0	8	0

all identified as the new subtype IIdA27G1, exhibited intra subtype sequence polymorphism. According to the nomenclature proposed by Sulaiman et al. (2005) and Xiao (2010), we assigned these new subtypes as IIdA21G1b (RB9), IIdA22G1b (RH1, RH2, RH7), IIdA22G1c (RH8, RB2, RB26), IIdA25G1b (RB6, RB7), IIdA25G1c (RB22), IIdA26G1b (RP2), IIdA27G1a (RB4, RB14, RB16, RB19) and IIdA27G1b (RB8, RB15, RB27). Moreover, the multiple alignment of GP60 sequences obtained shows that although all sequences of subtype family IId are identical to each other in the non-repeating region, sequences of seven isolates stand out from others due to the existence of at least one polymorphism in the non-repeating region. Phylogenetic analysis of the sequences confirmed that *C. parvum* isolates belonged to two subtype families (IIa and IId) and showed the existence of two genetically distinct types of sequences in the subtype IIdA22G1 (Fig. 1). One of them was described in three human isolates (RH1, RH2 and RH7), whereas the second type of sequence was detected in one human (RH8) and two bovine isolates (RB26 and RB2).

Subtype family IId was prevalent and widely distributed in the present study. Of the 22 isolates, 96% (21/22) belonged to this subtype family, being detected in humans (4 isolates), calves (16) and pigs (1). In contrast, subtype family IIa was less prevalent: of the 22 isolates, only one was detected in a calf. As shown in Table 1, IIdA22G1 was the only subtype detected in humans (4 isolates). In contrast, multiple subtypes were seen in calves: IIdA27G1 was the predominant (8 isolates from a total of 17 samples), followed by subtypes IIdA25G1 (5 isolates) and IIdA22G1 (2), whereas subtypes IIaA16G1R1 and IIdA21G1a were rare (only 1 isolate). The only specimen characterised in pigs had the subtype IIdA26G1.

Genetic characterisation of *Cryptosporidium* isolates from Timis County, Romania, revealed that *C. parvum* was the only species present in children. Our results are in agreement with several studies conducted in European countries (France, Portugal, Slovenia, Switzerland, UK), which have shown a common occurrence of *C. parvum* in humans (McLauchlin et al. 2000, Morgan et al. 2000; Alves et al. 2001, 2003, Guyot et al. 2001, Matos et al. 2004, Soba and Logar 2008). In contrast, *C. hominis* is more prevalent in other regions of the world, especially in developing countries, Australia and USA (Xiao 2010).

In calves, all isolates were characterised as *C. parvum*, as in previous studies conducted in the same region (Imre et al. 2011). Our results correlate with those on genotyping of *Cryptosporidium* spp. in calves of the same age, in which *C. parvum* was considered responsible for most infections (Xiao 2010). The high prevalence of *C. parvum* detected in this study is consistent with the age of the calves, which may serve as major reservoirs of the most important zoonotic species, *C. parvum*.

In pigs, a single isolate was identified as *C. parvum*, whereas the species most often detected in pigs are *C. suis* and *C. scrofarum* (see Kváč et al. 2013). *Cryptosporidium parvum* has been only detected in a few studies (Kváč et al. 2009, Xiao 2010, Garcia-Presedo et al. 2013, Němejc et al. 2013).

The present study reveals the existence of some genetic diversity in *C. parvum* in Romania. Overall, isolates of *C. parvum* were from two subtype families, IIa and IId, and six subtypes, namely IIaA16G1R1, IIdA21G1a, IIdA22G1, IIdA25G1, IIdA26G1 and IIdA27G1. All subtypes were only seen in calves, except subtype IIdA22G1 in both calves and humans, and subtype IIdA26G1, in a pig. Interestingly, the subtype family IIa was only seen in a single isolate of *C. parvum*, being identified as the subtype IIaA16G1R1. This subtype, which is an important etiological agent for bovine cryptosporidiosis (Xiao 2010), is predominant in countries neighbouring Romania (71% in Hungary, but only 21 samples examined; 33% in Serbia and Montenegro, 18 samples; 13% in Slovenia, 6 samples) (Plutzer and Karanis 2007, Misic and Abe 2007, Soba and Logar 2008), suggesting that in these countries as well as in Romania, this subtype is an important etiological agent for bovine cryptosporidiosis (Plutzer and Karanis 2007, Soba and Logar 2008, Imre et al. 2011). Besides calves, this subtype has also been found in lambs, pigs and humans and thus can be considered as a zoonotic pathogen (Soba and Logar 2008, Kváč et al. 2009, Imre et al. 2013). In our study, the subtype IIaA15G2R1, described as the most widely distributed in calves worldwide (Xiao 2010), was not identified.

Amongst the *C. parvum* IId subtype families characterised, five different subtypes were detected for the first time in Romania. Interestingly, some of these subtypes, namely IIdA21G1a, IIdA25G1 and IIdA26G1, have been found only occasionally in small ruminants (Xiao 2010), except for subtype IIdA22G1, which has also occasionally been implicated in bovine cryptosporidiosis in some European countries (Belgium, Germany, Hungary, Sweden – Silverlås et al. 2010, 2012, Xiao 2010). Apart from calves, this subtype was also detected in children with gastrointestinal symptoms in the present study. Subtype IIdA22G1 was also previously seen in humans in Portugal and in the United Kingdom (Alves et al. 2006, Chalmers et al. 2011). Results of phylogenetic analysis of the GP60 gene revealed the existence of two types of sequence within this subtype. One of these types from three human isolates (RH1, RH2 and RH7) was related to, but not identical with, isolates from lambs in Spain (GenBank Acc. No EU549715) and bovines in Sweden (GenBank Acc. No. FJ917374). The fact that this type of sequence was found only in humans in the present study indicates the possibility of anthroponotic transmission. In contrast, the second type of sequences was seen in a human (RH8) and two bovine isolates (RB26 and RB2). It should be noted that subtypes IIdA21G1a and IIdA26G1 were also already found in human isolates (Alves et al. 2006, Nazemalhosseini-Mojarad et al. 2011). The subtype IIdA27G1 was the most frequent subtype in calves in this study, and represents a new subtype.

In summary, our epidemiological analysis suggests that in Timis County, Romania, there is a high prevalence of *C. parvum* subtype family IId characterised in this study, in contrast to

the high prevalence of the family IIa in previous studies also in this region (Imre et al. 2011). Human cryptosporidiosis appears to occur primarily through zoonotic transmission and cattle appear to be a major zoonotic reservoir. Results of this and previous studies, however, need to be confirmed by analyses of more samples. These studies should also be performed in other regions of Romania and should include better collection of epidemiological data, with the purpose of more conclusive identification of infection sources, reservoir hosts and transmission dynamics of *Cryptosporidium* spp. in humans in this country.

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ORIGINAL SCIENTIFIC WORKS | LUCRĂRI ORIGINALE

IDENTIFICATION OF POTENTIAL ZOONOTIC PARASITIC ELEMENTS IN PARKS AND PLAYGROUNDS FOR CHILDREN IN TIMIŞOARA

IDENTIFICAREA UNOR ELEMENTE PARAZITARE POTENŢIAL ZOONOTICE ÎN PARCURI ŞI LOCURI DE JOACĂ PENTRU COPII DIN TIMIŞOARA

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ABSTRACT | REZUMAT

The diseases caused by parasites with zoonotic potential (ex. *Toxocara canis, Echinococcus spp.*) is a real problem for physicians and veterinarians, also.

For humans, the infestation possibilities are multiple, the major route being by direct contact with infected dogs which remove eggs in faeces. However, the distribution of parasitic elements in nature is not limited to places to deposit faeces, such as: parks, children playgrounds, gardens, sand pits.

The aim of this study is to identify potentially zoonotic parasitic elements by coprologic methods in samples of soil /sand /faeces collected from parks and playgrounds for children in Timişoara and to assess the risk of human contamination.

The study was conducted over a period of one year, and the samples were examined by flotation method and by direct examination (staining with Lugol solution). Parasitological examinations revealed the presence of parasitic elements belonging to the genera *Toxocara, Ancylostoma, Trichocephalus* in 88% of parks (22/25) and 100% in playgrounds (16/16).

Coproparasitological examinations revealed the presence of parasitic elements in 31 samples (37.80 %). The parasitic elements identified in fecal samples were: cyst *Giardia spp., Eimeria /Isospora* oocysts, *Taenia spp.* oncospheres, *Toxocara canis* eggs, *Ancylostoma/ Uncinaria* eggs, *Tricocephalus vulpis* eggs.

The contamination of parks and playgrounds for children with potential zoonotic parasitic elements from dogs is a public health problem. The enforcing of regulations regarding the movement of stray dogs which are a permanent source of environmental contamination, the education of dog owners about deworming dogs and feces collection, the information of the population about the danger of infestation are important links in the parasitological control implementation whose aim is to reduce significantly the risk of human infestation.

Key words: parasites, potential zoonotic, parks, children

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Bolile produse de paraziţi cu potenţial zoonotic (ex. *Toxocara canis, Echinococcus sp.*) reprezintă o reală preocupare pentru medicul uman şi cel veterinar, deopotrivă. Pentru om, posibilităţile de infestare sunt multiple, contactul direct cu câinii infectaţi care elimină ouăle prin fecale, reprezentând calea majoră. Dar distribuţia elementelor parazitare în natură nu este limitată la locurile de depunere a fecalelor, ele fiind găsite în parcuri, în locurile de joacă ale copiilor, în grădini, în gropile cu nisip.

Scopul studiului a fost identificarea prin metode coprologice a elementelor parazitare cu potenţial zoonotic din probele de sol/nisip/fecale recoltate din parcuri şi locuri de joacă pentru copii din oraşul Timişoara şi aprecierea riscului contaminării umane.

Au fost recoltate un număr 287 de probe de sol/ nisip şi un număr de 82 probe coprologice din 25 de parcuri publice (www.primariatimisoara.ro) şi 16 locuri de joacă pentru copii din oraşul Timişoara. Studiul s-a desfăşurat pe o perioadă de un an, iar probele au fost examinate prin metoda flotaţiei şi prin examenul direct (colorare cu soluţia Lugol).

Examenele parazitologice au relevat prezenţa elementelor parazitare aparţinând genurilor Toxocara, Ancylostoma, Trichocephalus în 88% din parcuri (22/ 25) şi 100% din locurile de joacă (16/16). Examenele coproparazitologice au relevat prezenţa elementelor parazitare în 31 probe (37,80%). Elementele parazitare identificate în probele de fecale au fost: chisturi de *Giardia spp.*, oochisturi de *Eimeria/Isospora,* oncosfere de *Taenia spp.*, ouă de *Toxocara canis,* ouă de *Ancylostoma/Uncinaria,* ouă de *Tricocephalus vulpis.*

Contaminarea parcurilor şi a locurilor de joacă pentru copii cu elemente parazitare potenţial zoonotice provenite de la câini reprezintă o problemă de sănătate publică. Reglementarea circulaţiei câinilor fără stăpân, sursă permanentă de contaminare a mediului, educaţia proprietarilor cu privire la deparazitarea câinilor şi colectarea fecalelor, informarea populaţiei privind pericolul infestării sunt verigi importante ale implementării unui plan de control parazitologic al cărui scop este reducerea în mod semnificativ al riscului infestării umane.

Cuvinte cheie: paraziţi, potenţial zoonotic, parcuri, copii

II.4. CONTRIBUȚII LA STUDIILE ETIOLOGICE, CLINICE, MICROSCOPICE ȘI TERAPEUTICE ÎN INFECȚIILE BACTERIENE

Anca Sofiana Surpat (Hulea), Viorel Herman, Iosif Marincu, Narcisa Mederle, **Ovidiu Alexandru Mederle,** Immunohistochemical method for identification of Lawsonia intracellularis infection in pigs Journal of Biotechnology, Volume 208, Supplement, 20 August 2015, Pages S100.

Complexul bolilor enterice ale suinelor reprezintă un grup de procese patologice cauzate de o serie de specii bacteriene comensale sau patogene, care în urma depășirii barierei imunologice determină apariția stării de boală, provocând pierderi economice traduse prin indici productivi reduși, corelați cu costuri de producție ridicată și profitabilitate scăzută.

Implicațiile speciilor bacteriene din genul *Salmonella*, *Lawsonia* sau *Escherichia* în etiologia complexului enteric suin și rolul acestora în dezvoltarea proceselor morbide au fost urmărite într-un studiu histologic și imunohistochimic realizate pe fragmente de ileon și jejun cu adenomatoză. Preparatele histopatologice au permis evidențierea modificărilor arhitecturii mucoasei intestinului subțire, care au constat în proliferarea enterocitelor din tunica epitelială a mucoasei cu depleția celulelor caliciforme, zonele de proliferare alterând cu zone de descuamări epiteliale. În lamina propria a mucoasei intestinale abundă infiltratul leucocitar, care ajungea până în tunica musculară.

Tehnica imunohistochimică a permis identificarea antigenului bacterian (*Lawsonia intracellularis*) cu localizare în macrofagele din lamina propria în proporție de 21,96% (27 probe); în macrofagele din lamina propria și în enterocitele din stratul epitelial al mucoasei intestinale în proporție de 61,79% (76 probe); în enterocite, macrofagele din lamina propria, dar și libere în lumenul glandelor intestinale în proporție de 8,95% (11 probe). Antigenul bacterian a lipsit în cazul a nouă probe (7,31%).

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COMPARISON OF SOME DIFFERENT METHODS FOR IDENTIFICATION OF *LAWSONIA INTRACELLULARIS* INFECTION IN PIGS

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Abstract

To compare different histopathological methods for diagnosis of Lawsonia intracellularis infection in pigs were taken in study 25 samples of ileum with specific lesions of intestinal adenomatosis. In order to perform slides were used Kinyoun, Green-Methyl-Pironine, Masson-Fontana, Schmitz, Diff-Quick methods and immunohistochemistry. The results showed that Green-Methyl-Pironine method has no value for diagnosis of porcine proliferative enteropathy, while Kinyoun coloration is capable to identify the bacteria only in 28% of samples. The argentic impregnation and Diff-Quick are able to highlight the aetiological agent in 44%, respectively 40% of the studied samples, so this methods have enlarge value of diagnosis. Immunohistochemistry demonstrated a high sensitivity and specificity and it was capable to emphasize the causative agent of intestinal adenomatosis in all 25 studied samples with proliferative ileitis.

***Key word**: intestinal adenomatosi, Lawsonia intracellularis, porcine proliferative enteropathy*

INTRODUCTION

Infection of *Lawsonia intracellularis*, the causative agent of proliferative enteropathy, occurs all over the world, in different types of production systems, affecting young breeding and growing-finishing pigs. The disease occurs in two major clinical forms including a chronic form, called porcine intestinal adenomatosis (PIA), and an acute form, named proliferative hemorrhagic enteropathy (PHE) (Gyles et al, 2010; McOrist and Gebhart, 2006; Moga Mânzat, 2001).

The economic impact of proliferative enteropathy on the swine industry is estimated to be very high. It was considered the most common problem in grower-finisher pigs in the 2000 National Animal Health Monitoring System survey, occurring on more than a third of all sites and reported on 75% of large sites (Guedes, 2004). The economic damage due the evolution of this morbid entity could not be stopped, as long as the aetiopathogenesis is unclear, as the earlier diagnosis methods of outbreaks are not established, it is impossible to determine appropriate measures against the disease and to control it.

MATERIALS AND METHODS

A number of 25 samples of ileum, with specific lesions of intestinal adenomatosis, were submitted to microscopic examination, using Kinyoun, Green-Methyl-Pironine, Masson-Fontana, Schmitz, Diff-Quick methods and immunohistochemistry.

Protocol for slides stained include few step (Șincai, 2003):

- Samples were paraffined, after keeping them for 7 days in 80° alcohol solution.
- The paraffin block was cut at 5 µm.
- Dewaxing involved 3 successive baths of toluene, 3-5 minutes each one.
- Dehydration in decrease concentration of alcohol (absolutely, 96° and 80°) was followed by hydration with distilled water for one minute.
- The slide were stained, noting that the staining technique depends by chosen method. In the present study we used Kinyoun, Green-Methyl-Pironine, Masson-Fontana, Schmitz, Diff-Quick methods.
- Before clearing with toluene (1 bath) and mounting, the samples were dehydrated with increase concentration of alcohol (80°, 90°, absolutely).

For immunohistochemical technique (IHC), initially, samples were subject to inclusion in

paraffin technique, sectioning, dewaxing and rehydrating, according to the above mentioned protocol. This method involves antigenic exposure and immunostaining. Antigenic exposure was performed by exposing of dewaxed and rehydrated sections to heat, into a sodium citrate solution at pH 6, for 30 minutes. To block endogenous peroxidase was used hydrogen peroxide 3% (Lin et al., 2011). Immunostaining involved use of work system NovoLink Max Polymer Detection (Novocastra, Newcastle UponTyne, UK). All steps were made using DakoCytomation Autostainer immunohistochemistry machine. Chromogen used consisted of 3.3-diaminobenzidine and for counter-stain was applied Lille haematoxylin. All samples were double staining using alcian blue coloration.
Microscopic evaluation was realized using Nikon Eclipse E 600 microscope and images were captured with LUCIA G system.

RESULTS AND DISCUSSIONS

Microscopic examination of intestinal fragments seems to be capable for highlight caracteristic lesions and causal agent of porcine proliferative enteropathy, depending of the chosen methods.
Comparison of different histopathologic methods results for diagnosis of porcine proliferative enteropathy, obtained in our study, are shown in table number 1.

Tabel no. 1
Comparison of some diagnostic methods of porcine proliferative enteropathy

Histopathologic methods	No. of examined samples	No. of positive samples	Diagnostic Value
Green-Methyl-Pironine	25	0	No value
Diff-Quick	25	10	Orientative
Masson-Fontana	25	11	Orientative
Schmitz	25	11	Orientative
Kinyoun	25	7	Orientative
IHC	25	25	Routine

Using Green-Methyl-Pironine method it was observed that epithelial proliferation of ileal mucosa associated goblet cell depletion alternate with epithelial desquamation (figure 1) and with lake of lesions areas. Characterization of inflammatory infiltrate it was not possible and also this method has not capacity to highlight the bacteria.

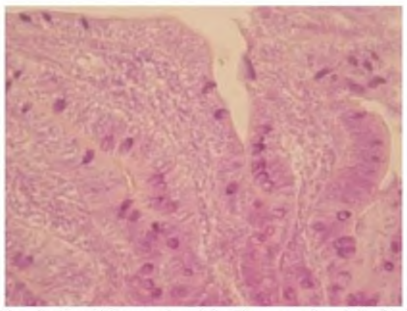

Fig. 1. Proliferated epithelium and epithelial desquamation (Green-Methyl-Pyronine, x400)

Diff-Quick coloration is a method capable to expose all characteristic lesions of porcine proliferative enteropathy, but not always the present of the bacteria, which was observed in 10 samples, that means 40%. It was observed areas with epithelial proliferation of ileal mucosa, goblet cell depletion, epithelial desquamation and inflammatory infiltrate in lamina propria of the mucosa characterized by mobilization of macrophages, lymphocytes and eosinophils (figure 2).

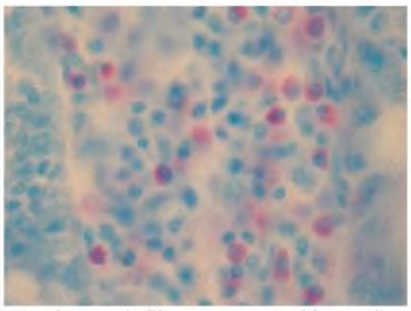

Fig. 2. Leukocyte infiltrate composed by eosinophils, lymphocytes and macrophage cells (Diff-Quick, x400)

The present of eosinophils as a cellular components, involved in antibacterial defense, characteristic of *Lawsonia intracellularis* infections, was first reported in this study, and may suggest an allergic reaction caused by the existence of protein LsaA in bacterial wall, a phenomenon that triggers edema as a

consequence of histaminic release by mast cells. On the other hand, eosinophils may play a role in bacterial neutralization, knowing the fact that they are attracted to the lipopolysaccharides from bacterial Gram-negative wall.

Argentic impregnation, Masson-Fontana and Schmitz, allowed emphasizing less the histological aspects, but, due agrophilic characteristic of *L.intracellularis* strains, the methods were able to highlight the presence of the bacteria (figure 3, figure 4) in 11 samples, which implies a rate of 44% positive samples. However, these methods are capable to exposure microscopic lesions of epithelial proliferation caused by multiplying immature enterocytes.

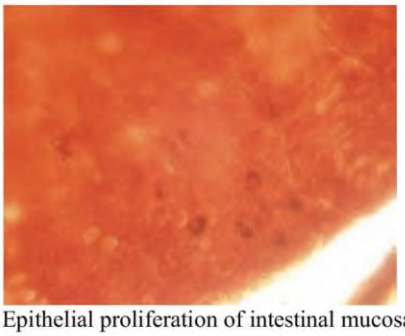

Fig. 3. Epithelial proliferation of intestinal mucosa with intracellular bacteria (Masson-Fontana, x1000)

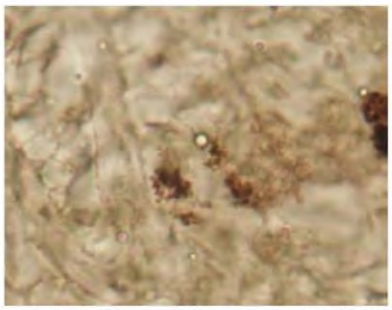

Fig. 4. Cluster of bacteria in cytoplasm of immature enterocytes from epithelial proliferated layer (Schmitz, x1000)

Concerning to Kinyoun coloration, 7 samples were positive, which implies a rate of 28% positive pigs. Bacteria could be highlighted in the cytoplasm of enterocytes from intestinal villi (figure 5), into enterocytes of the intestinal glands and in macrophages. Being an acid-fast stain, this technique is not capable to express microscopic lesions.

Fig. 5. Cluster of bacteria in cytoplasm of immature enterocytes from epithelial proliferated layer (Kinyoun, x1000)

Unlike all histological methods that we described, immunohistochemistry was able to identify the bacterial agent in all examined samples. Even it highlights only few microscopic lesions, mentioning depletion of goblet cell, epithelial desquamation, immature enterocytes proliferation (figure 6), this diagnostic method represents an important tools for postmortem diagnostic of porcine proliferative enteropathy.

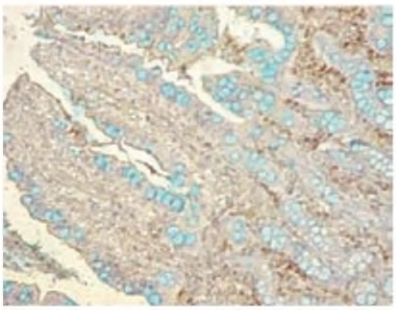

Fig. 6. The presence of bacterial antigen on the surface of intestinal villi and lamina propria between the intestinal glands (IHC – double staining with Alcian blue, x100)

Many studies were designed to compare some histopathological methods for diagnostic of swine proliferative enteropathy, but these were limited to H&E, Ziehl-Neelsen, Warthin–Starry technique and immunohistochemistry. Guedes et al. (2002) showed that all 14 pigs with microscopic lesions detectable by H&E staining were revealed the etiologic agent using Warthin-Starry methods, and of the 33 samples positive by IHC in only 19 specimens the bacteria was identified by silver impregnation (Guedes et al., 2002). Moreover, it seems that silver impregnation was able to highlight only a rate of 42% positive samples confirmed by PCR (Weissenbo et al., 2007). It seems that in

acute form of porcine proliferative enteropathy, Warthin-Starry and Ziehl-Neelsen stains are able to highlight the etiologic agent in all examined samples confirmed as positive by PCR (Dittmar et al., 2003). The low percentage of positive samples by Warthin-Starry and Ziehl-Neelsen stains which were obtained in our study may be due to the chronic form of this infectious disease.

Diagnosis of porcine proliferative enteropathy represents a problem faced by many researchers, but also by breeders. Earlier and low cost diagnosis remains a goal that seems to be difficult to achieve, as soon as there are still many questions about the etiopathogenesis of this disease.

CONCLUSIONS

Immunohistochemistry remains a precision diagnostic method of porcine proliferative enteropathy outbreaks.

Due to expedient technique and satisfactory results, Diff-Quick method can successfully replace the argentic impregnation. Poor results obtained in case of Green-methyl-pironine method recommend that these techniques are not used.

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III. BREVETE

Sunt coautor a trei cereri de brevet de invenție.

Gelul pentru tratarea leziunilor uscate din demodicoza canină

Patent nr. A/00075/1.02.2016

Mederle Narcisa, **Mederle Ovidiu**, Morariu Sorin, Morariu Florica, Dărăbuș Gheorghe, Oprescu Ion, Ilie Marius, Negrescu Adina

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GEL FOR TREATMENT OF DRY WOUNDS IN CANINE DEMODICOSIS gel consists, in mass percentage, of 25% honey

Patent Number(s): RO131619-A0

Inventor(s): DARABUS G, ILIE M S, MEDERLE N, MEDERLE O, MORARIU F, MORARIU S, NEGRESCU I A, OPRESCU I

Patent Assignee Name(s) and Code(s): UNIV TIMISOARA STIINTE AGRIC SI MEDICINA (UYTI-Non-standard)

Derwent Primary Accession Number: 2017-083608

Abstract: NOVELTY - The invention relates to a gel for the treatment of dry wounds in canine demodicosis. According to the invention, the gel consists, in mass percentage, of 25% honey, 25% propolis, 30% apple vinegar and 20% hydro-glycero-alcoholic extracts of buds of blackcurrant, walnut and dog-rose, cedar sprouts, rye radices, in a ratio of 1:5 for each of the five ingredients.

Show Documentation Abstract

International Patent Classification: A61K-035/644

Derwent Class Code(s): B04 (Natural products and polymers. Including testing of body fluids (other than blood typing or cell counting), pharmaceuticals or veterinary compounds of unknown structure, testing of microorganisms for pathogenicity, testing of chemicals for mutagenicity or human toxicity and fermentative production of DNA or RNA. General compositions.)

Derwent Manual Code(s): B04-A08; B04-A10; B04-A98; B04-D01A; B12-M02G; B14-B04A; B14-N17B; B14-S12; B14-S18

Patent Details:

Patent Number	Publ. Date	Main IPC	Week	Page Count	Language
RO131619-A0	30 Jan 2017	A61K-035/644	201724		English

Application Details:

RO131619-A0	RO000075	01 Feb 2016

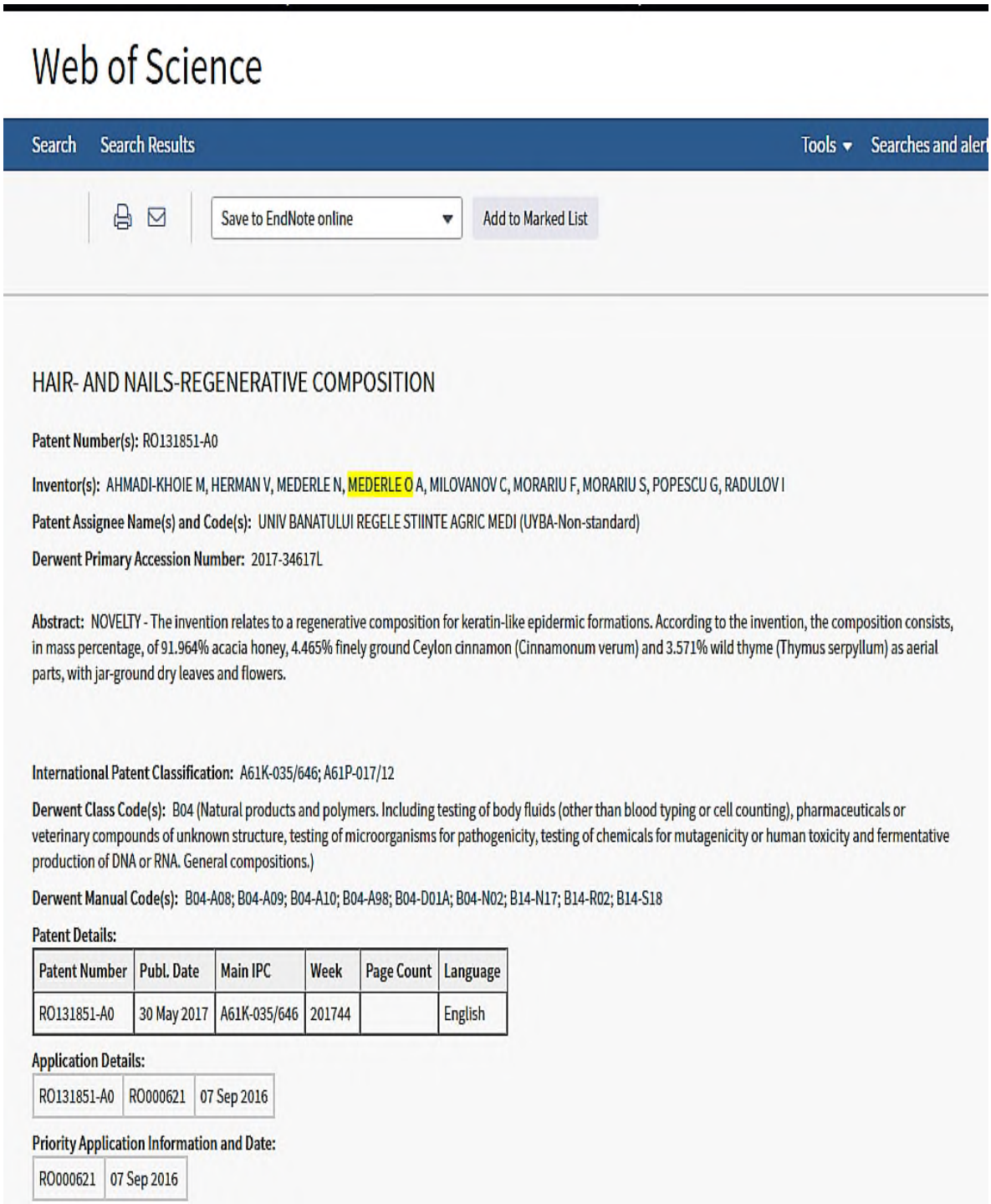
Priority Application Information and Date:

RO000075	01 Feb 2016

Compoziție regeneratoare pentru păr și unghii

Patent nr. A /00621/25.11.2016

Milovanov Cornelia, Mederle Narcisa, Ahmadi Mirela, Morariu Sorin, Popescu Gabriela, Morariu Florica, Herman Viorel, Radulov Isidora, **Mederle Ovidiu**



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HAIR- AND NAILS-REGENERATIVE COMPOSITION

Patent Number(s): RO131851-A0

Inventor(s): AHMADI-KHOIE M, HERMAN V, MEDERLE N, MEDERLE O A, MILOVANOV C, MORARIU F, MORARIU S, POPESCU G, RADULOV I

Patent Assignee Name(s) and Code(s): UNIV BANATULUI REGELE STIINTE AGRIC MEDI (UYBA-Non-standard)

Derwent Primary Accession Number: 2017-34617L

Abstract: NOVELTY - The invention relates to a regenerative composition for keratin-like epidermic formations. According to the invention, the composition consists, in mass percentage, of 91.964% acacia honey, 4.465% finely ground Ceylon cinnamon (Cinnamonum verum) and 3.571% wild thyme (Thymus serpyllum) as aerial parts, with jar-ground dry leaves and flowers.

International Patent Classification: A61K-035/646; A61P-017/12

Derwent Class Code(s): B04 (Natural products and polymers. Including testing of body fluids (other than blood typing or cell counting), pharmaceuticals or veterinary compounds of unknown structure, testing of microorganisms for pathogenicity, testing of chemicals for mutagenicity or human toxicity and fermentative production of DNA or RNA. General compositions.)

Derwent Manual Code(s): B04-A08; B04-A09; B04-A10; B04-A98; B04-D01A; B04-N02; B14-N17; B14-R02; B14-S18

Patent Details:

Patent Number	Publ. Date	Main IPC	Week	Page Count	Language
RO131851-A0	30 May 2017	A61K-035/646	201744		English

Application Details:

RO131851-A0	RO000621	07 Sep 2016

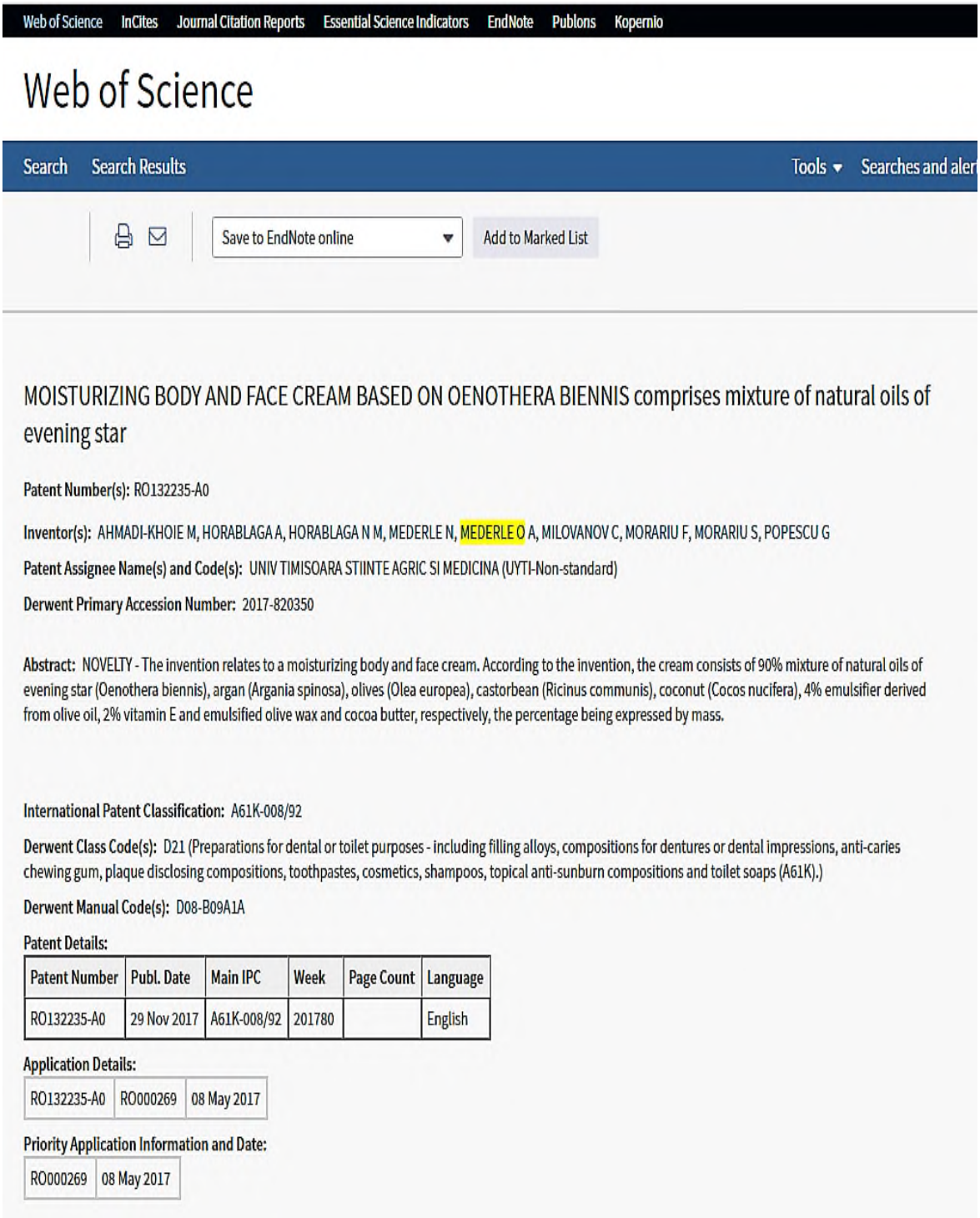
Priority Application Information and Date:

RO000621	07 Sep 2016

Cremă pentru hidratantă piele și ten pe bază de Oenothera biennis

Patent nr. A /000269/08.05.2017

Horablaga Adina, Milovanov Cornelia, Ahmadi Mirela, Mederle Narcisa, Morariu Sorin, Morariu Florica, Horablaga Marinel, Popescu Gabriela, **Mederle Ovidiu**



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MOISTURIZING BODY AND FACE CREAM BASED ON OENOTHERA BIENNIS comprises mixture of natural oils of evening star

Patent Number(s): RO132235-A0

Inventor(s): AHMADI-KHOIE M, HORABLAGA A, HORABLAGA N M, MEDERLE N, MEDERLE O A, MILOVANOV C, MORARIU F, MORARIU S, POPESCU G

Patent Assignee Name(s) and Code(s): UNIV TIMISOARA STIINTE AGRIC SI MEDICINA (UYTI-Non-standard)

Derwent Primary Accession Number: 2017-820350

Abstract: NOVELTY - The invention relates to a moisturizing body and face cream. According to the invention, the cream consists of 90% mixture of natural oils of evening star (Oenothera biennis), argan (Argania spinosa), olives (Olea europea), castorbean (Ricinus communis), coconut (Cocos nucifera), 4% emulsifier derived from olive oil, 2% vitamin E and emulsified olive wax and cocoa butter, respectively, the percentage being expressed by mass.

International Patent Classification: A61K-008/92

Derwent Class Code(s): D21 (Preparations for dental or toilet purposes - including filling alloys, compositions for dentures or dental impressions, anti-caries chewing gum, plaque disclosing compositions, toothpastes, cosmetics, shampoos, topical anti-sunburn compositions and toilet soaps (A61K).)

Derwent Manual Code(s): D08-B09A1A

Patent Details:

Patent Number	Publ. Date	Main IPC	Week	Page Count	Language
RO132235-A0	29 Nov 2017	A61K-008/92	201780		English

Application Details:

RO132235-A0	RO000269	08 May 2017

Priority Application Information and Date:

RO000269	08 May 2017

IV. CAPACITATEA DE A CONDUCE PROIECTE DE CERCETARE-DEZVOLTARE. CAPACITATEA DE A LUCRA ÎN ECHIPĂ ȘI EFICIENȚA COLABORĂRILOR ȘTIINȚIFICE ALE ACESTUIA

În cariera universitară, am urmărit să realizez o comunicare eficientă, rezultat al unor relații bazate pe respect, încredere, ascultare, atenție, oglindire, indiferent de natura contextului relațional. Multe descoperiri într-un domeniu au fost sugerate de soluțiile găsite în altă disciplină. În munca de cercetare, colaborarea interdisciplinară a fost benefică și eficientă.

Am colaborat cu cadrele didactice ale Facultății de Medicină și Farmacie „Victor Babeș" Timișoara, cu cadrele didactice ale Facultății de Medicină Veterinară și ale USAMVB Timișoara, cu cadrele didactice ale USAMV Iași, cu cadrele didactice ale Universității Politehnica București, cu cadrele didactice ale Universității Mustafa Kemal, Turcia, cu cadrele didactice și cercetătorii de la Universitatea Nova din Lisabona, Portugalia, Institutul Național de Cercetare și Dezvoltare – Textile și Pielărie, București, colaborare care s-a concretizat prin publicarea de lucrări științifice ISI/BDI și prin realizarea obiectivelor propuse în cadrul proiectelor de cercetare.

De-a lungul carierei universitare au fost depuse propuneri de proiecte în calitate de director sau responsabil, dintre care s-au materializat două. Am depus trei cereri de brevet de invenție, în calitate de colaborator. Toate cererile sunt înregistrate la OSIM și se regăsesc în Web of Science (WOS).

În derularea proiectelor finanțate, am îndeplinit cu succes obiectivele propuse, am lucrat cu echipa de cercetare în realizarea indicatorilor de proiect, am întocmit rapoartele științific și economic și am diseminat rezultatele cercetării.

Director proiect

1. Efectul degranulantelor mastocitare şi al inhibitorilor degranulării asupra angiogenezei tumorale - CNCSIS A/ 759/2006 - 2006-2007

Mastocitul este o celulă proprie ţesutului conjunctiv, cu rol atât în reacţia inflamatorie peritumorală, cât şi în stimularea angiogenezei în tumoră, ştiut fiind că mastocitele sunt, alături de macrofage şi limfocite T, surse principale de TNF-α (citokină cu rol inflamator antitumoral)

şi secretante de factori proangiogenici (VEGF/VPF, β-FGF, TGF-β^2) şi de heparină, care leagă FGF-1, FGF-2, pleiotropină, VEGF, PDGF, care au la rândul lor diverse roluri în angiogeneză. Migrarea mastocitelor la nivelul tumorii poate fi explicată prin ambele roluri majore, atât reacţia inflamatorie peritumorală, cât şi switch-ul proangiogenic promovat de mastocite au un prognostic nefavorabil (fig.5, 6).

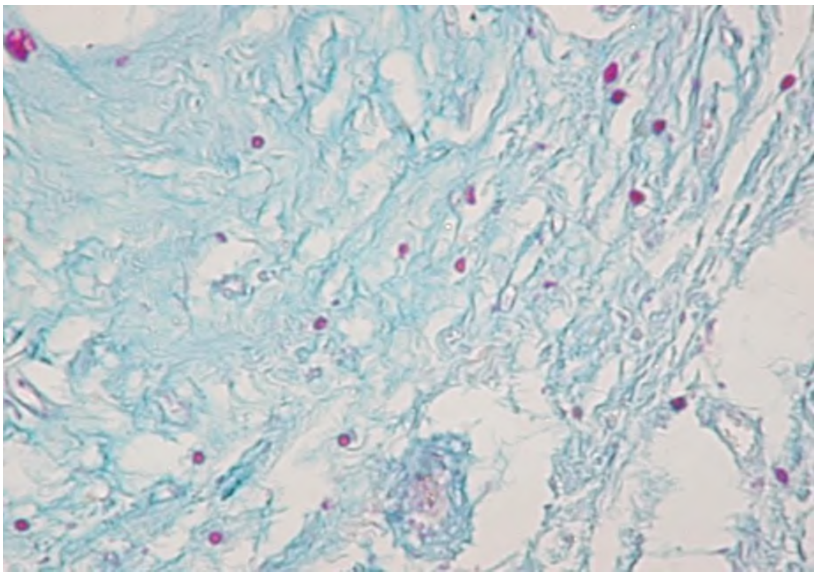


Fig. 5. Mastocite safraninofile în stromă.Ob 20x

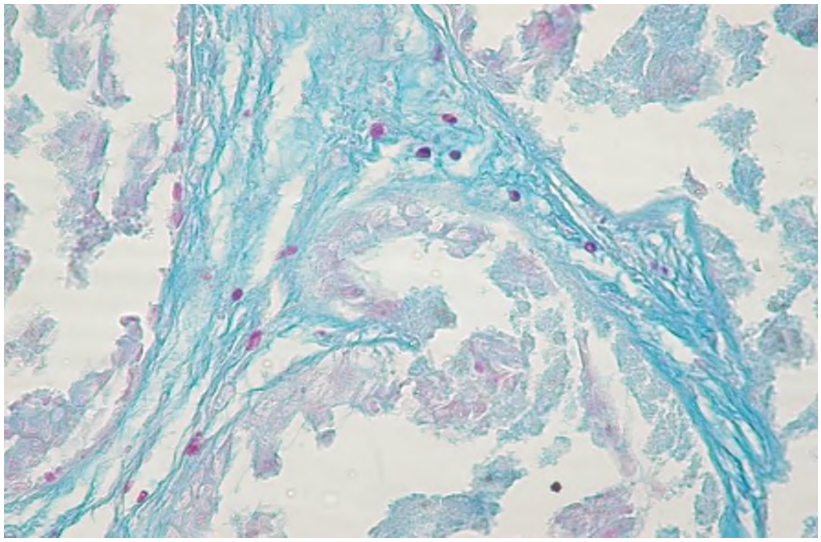


Fig. 6. Mastocite safraninofile dispuse peritumoral.Ob. 20x

Cea mai importantă problemă în acest studiu este rolul mastocitelor, atât reacţia stromală de tip inflamator (în care ar putea fi implicate mastocitele), cât şi microdensitatea vasculară (cu care MCD este corelată în numeroase studii) au prognostic nefavorabil. Switch-ul angiogenic promovat de mastocite are prognostic mai nefavorabil decât reacţia inflamatorie peritumorală, tumorile care dobândesc capacitate angiogenetică fiind mult mai agresive decât cele care nu pot încă să formeze neovase. Studiul de cercetare nu face această diferenţă, motiv pentru care răspunsul la această întrebare stă într-un studiu ulterior care să evalueze, alături de MCD, şi densitatea microvasculară (MVD), cu care MCD ar trebui să se coreleze dacă mastocitele din aceste tumori ar fi cu rol angiogenetic, şi, eventual, expresia VEGF în aceste mastocite. Dacă mastocitele din aceste tumori au rol inflamator, atunci cel mai util marker (expresie cantitativă) ar fi TNF-α.

2. Lanţuri epidemiologice posibile şi modalităţi de control în criptosporidioza la animale şi om - PN II 51-034/2007 - 2008-2010

Infecţia cu *Cryptosporidium* este o problemă importantă de sănătate publică, anual, ţările dezvoltate cheltuind sume semnificative pentru tratamentul apelor infectate, pe de o parte, şi al pacienților, pe de altă parte. Marele avantaj pe care îl prezintă acest agent parazitar este rezistenţa la mijloacele uzuale de dezinfecție a apei. Din perspectiva clinică, criptosporidioza se manifestă prin acuze digestive, pentru că parazitul infestează ileonul, iar la pacienţii imunodeprimaţi infecţia se poate extinde de la esofag până la rect. Principalul semn luat în considerare este diareea profuză, cu scaune frecvente şi apoase. Deshidratarea rapidă a pacientului şi tulburări electrolitice sunt repercusiunile acestui fenomen diareic. La pacienţii sănătoşi, boala are evoluţie autolimitantă, ceea ce scade frecvenţa prezentărilor la medic.

Spre deosebire de infecţia la animale, criptosporidioza la om nu are specificitate de vârstă, deşi incidenţa la tineri poate fi mai ridicată datorită faptului că, posibilităţile de expunere sunt mai mari iar probabilitatea unei imunități instalate datorate unei expuneri anterioare este mai mică.

În realizarea obiectivelor proiectului, au fost selectați pacienți din diferite categorii de vârstă (1 lună – 80 de ani), sexe diferite, proveniență geografică diferită (urban/rural; județe diferite din Sud-Vestul României), posibil cu o simptomatologie de boală diareică, pacienți imunosupresaţi (TBC, SIDA) sau parazitați (oxiuri, lamblii) şi copii tarați (malnutriţi, distrofici).

Rezultatele cercetării care s-a realizat pe o perioadă de doi ani au relevat o prevalență a criptosporidiozei, în partea de Vest a României, de 6,18 %,la pacienții proveniți din mediul rural, respectiv, de 4,54 %, la cei din mediul urban, grupul de risc fiind reprezentat de copiii cu vârsta cuprinsă între 2-6 ani.

Identificarea imunohistochimică a infecţiei naturale cu *Cryptosporidium spp*. la viţei este o tehnică modernă şi precisă care oferă importante date anatomopatologice despre puterea patogenă a criptosporidiilor.

Cercetările au fost realizate pe fragmente de intestin subțire și cec de vițel. S-a utilizat colorația morfologică uzuală cu hematoxilină eozină pentru stabilirea diagnosticului histopatologic, iar ulterior, pe secțiuni adiacente, s-a practicat imunohistochimie și, respectiv, imunofluorescență. La examinarea secțiunilor colorate cu hematoxilină eozină s-au identificat: edem la nivelul submucoasei, vase hiperemice, ulcerații și infiltrat inflamator in cantitate crescută. În imunofluorescență, au fost identificați agenții parazitari la nivelul vilozității intestinale, în glandele dilatate chistic și la nivelul laminei propria.

Infecția cu *Criptosporidium parvum* este răspunzătoare de apariția diareii apoase și instalarea decesului la un mare număr de erbivore. Histopatologia intestinului subțire are un rol esențial în susținerea diagnosticului cu utilizarea imunohistochimiei și imunofluorescenței atunci când există dubii în diagnosticul histopatologic uzual.

2. REALIZĂRI PROFESIONALE ȘI ACADEMICE

2.1. COMPETENȚELE DIDACTICE

Cultivarea gândirii inovatoare în învățământul universitar a devenit o permanentă preocupare. Pe lângă efortul tradițional de educare a gândirii critice, stimularea fanteziei apare și ea ca un obiectiv major. Aceasta implică schimbări importante, atât în mentalitatea profesorilor, cât și în abordarea metodele de educare și instruire.

Am încercat de-a lungul celor 25 de ani petrecuți în mediul universitar să împletesc munca didactică cu eforturile pe care le presupune cercetarea, imaginația cu acumularea de cunoștințe și să implic studenții, oricând a fost posibil, în acest fenomen complex al creativității.

Coordonez activitatea practică a studenților anului II – disciplina de Histologie/Departamentul de Morfologie Microscopică, din anul 1994 și sunt implicat în organizarea și susținerea stagiilor clinice și a gărzilor efectuate de studenții anului IV – disciplina Urgențe/Departamentul Chirurgie I, din anul 2017. De la înființarea liniilor engleză și franceză, sunt membru al colectivului didactic cu predare în aceste limbi străine.

Sunt titularul cursului de Histologie – limba română și franceză, respectiv al cursului de Urgențe – limba română, engleză, franceză.

La Disciplina de Histologie, studenții au posibilitatea să consulte bibliografia de referință, cărți/manuale didactice și îndrumătoare de lucrări practice la care sunt prim autor sau colaborator.

Prim autor

1) **Mederle O**, Raica M – *Histologia cavităţii bucale*. Editura Mirton, Timişoara, ISBN 973-585-299-3, 146p., 2001.

2) **Mederle O**, Mederle C, Raica M -*Histologia şi fiziologia rinichiului*, Editura Mirton, Timişoara, ISBN 973-578-717-2, 174 p., 1999.

3) **Mederle Ovidiu**, Raluca Amalia Ceausu, Sorin Ioanovici *Histologie de la biologie a la clinique* Editura Eurostampa, 2016, 305p. ISBN 978-606-32-0158-5

Colaborator

4) Bernard Elena, M Craina, **Ovidiu Mederle** *Ghidul Furnizorilor in cadrul programului de screening al cancerului de col* Editura Mirton, Timişoara, 2014, 183p ISBN 978-973-5214388

5) Gaje Pusa, M Raica**, Ovidiu Mederle**-Histologie orala-Lito UMF Timisoara 2010

6) Mederle C, **Mederle O** -*Mecanisme patogenice în astmul bronşic*, Editura Mirton, Timişoara, ISBN 973-578-432-7, 134 p., 1998.

7) Raica M, Alexa A, Iacovliev M, Lighezan R, Sârb S, **Mederle O** -*Histologie* – lucrări practice vol II, Editura Mirton, Timişoara, ISBN 973-578-449-4, 129 p., 1997.

8) Raica M, Dumnici A, Mederle C, **Mederle O -** *Leziunile precanceroase gastrice,* Editura Mirton, Timişoara, ISBN 973-578-250-2, 133 p., 1997.

9) Raica M, Hereţiu D, **Mederle O** -*Interpretarea biopsiilor hepatice,* Editura Mirton, Timişoara, ISBN 973-578-320-7, 147 p., 1997.

10) Raica M, Iacovliev M, Alexa A**, Mederle O**, Sârb S - *Histologie medicală*, Editura Mirton, Timişoara, ISBN 973-578-316-7, 432 p., 1997.

11) Raica M, **Mederle O** et al- *Citologie clinică,* Editura Mirton, Timişoara, ISBN 973-578-442-4, 298 p., 1998.

12) Raica M**, Mederle O**, Căruntu I D, Pintea A, Chindriş A M - *Histologie teoretică şi practică*, Editura Brumar, Timişoara, ISBN 973-602-047-9, 491p., 2004.

13) Raica M**, Mederle O**, Grigoraş A -*Histologie stomatologică*, Editura Mirton, Timişoara, ISBN 973-578-147-6, 183 p.,1996.

Contribuțiile la dezvoltarea activităților didactice s-au concretizat în elaborarea, ca prim sau coautor, a mai multe lucrări destinate instruirii universitare și postuniversitare, publicate în format tipărit. Dintre aceste pot fi amintite:

Elaborarea capitolelor „Sistemul tegumentar"(15 pagini) si „Organele de simt" (24 pagini) din volumul „*Histologie teoretică și practica*", Editura Brumar, Timișoara, ISBN 973-602-047-9, 2004, coordonat de Prof. Univ. Dr. Marius Raica. Lucrarea, dedicată în exclusivitate sistemelor și organelor, este redactată în conformitate cu bazele moderne ale histologiei, constituind produsul colaborării dintre autori aparținând unor școli de histologie cu tradiție (Timișoara, Cluj, Iași). Elementele de noutate constau în actualizarea datelor, terminologiei și interpretărilor, detalierea variantelor structurale normale, corelarea cu semnificația clinică și introducerea notelor practice. Maniera de abordare profundă, cu acces la numeroase detalii, recomandă lucrarea drept un tratat, utilizabil la diferite nivele de instruire, de la cursurile obligatorii de histologie existente în planurile de învățământ, până la pregătirea in cadrul rezidențiatului, în principal în specializarea anatomie patologică.

Elaborarea volumului „*Histologia cavității bucale"*. Editura Mirton, Timișoara, 2001 ISBN 973-585-299-3, 146p, in colaborare cu Prof. Univ. Dr. Marius Raica. Lucrarea are doua obiective: pe de o parte sistematizează noțiunile teoretice de microscopie a țesuturilor și organelor orale strict necesare studenților, și pe de altă parte actualizează materialul in funcție

de datele noi apărute în domeniu. Noutatea lucrării constă în introducerea la fiecare capitol a noțiunilor de histologie clinică. Sunt prezentate noțiuni de factură clasică și de dată foarte recentă, care permit dezvoltarea unui cadru mult mai cuprinzător decât cel existent în cărțile de histologie a cavității bucale apărute în ultimul deceniu în literatura medicală din România. Informația acoperă integral tematica orelor de curs obligatorii privind histologia cavității bucale și totodată, oferă un bogat material de studiu pentru programele de masterat.

Elaborarea volumului *„Histologie* – lucrări practice" vol II, Editura Mirton, Timișoara, ISBN 973-578-449-4, 129 p., 1997, coordonat de Prof. Univ. Dr. Marius Raica. Manualul pune in valoare experiența ședințelor de aplicații practice desfășurate in anii universitari precedenți, pe baza unor referate care au fost amendate de la o serie de studenți la alta. Materialul urmărește eficientizarea modalității de transmitere a cunoștințelor, prin definirea unui protocol de desfășurare a aplicațiilor practice, in care activitatea practica propriu-zisa este precedata de o sinteză a elementelor teoretice esențiale, cu accent pe latura imagistică.

Am studiat și aspectele morfopatologice ale glomerulonefritelor cronice pe fragmente realizate prin puncție biopsie renală. Corelarea modificărilor histopatologice cu datele clinice și cu cele paraclinice care permit evaluarea funcției renale ne-au permis să stabilim valoarea parametrilor histopatologici în contextul elaborării diagnosticului și prognosticului afecțiunilor renale. În același timp am elaborat un algoritm de investigații histochimice și imunohistochimice pentru a urmări utilitatea acestor metode în stabilirea diagnosticului etiologic și în urmărirea eficienței terapeutice. Rezultatele acestor studii s-au concretizat în editarea unei monografii ca prim autor "Histologia și fiziologia rinichiului", Ed. Mirton, Timișoara, 1999.

Elaborarea monografiei „*Mecanisme patogenice în astmul bronşic"*, Editura Mirton, Timişoara, ISBN 973-578-432-7, 134 p., 1998, coautor. Introducând in literatura medicală românească numeroasele achiziții din domeniu, apărute in ultimii ani, lucrarea poate reprezenta punctul de plecare pentru numeroase cercetări. Pentru ilustrarea si înțelegerea noțiunilor prezentate au fost introduse scheme si imagini – care in cea mai mare parte reprezintă contribuții personale. Prin modalitatea de abordare fizio-histologică, lucrarea reflectă una dintre tendințele dominante ale cercetării științifice medicale: atrage atenția asupra caracterului obligatoriu multidisciplinar, singura modalitate prin care se pot obține rezultate de performanță – invariabil utile pentru practica medicală.

Elaborarea capitolului „*Citodiagnosticul afecțiunilor hepatice*"(15 pagini) din volumul „*Citologie clinică",* Editura Mirton, Timișoara, ISBN 973-578-442-4, 298 p., 1998, coordonat de Prof. Univ. Dr.. Marius Raica. Lucrarea tratează problemele majore ale diagnosticului

citologic. Sunt prezentate teme mai rar abordate, dar cu utilitate reală pentru practică, așa cum sunt citologia gastrică, sinovială și oculară. În acest mod, lucrarea acoperă o mare parte din spectrul investigațiilor citologice, fiind utilă nu numai pentru că este prima lucrare de acest fel din țara noastră.

Am contribuit la realizarea unui site www „Laborator de imunohistochimie", acesibil la adresa http://www.umft.ro/en/faculties/histology/imuno.html, cu documentare în limbile română și engleză. Site-ul a fost conceput pentru eficientizarea studiului individual, cuprinzând imagini comentate ale unor preparate histologice proprii. La momentul creării, a constituit o premieră la UMF "Victor Babeș" Timișoara, în direcția utilizării performante a tehnicii de calcul pentru modernizarea și diversificarea actului educațional.

2.2. CAPACITATEA DE A ÎNDRUMA STUDENȚI SAU TINERI CERCETĂTORI

Cea mai puternică și frumoasă colaborare: colaborarea între profesor și student, *flux permanent și reciproc de informație, noutate și progres!*

Comunicarea educațională este o formă fundamentală de interacțiune între profesor și student. Ca în orice proces de comunicare umană, în comunicarea educațională, relaționarea presupune un veritabil sistem de simboluri și semnificații cu ajutorul căruia, mesajul să poată fi transmis. Calitatea comunicării educaționale depinde de calitatea acestui sistem, dar și de gradul de stăpânire a acestuia. Dacă relaționarea educațională se bazează pe un schimb de informații, în care inițierea comunicării se realizează spontan și liber, iar gradul de interacțiune profesor - studenți și studenți - studenți este puternic, atunci, implicarea în activitate este intensă, efectele formative sunt puternice, cu condiția fundamentării lor științifice și morale pronunțate.

Consider că rolul profesorului este de a pune la dispoziția studentului cele mai relevante informații (conținut) într-un cadru (proces) care să producă motivarea, entuziasmul și implicarea studenților. În aceste condiții, progresul studenților devine evident.

Am încercat pe tot parcursul activității mele didactice și de cercetare, să implic studenții, să-i motivez, să le încurajez entuziasmul spre a crea, a gândi, a inova, a lega punți între informație și practică.

Se cunoaște efortul pe care l-am depus în implicarea și coordonarea studenților în studiile de cercetare care s-au derulat și se derulează la Disciplina de Histologie, rezultatelor

obținute împreună au fost valorificate în elaborarea lucrărilor de licență, dar și în publicarea în reviste de specialitate.

Doar câteva exemple argumentează aceste afirmații:

1. Histochimia parenchimului renal normal – Michalopoulou Fani, 1999
2. Histochimia glandelor salivare – Isabela Tăutu, 1999
3. Implicațiile markerilor imunohistochimici în prognosticul tumorilor vezicale – Ziad Abdallah Al Qasem, 2001
4. Particularități histochimice și imunohistochimice ale leziunilor precanceroase gastrice – Abdelfattah Abushammala, 2001
5. Factori histochimici și imunohistochimici de prognostic în hepatopatiile cronice – Mohammad Ibrahim Qasad, 2001
6. Corelația dintre expresia imunohistochimică a receptorilor hormonali și microdensitatea mastocitară în cancerul mamar – Marinescu Georgeta, 2004
7. Factori histochimici și imunohistochimici de prognostic în leziunile precanceroase gastrice – Nteligiorgios Christos, 2004
8. Particularități histochimice și imunohistochimice ale hepatopatiilor cronice – Petrogianos Charalampos, 2004
9. Histologia glandelor salivare – Beschiu Ioan Luca, 2004
10. Markeri imunohistochimici de prognostic în adenocarcinomul gastric – Stoichițoiu Nicoleta, 2005
11. Angioarhitectura placentei – Matiș Bianca, 2005
12. Microdensitatea mastocitară factor de prognostic în cancerul mamar – Grigoraș Mirela, 2005
13. Microvascularizația placentară – Bilal Ahmad Rather, 2005
14. Corelația dintre expresia imunohistochimica a VGEF și densitate mastocitară în carcinomul gastric - Barbu Catalin, 2006

15. Microdensitatea mastocitară factor de prognostic în cancerul mamar - Dimeny Zoltan Cristian, 2008
16. Markeri imunohistochimici implicați în tumorile glandei mamare - Carmen Pădurean, 2009
17. Expresia markerilor endoteliali și cuantificarea microdensității vasculare sanguine în leziunile hepatice - Alina Tatomir, 2009
18. Expresia imunomorfologică a limfonodulului - Buligă Cristian, 2010
19. Expresia markerilor endoteliali și cuantificarea microdensității vasculare în metastazele hepatice asociate adenocarcinoamelor de colon - Haralambie Elena, 2010
20. Precondiționarea ischemică a mușchiului striat scheletal - Lula Oana Diana, 2012
21. Studiu privind prevalența criptosporidiozei la copii in judetul Timiș - Amina Tina Al-Shammat, 2015
22. Enterita proliferativă bacteriană a suinelor. Studiu Experimental - Miuca Milena, 2017.

Implicarea doctoranzilor în echipa de cercetare a proiectelor pe care le-am directorat, dar și implicarea acestora în derularea studiilor de cercetare care susțin obținerea celor trei cereri de brevet de invenție au fost posibile prin directa mea coordonare. Alături de conducătorul de doctorat, am coordonat doctoranzii în derularea experimentelor și în interpretarea rezultatelor (Dragoș Izvernariu, Cristian Suciu, Gartner Andreea, Surpat Hulea Anca). Colaborarea cu tinerii cercetători s-a concretizat în publicarea de lucrări științifice, iar această colaborare a continuat și după finalizarea tezei de doctorat.

Suciu C, Mederle O - *Imunoexpresia vimentinei în celulele dendritice foliculare*, A XXVII-a Conf Naţ de Imunologie, Timişoara, 1998.

Raica M, Mederle O, Suciu C, Niculescu M – *Secretory granules in the ductal cells of the human submandibular glands*, 15th IFAA, Rome, 1999.

Raica M, Mederle O, Suciu C – *Immunohistochemical expression of anti-mesothelioma HBME-1 in pleural effusions*, Cytopatology, 140-141, Belgium, 2002

Gaje N, Izvernariu D A, Raica M, Mederle O, Vasile Liliana – *Dinamica subpopulatiilor mastocitare in leziunile benigne si maligne mamare*, CEMC; 4:178-83, 2005.

Mederle O, Izvernariu D A – *Qualitative and quantitative quantificaton of the chronic hepatitis*, Rev Rom Anat Clin Antrop; III(1):120-123, 2004

Mederle O, Izvernariu D A, Raica M, Anghel A: *Corelatia dintre expresia imunohistochimica a receptorilor hormonali si microdensitatea mastocitara in cancerul mamar*, Craiova Med; 6(4):517-522, 2004.

Mederle O, Izvernariu D A, Raica M, Anghel A: *Microdensitatea mastocitara in cancerul mamar*, CEMC; 4:178-83, 2004.

Suciu C, Raica M, Mederle O – *Histologia glandei mamare*, Jurnalul Român de Patologie, 2005

Mederle O, Suciu C, Raica M, Cimpean A M, Ioanovici S – *Markeri imunohistochimici de prognostic in adenocarcinomul gastric*, Jurnalul Român de Patologie, 2005

Gartner Andreea, Mederle O., Mederle Narcisa Demodex folliculorum and Demodex brevis – A cause of facial dermatitis and blepharitis, Journal of Biotechnology, 2014, 0168-1656; 185S, S100.

Anca Sofiana Surpat (Hulea), Viorel Herman, Iosif Marincu, Narcisa Mederle, Ovidiu Alexandru Mederle - Immunohistochemical method for identification of Lawsonia intracellularis infection in pigs Journal of Biotechnology, Volume 208, Supplement, 20 August 2015, Pages S100.

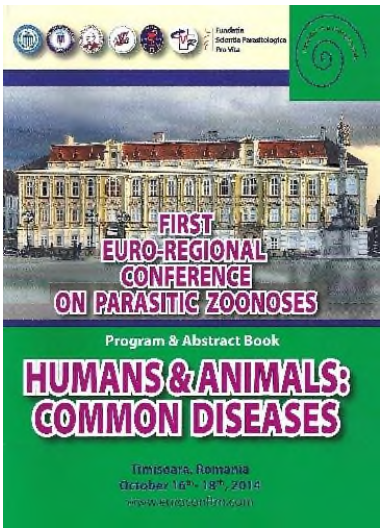
Gartner Andreea Ionela, Mederle Narcisa Geanina, Darabus Gheorghe, Marincu Iosif, Mederle Ovidiu Alexandru - A case report of Cheyletiella blakei infestation in an asymptomatic cat and skin lesions of her owner Journal of Biotechnology 2016 231 Supplement S107.

Mederle Narcisa, Mederle A. O., Dărăbuș Gh., Gartner Andreea, Ioanoviciu S.- Immuno-fluorescence and immuno-histochemistry methods in diagnose of human

cryptosporidiosis, Journal of Biotechnology, 0168 - 165 6, 256, 2017 https://www.sciencedirect.com/science/article/pii/ S0168165617313779

În întreaga mea activitate didactică, am folosit cea mai la îndemână „unealtă”, actul nobil al predării, în stimularea și încurajarea studenților de a participa activ, constructiv și inițiativ în procesul de formare profesională. Imaginația, uneori chiar fantezia, originalitatea ideilor le-am apreciat corespunzător, alături de temeinicia cunoștințelor și de raționamentul riguros și responsabilitatea actului efectuat.

Am participat împreună cu studenții și doctoranzii la diferite evenimente științifice: Simpozionul Științific internațional dedicat tinerilor cercetători, Simpozionul Științific Internațional FMVT, EuroConferința de Zoonoze Parazitare Timișoara, Conferința Internațională Balkan Fungus, Saloanele Naționale de Invenții: Pro Invent Cluj Napoca, „Traian Vuia” Timișoara, EuroInvent Iași.



STUDY ON CRYPTOSPORIDIOSIS PREVALENCE IN CHILDREN FROM TIMIS COUNTY - RECENT UPDATES

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Abstract. Cryptosporidiosis as a parasitic disease found all over the world is a major health issue. According to some researchers, *Cryptosporidium sp.* is among the first three or four enteric pathogenes in humans. The most serious problems occur in paedriatics, mainly in the countries where hygienic conditions are poor. The aim of the study was to collect information related to epidemiology from patients in hospitals, clinics and foster institutions for children and to examine faeces samples using ELISA method. Following the examination of 212 coprological samples taken from children with diarrhea symptoms, tuberculosis, AIDS, dystrophy or infested with *Giardia spp.* the results show a cryoptosporidiosis prevalence of 7.54% (16/2120).
Key words: Cryptosporidiosis, children, prevalence

Am îndrumat studenţi pentru realizarea de lucrări la sesiunile de comunicări studenţeşti:

- Medis, 1997,1998,
- Medis, 2003, lucrare premiata cu preiul III, la sectiunea preclinic
- În 2018 și 2019 am coordonat, în cadrul sesiunii științifice studențești MEDIS, workshop-urile cu tema ALS și BLS
- În 2018, am susținut în cadrul HEART, workshop-ul cu tema FAST in trauma
- În 2019, am coordonat în cadrul GALMED, șase workshop-uri
- Am prezentat studenţilor, în cadrul şedinţelor de referate, lucrări din domeniul histochimiei şi al imunohistochimiei.

2.3. CAPACITATEA DE A TRANSFERA CUNOȘTINȚELE ȘI REZULTATELE CĂTRE MEDIUL ECONOMIC SAU SOCIAL, ORI DE A POPULARIZA PROPRIILE REZULTATE ȘTIINȚIFICE

♣ Una dintre activitățile principale ale societății Primosal S.R.L. este aceea de a investi în activități de cercetare – dezvoltare pentru lansarea în piață a unor produse și servicii inovative pe baza tehnologiilor deja existente. SC Primosal este implicată în realizarea unei linii de fabricație performante în vederea obținerii kit-ului Demosimcan - șampon și gel folosit în medicina veterinară, precum și în dezvoltarea unor parteneriate durabile și sustenabile între agentul economic și instituțiile academice. Unul dintre componentele kit-lui, gelul, este cererea de brevet al cărei coautor sunt și este rezultatul colaborării interinstituționale UMFT, USAMVBT, Institutul de Cercetare - INCDT București și S.C. Primosal București.

♣ Cea mai eficientă formă de promovare se află la îndemâna fiecăruia dintre noi și constă în reliefarea permanentă a caracteristicilor Universității căreia îi aparținem, cu ocazia tuturor contactelor cu mediul extern, fie acestea personale sau profesionale. Acest lucru presupune, însă, cunoaștere, crez și motivație!

Sunt motivat să promovez instituția căreia îi aparțin, Universitatea de Medicină și Farmacie ”Victor Babeș” Timișoara, pentru că aici am găsit suportul și mediul pozitiv pentru întreaga mea activitate didactică și de cercetare.

Am promovat prin participările la evenimente științifice naționale și internaționale, instituția de unde provin ca pe o instituție deschisă comunicării, cu o viziune evolutivă care promovează inovația și nu, rutina, orientată spre finalitatea procesului de cercetare și aplicabilitatea acestuia, responsabilă și dinamică, pentru a putea crea prin intermediul informării, specializării și comunicării, potențialități interne și mijloace de a le transforma în realitate.

Prin participarea mea consecventă cu cele trei cereri de brevet de invenție la Saloanele Naționale și Internaționale de Inventica, am avut oportunitatea să prezint și să promovez rezultatele cercetării, dar și să reprezint Universitatea în care m-am dezvoltat.

Participarea la Salonul Internațional de invenții de la Geneva, 2017, a însemnat și un demers în colaborarea cu reprezentanții Ambasadei României, Misiunea Permanentă a României pe lângă Oficiul Națiunilor Unite de la Geneva și Organizațiile Internaționale cu sediul în Elveția.

2.4. ACTIVITATEA MEDICALĂ

”Să fi specialist nu e de ajuns.

Ca medic trebuie să înțelegi și emoția. Să observi semnele subtile, micile detalii, nu doar analizele. Să reacționezi la muzica întregului corp omenesc, nu doar la simptome.

Medicina este o știință, dar doctorul are subtilitatea unui artist. Cunoaște empatia, nu doar tehnologia viitorului. Își dă timp pentru a fi inspirat de viață, nu doar pentru a se lupta cu degradarea ei.

Medicina este cunoașterea vieții de la știință la emoții”.

1991-1993 - Medic Stagiar, Spitalul Județean - Secția Urologie

1993 -1994 – Medic, Medicină Generală, DMC Seleuș/ DMO Pîncota, Județul Arad

1994 -1996 - Medic rezident, Medicină De Urgență

1996 - Medic specialist, Medicină de urgență, Spitalul Clinic Municipal Timișoara

2001 – prezent, Medic primar, Medicină de urgență, Spitalul Clinic Municipal Timișoara

3. RECUNOAȘTEREA PROFESIONALĂ, ȘTIINȚIFICĂ ȘI ACADEMICĂ

3.1. MEMBRU ÎN COMISII ȘI ASOCIAȚII PROFESIONALE ȘI ȘTIINȚIFICE

Sunt membru în asociații profesionale și științifice, iar în cadrul acestora, rezultatele cercetării sunt prezentate, discutate și popularizate.

1. Societatea Română de Medicină de Urgență
2. European Society for Emergency Medicine (EUSEM) NR: M-03700
3. Societatea Română de Morfologie normală și patologică
4. Colegiului Medicilor

Am fost referent specialist în comisii de susținere a tezei de doctorat:

- Cioloca Romeia, 2009, Corelaţii radioimagistice şi biologice în cancerul mamar.
- Ioanoviciu Dumitru Sorin, 2009, Implicațiile diagnostice, prognostice și potențial terapeutice ale angiogenezei și limfangiogenezei în cancerul gastric.
- Surpat Hulea Anca, 2013, Contribuții la studiul etiopatogenic al unor enterite bacteriene ale suinelor după înțărcare.

Am fost membru (secretar) al Comisiei de examen pentru obţinerea titlului de medic primar şi, respectiv, medic specialist anatomo-patolog; membru al comisiilor de examen pentru promovarea pe postul didactic de asistent universitar și șef lucrări și membru în comisii de examene pentru pregătirea tezei de doctorat.

În perioada, 2012 – 2016, am fost membru al Senatului Universității de Medicină și Farmacie „Victor Babeș" Timișoara.

Din anul 2017, am fost numit Șef interimar al Disciplinei de Urgențe/Chirurgie 1 Departamentul IX, UMF Timișoara, prin ordinul Senatului UMFT.

3.2. PARTICIPARE/ORGANIZARE - EVENIMENTE ȘTIINȚIFICE

Am participat la conferințe și simpozioane științifice unde am susținut prezentări orale sau poster:

- Simpozion Ştiinţific Anual FMV Timișoara, București, Iași, Cluj
- First Balkan Conference of Medical Mycology and Mycotoxicology – Balkan Fungus, Timișoara, 2018
- Euro-Regional Conference on Parasitic Zoonoses, Timișoara, 2014, 2016, 2018
- International Symposium Young People and Veterinary Medicine, Timișoara, 2017, 2018
- Saloanele de invenții naționale și Internaționale: Timișoara, București, Iași, Cluj, Geneva, Barcelona, Bruxelles.
- Conferința „Învățământul medical românesc: între tradiție, noutate și necesitate", Timișoara, 2006

- Conferința „Celula endotelială și angiogeneza tumorală”, Timișoara, 2007
- Conferința „Present and future in medical education”, Timișoara, 2008
- Conferința Internațională „Angiogenesis: present and future”, Timișoara, 2009
- XX International Symposium on Morphological Sciences, 2008
- III International Giardia & Cryptosporidium Conference, Orvieto, Italia, 2009
- EMOP, Cluj Napoca, 2012
- European Regional Conference on Goats, Debrecen, Ungaria, 2014
- EuroBiotech, 2015, 2016
- Simpozionul UV Timișoara, 2017
- SRBBM Timișoara, 2017
- 15th IFAA, Rome, 1999
- 19 th European Congress of Pathology, Ljubljana, Slovenia, 2003.
- 28 th European Congress of Cytology, Antwerp, Belgium, 2002.

Am fost membru al comitetului de organizare al diferitelor manifestări științifice, precum și al cursurilor postuniversitare care au urmărit dezbaterea diferitelor subiecte de histologie și citologie:

- Conferința „Învățământul medical românesc: între tradiție, noutate și necesitate”, Timișoara, 2006
- Simpozion Ştiinţific Anual FMV Timișoara, 2017, 2018
- First Balkan Conference of Medical Mycology and Mycotoxicology, Timișoara, 2018
- Curs de citologie normală și patologică, 1998
- Cursul Histologia pentru patolog, Histologia sistemului digestiv, 2001
- Curs de Citologie clinică, 2001
- Tehnici de citologie, morfohistochimie și imunohistochimie, 2003
- Curs de Citologie clinică, 2003
- Curs postuniversitar de Imunohistochimie, 2004
- Histologia pentru patolog, 2006.

3.3. PREMII

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- **Gelul pentru tratarea leziunilor uscate din demodicoza canină**
  Patent nr A 00075/1.02.2016

  Autori - Mederle Narcisa, **Mederle Ovidiu**, Morariu Sorin, Morariu Florica, Darabus Gheorghe, Oprescu Ion, Ilie Marius, Negrescu Adina

  - Medalia de aur – Salonul Internațional de Invenții, Geneva, 2017
  - Premiu Special – Taiwan Invention Association, Salonul Internațional de Invenții, Geneva, 2017
  - Mențiune Specială – China Delegation, Salonul Internațional de Invenții, Geneva, 2017
  - Medalia de aur – Salonul Internațional de Invenții, Barcelona, 2017
  - Premiu Special – Spania Delegation, Salonul Internațional de Invenții, Barcelona, 2017
  - Diploma de Apreciere –Bursa Nationala a Inventiilor Romanesti, 2017, Palatul Parlamentului, Bucuresti

- Diploma de Apreciere –Salonul Cercetării Românești, 2017, Palatul Parlamentului, Bucuresti
- Medalia de argint - Salonul Internațional de Invenții, Chisinau, 2017

❖ **Compoziție regeneratoare pentru păr și unghii**

Patent nr A 00621/1.02.2016

Autori - Milovanov Cornelia, Mederle Narcisa, Ahmadi Mirela, Morariu Sorin, Popescu Gabriela, Morariu Florica, Herman Viorel, Radulov Isidora, **Mederle Ovidiu**

- Diplomă de Excelență și Medalia de Aur cu menţiune specială – PRO INVENT, Cluj-Napoca, 2017
- Diplomă de Excelenţă acordată de „Asociaţia Iustin Capră" și Premiul Special din partea Romanian Association for Nonconventional Technologies, Bucharest, PRO INVENT, Cluj-Napoca, 2017
- Diplomă şi Medalie EuroInvent acordată de European Exhibition of Creativity and Inovation Iaşi, Romania, PRO INVENT, Cluj-Napoca, 2017
- Diplomă şi Medalie argint la Salonul internațional EUROINVENT, Iași, 2017
- Diplomă şi Medalie de aur la Salonul Internațional de Invenţii şi Inovaţii "Traian Vuia" Timișoara, 2017
- Diplomă "Bronze Medal" International Specialized Exhibiton „INFOINVENT" Chisinău, Republica Moldova, 2017
- Diplomă de Excelenţă de la EIS „INFOINVENT" Chisinău, Republica Moldova, 2017

❖ **Cremă pentru hidratantă piele și ten pe bază de Oenothera biennis**

Patent nr A 000269/1.02.2016

Autori - Horablaga Adina, Milovanov Cornelia, Ahmadi Mirela, Mederle Narcisa, Morariu Sorin, Morariu Florica, Horablaga Marinel, Popescu Gabriela, **Mederle Ovidiu**

- Diploma de excelență cu mentiune speciala – Universitatea de Stat de Medicină si Farmacie „Nicolae Testemitanu" din Republica Moldova, Salonul Pro Invent, 2018
- Diploma de Excelență – Academia Justin Capra, nr 107 / 23.03.2018, Salonul Pro Invent, 2018
- Diploma de Excelenta si Medalia de Aur - Salonul Pro Invent, 2018
- Diplome Inventions Geneva and Silver Medal – Salon des Inventions Geneve, 2018
- Honorable Mention and Medal from China Delegation –Geneva, 2018
- Diploma and Silver Medal – EuroInvent Iasi, 2018

- Diploma si Medalie de Aur –Salonul International de Inventii si Inovatii „Traian Vuia" Timisoara, 2018
- Silver Medal – International Exhibition of Technical Innovation, Patents and Inventions, 2018, Werk Arena Trinec, Czech Republic

Activitatea de cercetare desfășurată în direcția inventicii a fost recunoscută prin:

- Diploma de Excelență și cupa din partea Societății Inventatorilor din Banat – pentru merite aduse cercetării, Salonul Internațional de Invenții Traian Vuia, Timișoara, 2017.

C. PLANUL DE EVOLUȚIE ȘI DEZVOLTARE A CARIEREI

"Jocul este forma cea mai elevată a cercetării."

Einstein

Consider că implicarea sistemică, progresivă, continuă și relațională a studenților și a rezidenților în activitatea mea didactică este o cale sigură de a răspunde cerințelor în permanentă creștere ale pieței muncii, ale creșterii calității pregătirii profesionale în perspectiva integrării lor ulterioare în diverse domenii ale sistemului sanitar european.

Misiunea didactică este o responsabilitate dar mai ales o „instigare" pentru a menține echilibrul între caracterul instructiv-educativ al procesului de pregătire profesională, pe de o parte, și calitatea competențelor dobândite în timpul școlii, pe de altă parte, ca singură premisă pentru un onorant viitor al absolvenților.

Corelarea ofertei învățământului universitar cu cererea pieței de muncă din domeniu urmărește obținerea unei corespondențe adecvate între investiția materială și umană în procesului de învățământ și rezultatul acestei investiții cuantificat prin viitorii specialiști care se vor integra în domeniul vizat. Caracteristicile studiului actului didactic sunt premisa necesară ce asigură o bună pregătire și o motivație puternică pentru studiul individual.

Contextul internațional actual, dar mai ales apartenența la Uniunea Europeană dă noi valențe ale actului educațional. Ofertele economice din plan european, motivează o serie de absolvenți să plece în state ale Uniunii Europene, unde salarizarea este mai bună comparativ cu opțiunile autohtone. În domeniul medicinei, facultatea din Timișoara se află în aceeași arie geografică cu Universitățile din Novisad, Belgrad (Serbia), cu Universitățile din Szeged și din Debrecen, Semmelweis din Budapesta (Ungaria). Toate aceste centre universitare au un cuvânt de spus în competiția pentru atragerea tinerilor absolvenți de valoare din blocul european de est. Ofertele lor tentante și opțiunea pe care tinerii o au de a alege una din aceste universități culminează cu șansa reală de a concura direct pe o piață europeană la standarde ridicate. Ei provin mai ales din Germania, Italia, Olanda, Austria. Existența unor astfel de oportunități

oferite dinspre exterior trebuie egalate de posibilitatea absolventului român de a se ridica la standardele înalte necesare unui salariat european, respectiv este imperios necesar ca absolventul nu doar să fie familiar cu tehnicile de vârf existente, ci să le fi folosit deja, dotările unor astfel de locuri de muncă fiind la standarde ridicate.

Pentru a rezista concurenței, toate aceste facultăți au o puternică și permanentă colaborare cu mediul economic.

În întreaga lume, cercetarea fundamentală este strâns legată de sistemele de „spin-out" care eficientizează din punct de vedere economic ideile universitare în domeniul cercetării, dovedind caracterul antreprenorial al acestora.

Tot mai mult, cercetarea trebuie să se regăsească în produse/servicii/componente ale acestora care să aducă un plus valoare concret domeniului vizat. Sunt tot mai populare și încurajate echipele de cercetare create atât fizic dar mai ales virtual. Internetul, video-conferințele, testele virtuale și proiectele europene/internaționale pot ține în legătură și genera echipe de diferite specializări, din zone diferite ale lumii, din domenii diferite, fără a fi nevoie ca acestea să se întâlnească fizic decât atunci când este neapărată nevoie și totuși să fie într-o permanentă interrelație. În prezent, accesul la infrastructuri importante de cercetare este deschis, acestea fiind promovate internațional, iar așteptările sunt legate de rezultate cu proiecții economice cât mai rapide și mai eficiente.

Astfel se explică tendința universităților de a devin actori principali în activitatea de cercetare cu reflectare directă în domeniul economic.

Contextul National

Tot ansamblul de schimbări, de la cele politice până la cele economice se reflectă în plan național și implicit, în plan educațional și de cercetare. Un alt aspect este legat de apariția unor centre universitare private, cu consecințe majore asupra calității învățământului și performanțelor în cercetare. Astfel, studenții au posibilitatea sa „aleagă" nivelul exigențelor și calitatea actului didactic.

Tocmai de aceea, Facultatea noastră a încercat și consider că a reușit să dețină o dotare a laboratoarelor, ceea ce permite studenților o practică la nivel european. Acest lucru permite ca absolvenții noștri să aibă o bună experiență dobândită chiar din timpul Facultății, căci pe tot parcursul studiilor studenții devin niște colegi de-ai noștri, iar realizările sunt de fapt oglinda muncii noastre.

În domeniul cercetării, consider că lipsește încă elementul aplicativ, elementul de proiecție în producție. Tinerii sunt tot mai greu de cooptat în activitățile de cercetare, fiind

demotivați financiar. Este îmbucurător interesul studenților străini față de universitatea noastră și sperăm la un număr în creștere al acestora.

Plan de dezvoltare academică

Planul meu de dezvoltare academică cuprinde două părți: prima parte se referă la direcțiile pe care le vizez în domeniul didactic, iar cea de-a doua parte conturează țintele mele în domeniul cercetării, ambele planuri vizând creșterea prestigiului meu profesional.

Obiectivele didactice și de cercetare pe care le-am urmat până în prezent consider că sunt oportune și vor reprezenta direcția pentru dezvoltarea mea academică viitoare. Ele se încadrează în obiectivele Universității de Medicină și Farmacie „Victor Babeș" Timișoara.

1. Plan de dezvoltare în domeniul didactic

Intențiile viitoare raportate la cariera didactică vor fi concentrate în continuare pe student și pe nevoile sale de dezvoltare. Am în vedere o bună armonizare între predarea cunoștințelor teoretice de specialitate și partea de activități practice, respectiv: actualizarea planului de învățământ conform cu cerințele actuale cu cunoștințele noi din domeniu, prin stimularea up-datării programelor analitice, a bibliografiei cursurilor de specialitate. Cursurile se vor preda cu mijloace moderne și atractive, cu trimiteri la exemple, comparații, situații reale, menite să faciliteze înțelegerea de către studenți a subiectelor abordate. Optez pentru o metodă interactivă bazată pe comunicare și experimentare urmată firesc de abstractizarea și generalizarea datelor practice. Permanenta mea pregătire prin reactualizarea cunoștințelor va fi suportul pedagogic pentru actul de predare-învățare-evaluare. Strategia mea didactică pe care o consider de viitor este focalizată pe asigurarea unei oferte educaționale competitive pe plan național și european, în vederea formării unor profesioniști în acest domeniu și creșterea semnificativă a atractivității studiilor universitare.

Planul de dezvoltare în domeniul didactic vizează linii de dezvoltare în domeniul colaborării cu studenții, strategii de îmbunătățire a curiculei universitare în domeniul de interes, îmbunătățirea sistemului de evaluare, îmbunătățirea continuă a infrastructurii specifice activității didactice, asigurarea materialelor consumabile pentru activitățile didactice, precum și modernizarea învățământului postuniversitar.

Dezvoltarea relației cu studenții se bazează în primul rând pe reconfigurarea, completarea și menținerea programelor de studiu în consens cu cele de la nivel european. Existenta tehnologiilor vechi va face subiectul istoriei în domeniu. Studenții trebuie să cunoască, în primul rând, teoretic și apoi practic noile tehnologii apărute, care vor fi promovate

în contextul tematicii de predare. Pot confirma că, până în acest moment, tematica urmată a respectat aceste cerințe. În concordanță cu tematica disciplinei, voi încerca să completez permanent noutățile apărute în acest domeniu, astfel încât absolvenții să poată să profeseze cu succes oriunde în Europa sau în lume.

Toate aceste noutăți vor face obiectul următoarelor cursuri și manualelor de lucrări practice, cu scopul de a informa la zi studenții. Suportul virtual este binevenit din motivul costului scăzut și al posibilității adăugării facile, continue, de informații la zi.

Dezvoltarea abilităților practice ale studenților necesită dotări corespunzătoare, necesare în contextul menținerii universității la nivelul unui pol real de cunoaștere academică. Voi urmări ca permanent, o parte din fondurile de cercetare, pe care voi încerca să le obțin, să le utilizez pentru îmbunătățirea permanentă a infrastructurii specifice activității didactice dedicată studenților de a căror viitor mă consider și eu răspunzător.

Aș dori să pun bazele unui cerc științific în domeniul urgențelor medico-chirurgicale, iar într-o fază ulterioară a unui centru dedicat descoperirii și dezvoltării aptitudinilor practice ale studenților pentru efectuarea unor brevete de cercetare.

Un aspect important este legat de îmbunătățirea sistemului de evaluare a studenților, în conformitate cu noul sistem implementat la nivelul universității, cu accent pe aspectul practic, dar și pe stimularea însușirii conceptelor teoretice care guvernează interrelațiile dintre domeniile medicinei care definesc abordarea pacientului ca un tot unitar.

Voi urmări de asemenea să sprijin dezvoltarea învățământului postuniversitar, prin cursuri postuniversitare si workshop-uri care să permită specializarea absolvenților.

2. Plan de dezvoltare în domeniul cercetării

Creativitatea este un proces complex, intuitiv și spontan. Elementul cheie în procesul creativității este omul!

Spiritul creativității, al progresului, al responsabilității pentru sănătatea oamenilor mă vor ajuta, în continuare, să dezvolt activitatea de cercetare. Scopul meu în cercetare va fi o conectare permanentă la noutate, la inovare și competiție științifică.

Cercetarea universitară, ca și punctajele prin care suntem apreciați pentru această activitate, la nivel național, au în vedere publicații științifice în reviste relevante din fluxul național și internațional, accesarea de proiecte, publicarea de cărți de specialitate, vizibilitate națională/europeană prin prezentarea orală sau sub formă de poster de lucrări științifice în cadrul manifestărilor științifice. și a numeroase articole publicate în reviste cotate ISI și BDI, precum și într-o serie de lucrări științifice prezentate la congrese de profil.

Dezvoltarea activității mele de cercetare se bazează și pe conceptul de colaborare interdisciplinară. Voi întreține relațiile de parteneriat cu instituțiile cu care am realizat studii de cercetare și voi dezvolta noi relații de colaborare în țară și în străinătate.

Pentru activitatea mea ulterioară în domeniul cercetării consider oportună urmărirea următoarelor aspecte:

- **dezvoltarea echipelor din care am făcut parte până în prezent,** precum și extinderea acestora. În proiectele de cercetare conduse de mine sau în care am fost membru am contribuit la formarea de tinere echipe compuse din studenți sau/și tineri cercetători. Rezultatele obținute vor face, ca și până acum, obiectul congreselor studențești, pentru tinerii medici sau cercetători, cu dorința obținerii de premii, atât la nivel național cât și internațional. Voi continua să sprijin organizarea manifestărilor științifice studențești desfășurate în Timișoara sau ale centre universitare
- **accesarea și utilizarea bazelor de date internaționale** recunoscute, site-urilor naționale și europene, specifice cercetării, care permit accesul la informație recentă. Conectarea permanentă aduce beneficii în plan profesional și oportunitatea de a accesa la timp un call compatibil cu posibilități efective de înscriere în competiție
- **accesarea de proiecte naționale și europene**. Recunoașterea, vizibilitatea și prestigiul profesional pe plan național și internațional se poate obține și prin proiecte de cercetare. Până în prezent am făcut parte din colective de cercetare pentru 2 proiecte în calitate de director de proiect
- **implicarea în organizarea de manifestări științifice internaționale**, care îmi pot crește prestigiul profesional și vizibilitatea internațională, dar și relații științifice personale care ulterior pot fi fructificate prin schimburi profesionale, participări la publicații sau proiecte comune. Voi încerca, ca și până acum, invitarea, în cadrul următoarelor manifestări științifice la a căror organizare voi colabora, a unor personalități recunoscute din domeniu, din țară și din străinătate ca invited speaker sau lectori
- **efectuarea de cursuri sau stagii în străinătate** în vederea dezvoltării profesionale prin contactul cu colegii din străinătate, în domeniul meu de activitate. Dacă până în prezent, activitatea mea didactică a fost secondată de cea de cercetare, consider, că în viitor cele două direcții (didactică și de cercetare) se vor complete reciproc. În viitor, sper ca studenții mei să-mi devină adevărați parteneri în activitatea de cercetare
- **publicarea de lucrări în reviste indexate BDI și ISI**. În acest context, un aspect important al planului de dezvoltare personală în domeniul cercetării îl reprezintă

publicarea rezultatelor cercetării în reviste cotate ISI cu factor de impact, care asigură o mai bună vizibilitate la nivel internațional și, implicit, o oportunitate de creștere a prestigiului meu profesional

- **publicarea de tratate și monografii în domeniu**, în edituri naționale recunoscute sau edituri internaționale. Experiența acumulată mă obligă să abordez și alte teme din domeniu și să urmăresc publicarea în afara țării.

Diferitele roluri profesionale asociate celui de cadru universitar, respectiv de medic practician, cercetător, trebuie să fie armonios articulate pentru a nu neglija niciuna dintre aceste activități.

Sunt convins că experiența mea profesională, susținută de elanul și încrederea lor vor face posibilă realizarea unor cercetări deosebite în domeniul de interes.

Căci nici o descoperire nu are valoare dacă nu este împărtășită și rămâne uitată într-un sertar fără o aplicabilitate directă.

Desigur că întreaga mea activitate de cercetare, alături de colegi, studenți și rezidenți se va oglindi în activitatea mea didactică viitoare și va contribui la creșterea prestigiului meu internațional, dar și al Universității noastre.

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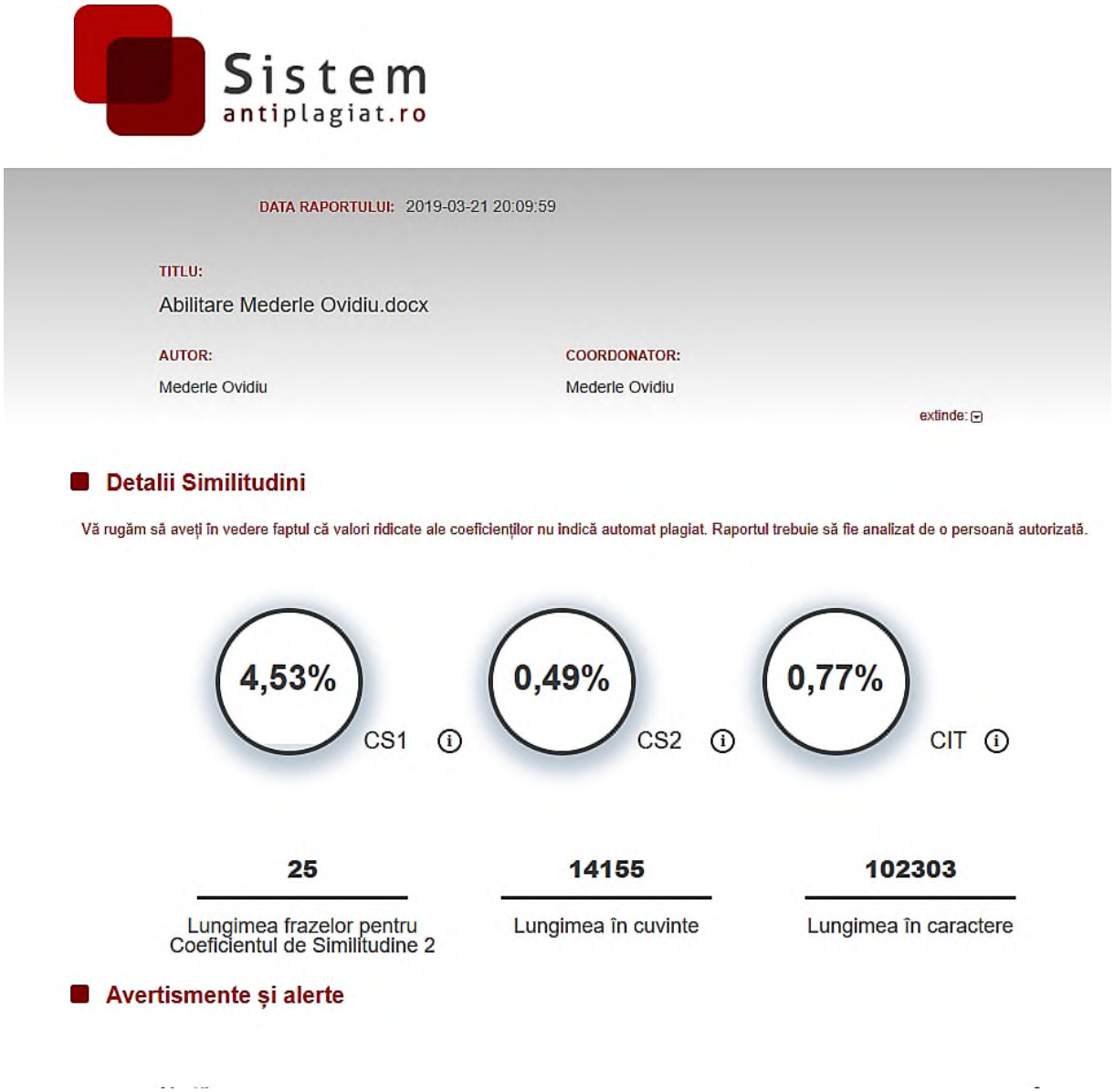
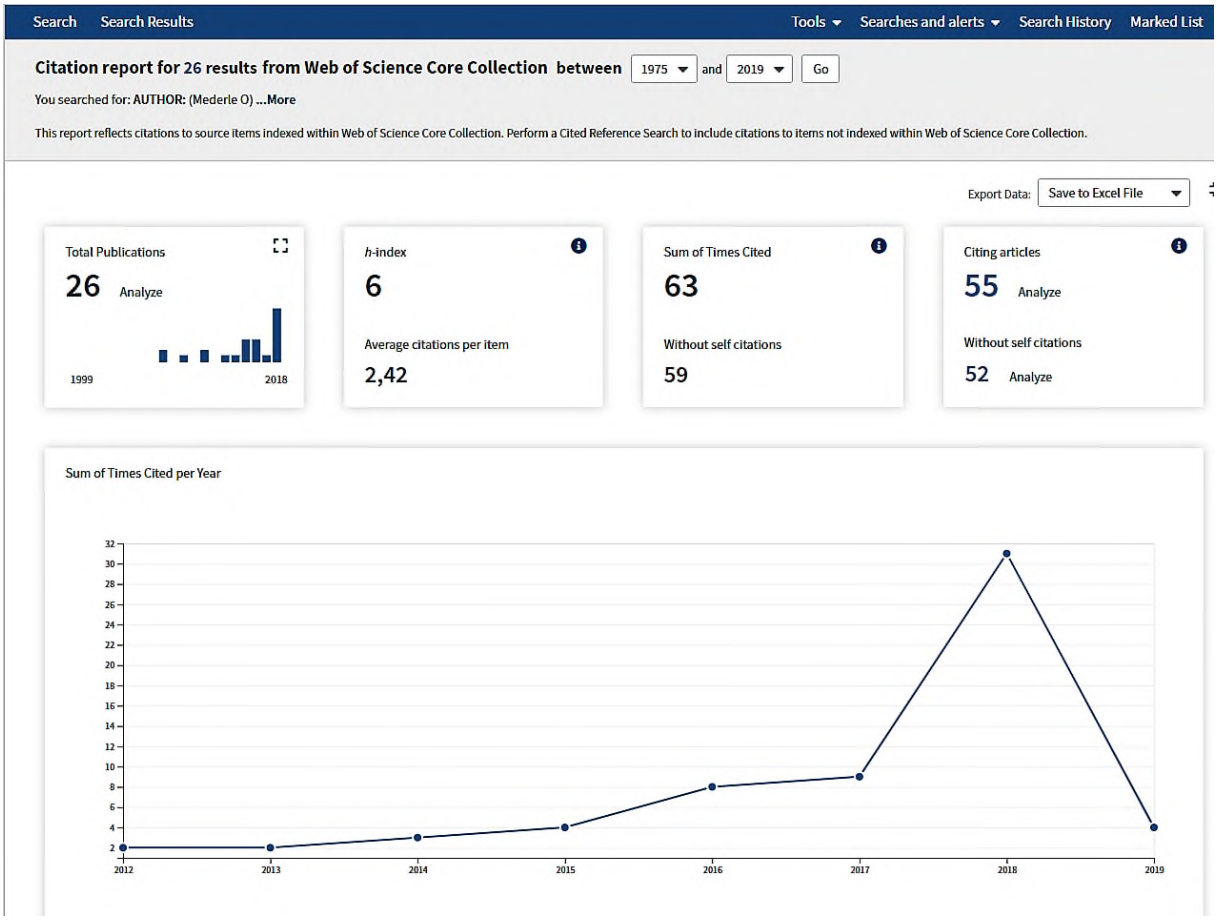
E. ANEXE

Anexa 1



Anexa 2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Anexa 3

DIPLOME REPREZENTATIVE

DIPLOMA

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OPRESCU Ion, ILIE Marius, NEGRESCU Adina

Banat's University of Agriculture Sciences and Veterinary Medicine
"King Michael I of Romania" from Timisoara/ Faculty of Veterinary Medicine

for the innovation

Gel for treatment of dry lesions in canine demodicosis

Gold medal

THE PRESIDENT OF
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Brussels, 19th November 2016

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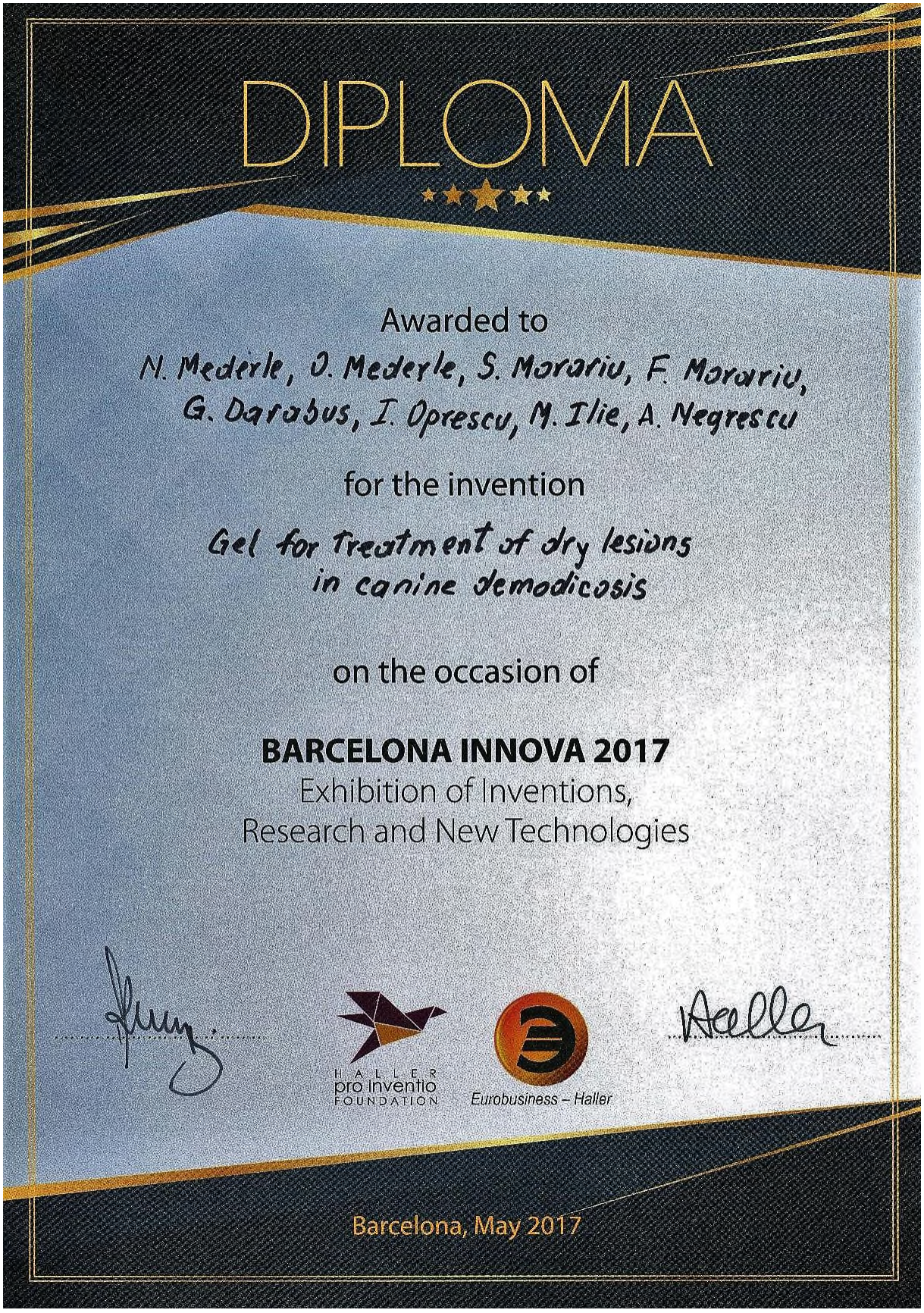

DIPLOMA

Awarded to

N. Mederle, O. Mederle, S. Morariu, F. Morariu, G. Darabus, I. Oprescu, M. Ilie, A. Negrescu

for the invention

Gel for treatment of dry lesions in canine demodicosis

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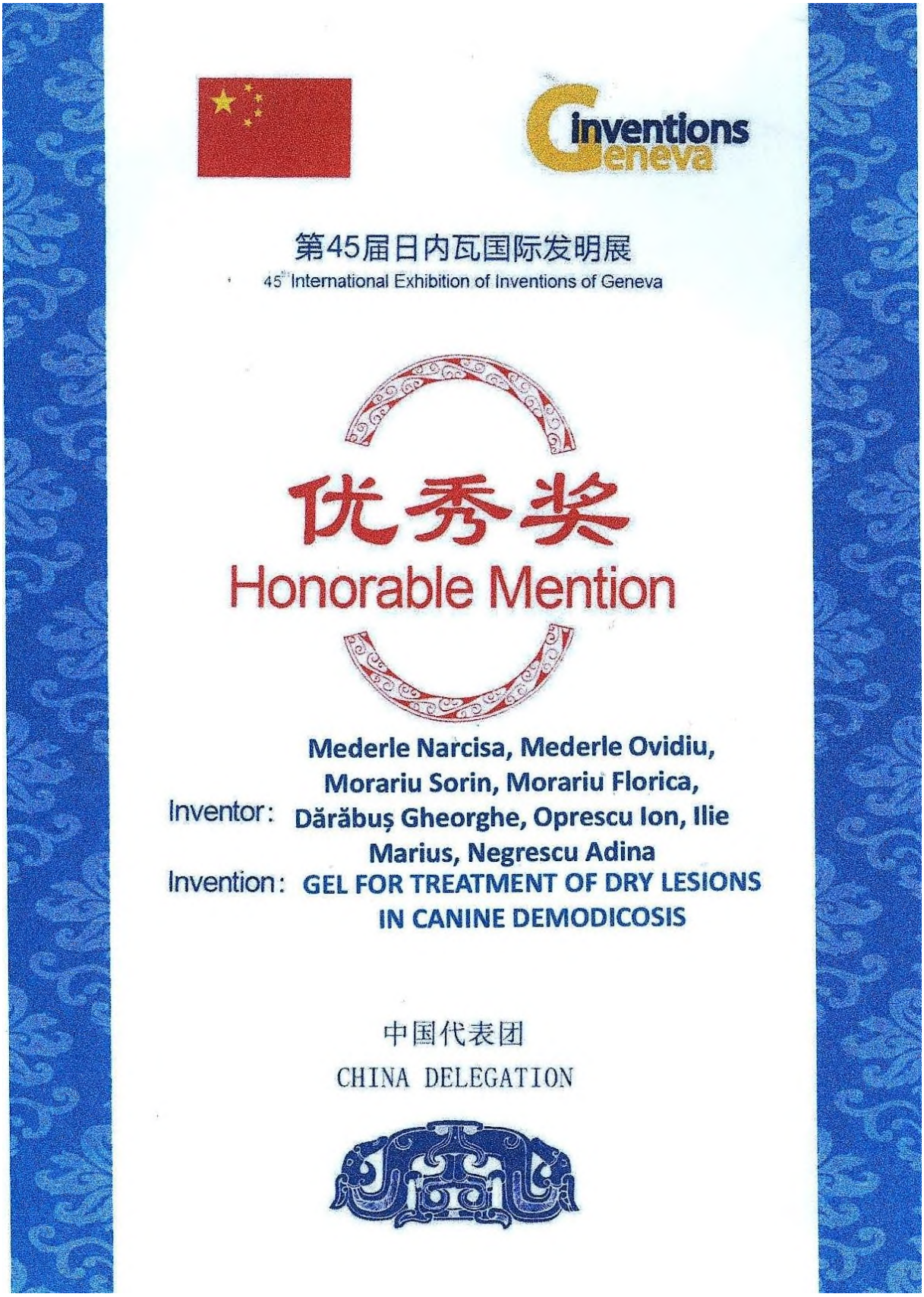
Eurobusiness – Haller

Barcelona, May 2017



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Invention: **GEL FOR TREATMENT OF DRY LESIONS IN CANINE DEMODICOSIS**

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CHINA DELEGATION

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SALON INTERNATIONAL DES INVENTIONS

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Après examen, le Jury International a décidé

de remettre à: MEDERLE Narcisa, MEDERLE Ovidiu, MORARIU Sorin, MORARIU Florica, DARABUS Gheorghe, OPRESCU Ion, ILIE Marius, NEGRESCU Adina

pour l'invention: Gel pour le traitement des lésions démodécie canine sèche

MÉDAILLE D'OR
GOLD MEDAL
GOLDMEDAILLE

Le Président du Jury: David Taji

Le Président du Salon: Jean-Luc Vincent

DIPLÔME

inventions Geneva

SALON INTERNATIONAL DES INVENTIONS

GENÈVE

Après examen, le Jury International a décidé

de remettre à: Adina HORABLAGA, Cornelia MILOVANOV, Mirela AHMADI-KHOIE, Narcisa MEDERLE, Sorin MORARIU, Florica-Emilia MORARIU, Nicolae Marinel HORABLAGA, Ovidiu Alexandru MEDERLE, Gabriela POPESCU

pour l'invention: Crème hydratante pour le corps et le visage basée sur biennis oenothera

MÉDAILLE D'ARGENT
SILVER MEDAL
SILBERMEDAILLE

Genève, le 13 avril 2018

Le Président du Jury: David Taji

Le Président du Salon: Jean-Luc Vincent

Inventions Geneva

第46届日内瓦国际发明展

46th International Exhibition of Inventions of Geneva

优秀奖

Honorable Mention

Inventor: **Adina HORABLAGA, Cornelia MILOVANOV , Mirela AHMADI-KHOIE, Narcisa MEDERLE, Sorin MORARIU, Florica-Emilia MORARIU, Nicolae Marinel HORABLAGA, Ovidiu Alexandru MEDERLE, Gabriela POPESCU**

BANAT'S UNIVERSITY OF AGRICULTURE SCIENCES AND VETERINARY MEDICINE "KING MICHAEL I OF ROMANIA" FROM TIMIȘOARA

Invention: **MOISTURIZING CREAM FOR BODY AND FACE BASED ON OENOTHERA BIENNIS**

中国代表团

China Delegation

invent arena

INTERNATIONAL EXHIBITION

OF TECHNICAL INNOVATIONS, PATENTS AND INVENTIONS

20TH – 22ND JUNE 2018, WERK ARENA TŘINEC, CZECH REPUBLIC

SILVER MEDAL

INVENTION 5
5005

OENOTHERA BIENNIS SKIN-MOISTURIZING CREAM

INVENTOR

HORABLAGA ADINA, MILOVANOV CORNELIA, AHMADI-KHOIE MIRELA, MEDERLE NARCISA, MORARIU SORIN, MORARIU FLORICA-EMILIA, HORABLAGA NICOLAE MARINEL, MEDERLE OVIDIU ALEXANDRU, POPESCU GABRIELA

USAMVB TIMISOARA, ROMANIA

JOSEF KRATOCHVÍL
PRESIDENT OF JURY

JAN KOBIELUSZ
CHAIRMANN OF THE INVENT ARENA ORGANIZING COMMITTEE

LUKÁŠ ZMEŠKAL
PRESIDENT OF THE CZECH UNION OF INVENTORS AND RATIONALIZERS

TŘINECKÉ ŽELEZÁRNY · ČESKÁ HUTNICKÁ SPOLEČNOST, Z.S. · IFIA INTERNATIONAL FEDERATION OF INVENTORS' ASSOCIATIONS · ČESKÝ SVAZ vynálezců a zlepšovatelů · ÚŘAD PRŮMYSLOVÉHO VLASTNICTVÍ