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Experimental and Applied Biomedical Research, Faculty of Medical Sciences,
University of Kragujevac 69 Svetozara Markovica Street, 34000 Kragujevac, PO Box 124, Serbia

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ORAL DISORDERS IN SJÖGREN'S SYNDROME

Mirjana Sijan Gobeljic[1], Vera Milic[2], Nada Pejnovic[1] and Nemanja Damjanov[2]

[1]*Institute of Rfleumatology, Belgrade*
[2]*University of Belgrade Medical Scflool, Institute of Rfleumatology, Belgrade, Serbia*

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Corresponding author:

Nada Pejnovic, M.D., PhD

Institute of Rheumatology, Resavska 69, 11000 Belgrade, Republic of Serbia

Phone: +381 645773282

E-mail: nada.pejnovic@gmail.com

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ABSTRACT

Sjögren's syndrome (SS) is a complex, chronic, systemic, autoimmune disease that mainly affects the exocrine glands, especially the salivary and lacrimal glands, leading to the dryness of the mouth and eyes, along with fatigue, joint and muscle pain. The prevalence of SS is estimated to be between 0.05% and 1% in European population. Diagnosis of SS is based on the revised criteria of the American-European consensus group (AECG). Sjögren's syndrome can be subclassified into primary disease (primary Sjögren syndrome, pSS) and a secondary disease (secondary Sjögren syndrome, sSS) when present with rheumatoid arthritis (RA), systemic lupus erythematosus (SLE) and systemic sclerosis. The decrease in salivary flow and qualitative alterations in saliva could explain many of the oral manifestations frequently present in patients with SS. Low salivary flow may affect chewing, swallowing, speech and sleeping in pSS patients. Oral manifestations include dental erosion, dental caries, mucosal infection, ulcers and oral candidiasis. Recent studies reveal that pSS patients experience impaired olfactory and gustatory functions and have higher occurrence of oral complications such as dysgeusia, burning sensation in the tongue (BST) and halitosis. The exocrine manifestations and systemic involvement in SS significantly impact the patient's perception of oral health related quality of life (OHRQoL).

Keywords: *Sjögren's syndrome, xerostomia, oral manifestations, chemosensory disorders.*

INTRODUCTION

Sjögren's syndrome (SS) is a complex systemic autoimmune disease in which body's immune system *attacks* exocrine glands, most commonly the salivary and lacrimal glands. The hallmarks of this autoimmune disease are lymphocytic infiltration of the exocrine glands and thepresence of circulating autoantibodies (anti-Ro/SS-A and anti-La/SS-B). The most common symptoms that occur inpatients with SS are dryness of the mouth and eyes, along with fatigue, muscle and joint pain (1).

This common rheumatic disease has insidious onset and affects primarily older women. Genetic traits and triggering factors such as infections, stress and hormonal factors play a role in the pathogenesis of Sjögren's syndrome (SS). Sjögren's syndrome can be subclassified into primarydisease (primary Sjögren syndrome, pSS), and a secondarydisease (secondary Sjögren syndrome, sSS) when present with other autoimmune rheumatic diseases such as rheumatoid arthritis (RA), systemic lupus erythematosus (SLE)and systemic sclerosis. The overall prevalence of pSS is between 0.05-1% in European countries (1, 2).

The impaired function of salivary glands accompanied with insufficient secretion of saliva results in many oral complications such as infections, periodontal disease, caries and gustative and olfactory dysfunctions that affect oralhealth-related quality of life (OHRQoL) in patients with pSS. Patients with pSS commonly experience oral disorderson a daily basis which indicates that dental professionals have to pay more attention and recognize initial symptoms and various oral manifestations of Sjögren's syndrome andto keep close communication with rheumatologists (3).

PATHOPHYSIOLOGY OF SJÖGREN'S SYNDROME

Although the pathogenesis of pSS in not completely understood the convincing data demonstrate the important role of genetics, innate and adaptive immune cells such as dendritic cells (DCs), T and B cells in this autoimmune disease. Circulating autoantibodies form immune complexes which when precipitated cause damage of affected tissues and organs (4).

Genetic predisposition to Sjögren's syndrome

There is strong evidence that genes involved in the function of innate and adaptive immune cells are associated with the susceptibility to primary SS including genes involved in the interferon (IFN) signaling pathway, antigenpresentation, lymphocyte signaling and NF-kB pathway. Genetic studies in pSS patients revealed single nucleotide polymorphisms (SNPs) in HLA alleles (HLA-DRA, HLA-DQB1, HLA-DQA1), STAT4, IRF5, IL-12A and TNIP1.

The strongest genetic link apart from HLA antigen region is in the IRF5 gene locus. In response to type IFN-α, IRF5 gene encodes IRF5 protein in monocytes which promotes production of type 1 IFN, interleukin (IL)-6, IL-12, and tumor necrosis factor alpha (TNF-α). The genetic variants ofSTAT4 and IL-12A (p35 subunit of IL-12) genes involved in regulation of IFN-γ secretion are associated with heritable predisposition to pSS. Polymorphisms within thegene for B lymphocyte tyrosine kinase (BLK), the kinase involved in B cell development and differentiation and in gene for TNFAIP3-interacting protein 1 (TNIP1) of the NF-kB pathway have also shown associations with the susceptibility to pSS (5). Recent study using novel approach which combines concept profile analysis (CPA), an online tool that retrieves literature based on the concepts of interest and gene expression data on peripheral blood cells and exocrine glands in female patients with SS identified several candidate genes that regulate the expression ofmatrix metalloproteinase (MMP) 9. The identified genes are CHEK1 (Checkpoint kinase 1), ETS1 (avian erythroblastosis virus E26 oncogene homolog 1), LEF1 (Lymphoid enhancer-binding factor-1), X-linked gene TIMP1 (MMPsinhibitor), and CXCL10. CHEK1 gene encodes serine/ threonine-specific protein kinase and together with the ETS1 regulates development of tumors through the transcriptional regulation of MMPs. LEF1, a member of high mobility group (HMG) protein family is expressed in pre-B and T cells and plays an important role in cancer development. CHEK1, ETS1, LEF1 and CXCL10 increase the expression of MMP9, the enzyme that has been implicated inthe pathogenesis of SS. MMP9 could deteriorate the structural integrity of the salivary glands resulting in decreased salivary flow rate in patients with SS (6).

Innate immunity in Sjögren's syndrome

Emerging evidence suggest an important role for the innate immune system in the pathogenesis of pSS. The pattern recognition receptors (PRRs) including TLRs and nucleotide oligomerization domain (*NOD*)-like *receptors* (NLRs) in innate immune cells detect pathogenassociated molecular patterns (PAMPs) and endogenous molecules from damaged cells (DAMPs). Initial events in pSS might be viral infection and hypoestrogenism that lead to activation of salivary and lacrimal gland epithelium. The activation of salivary gland epithelial cells (SGECs) and antigen presenting cells (DCs and macrophages) via PRRs result in the upregulation of MHC class I and II and co-stimulatory molecules and secretion of proinflammatory cytokines. Under the influence of IFN-γ and TNF-α secreted from innate immune cells tight junction proteins in SGECs lose their integrity causing decreased saliva production.

The activated plasmocytoid DCs (pDCs) via TLRs in salivary glands produce high levels of IFN-α particularlyin individuals with the IRF5 and STAT4 risk alleles. DCs function as antigen presenting cells in salivary tissue and promote expansion of autoreactive T cells and somatic hypermutation of B cells. On the other hand, DCs may exert tolerogenic activities by promoting expansion of SS-A autoantigen specific regulatory T cells (Trges) inpSS. Macrophages are recruited to salivary glands prior to lymphocytes and express proinflammatory IL-18 and IL-12 thereby amplifying ongoing

inflammation. The role of natural killer (NK) cells in pSS remains elusive. NK cells express NCR3/NKp30, an activating receptor that after ligation to NKp30 ligands on SGECs promotes IFN-γ secretion and polarization of Th1 cells.

TLR2 and TLR4 expression is increased in SGECs in patients with pSS and ligation of these PRRs results in production of high levels of the proinflammatory IL-17A. TLR3 binds double-stranded RNA of viral origin and leadsto secretion of proinflammatory cytokines and BAFF fromSGECs. In pSS, antibodies against SS-A in complex with hYRNA can bind and stimulate TLRs causing an inflammatory response. Microbial or tissue components may also be recognized by cytoplasmic receptors NLRs. Oneof the NLRs, NLRP3 oligomerise to form inflammasome multiprotein complex that activates caspase proteins and subsequent secretion of IL-1 and IL-18. In pSS, the NLRP3inflammasome is activated by concomitant stimulation of TLRs and purinergic P2X4/P2X7 receptors. Upregulation of P2X7, NLRP3 and caspase-1 genes correlate with focal lymphocytic sialadenitis, and increased expression of bothIL-1 and IL-18 indicate inflammasome hyperactivity in patients with pSS (7-9).

Adaptive immunity in Sjögren's syndrome

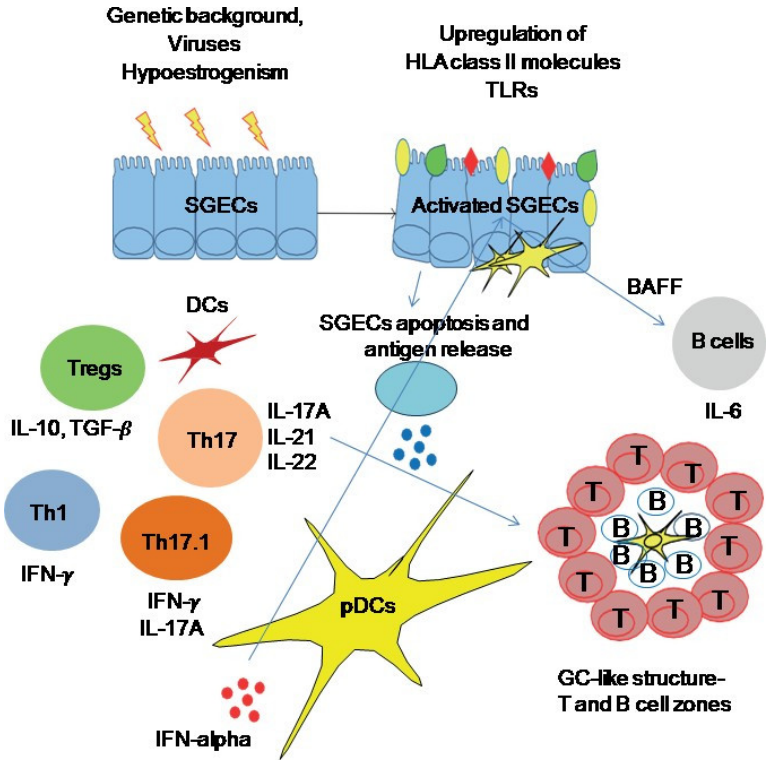
$CD4^+$ T cells, but also $CD8^+$ T cells, B cells and DCs are present in salivary gland infiltrates in patients with pSS. These immune cells form tertiary ectopic lymphoid structures with T- and B-cell zones in advanced lesions. The pathogenic role of various $CD4^+$T helper (Th) subsets,Th1, Th2, Th17, Treg cells and follicular helper T (Tfh) cells in pSS are incompletely understood. Naive $CD4^+$T lymphocyte differentiate towards Th17 cells in the concurrent presence of IL-6, TGF-β, IL-21, IL-1β, and IL-23. Th17 cells have upregulated retinoic acid orphan receptor(ROR)γt transcription factor and express IL-17, but also IL-21 and IL-22. High and consistent expression of IL-17 in the periductal infiltrates of all minor salivary glands hasbeen documented in patients with pSS. Th17 are the mainsource of IL-17 in affected exocrine glands, but γδ T cells, NK cells, innate lymphoid cells (ILCs) and CD8+ T cells also produce IL-17. Th17 cells in salivary glands may be locally differentiated from naive T cells or recruited from theblood. Th17 cells are elevated in patients with moderate-to-high systemic disease activity, the findings that suggest its association with disease severity and/or with certain stages of the disease. Th17 cells in affected exocrine glandsmight promote B cell activation and formation of germinalcenters. Abundant expression of IL-17 in salivary glandsin patients with pSS may drive the transformation of Th17cells towards both IFN-γ single producing Th1 cells and Th17.1 cells which produce both IL-17 and IFN-γ and coexpress CXCR3 and CCR6 receptors. Th17.1 cells may contribute to disease progression in pSS considering the well-established role of IFN-γ in the pathogenesis of pSS. In addition, most of the cytokines that support the Th17 phenotype such as IL-6, IL-21, IL-22, and IL-23 and their receptors, are consistently expressed in salivary glands of patients with pSS (10, 11).

Recent evidence suggests the pathogenic role of IL-17 in the initiation and progression of pSS. IL-17 stimulates the production of proinflammatory cytokines and MMPs that may cause destruction of salivary glands. The recent meta-analysis revealed that IL-17 is increased in the serum,tears, saliva, and salivary glands of pSS patients, particularly in RF positive patients and in patients without immuno-suppressive therapy. The levels of IL-17 correlated with thedisease severity of pSS. Moreover, pSS disease severity hasbeen linked to increased co-expression of IL-17 and IL-18 in pSS. In addition, IL-17 could be a key prognostic factor for lymphoma development in patients with pSS (12, 13).

Treg cells express high levels of CD25 and forkhead box protein P3 (FoxP3) transcriptional factor and suppress autoreactive lymphocytes via either cell-cell contact or the release of IL-10 and transforming growth factor β (TGF-β). TGF-β is required for differentiation of bothTregs and pathogenic Th17 cells, but the concurrent presence or absence of IL-6 leads to the generation of either Th17 or Tregs, respectively. The role of Treg cells in pSS pathogenesis is not clear. Several studies reported an overall reduction of $CD25^{high}$Treg cells in peripheral blood of pSS patients, whereas other studies reported an increaseof circulating Tregs. Similarly, no definitive conclusioncan be made regarding the role of Tregs in salivary gland tissue. FoxP3 positive Tregs were detected in minor salivary glands in pSS and their expression correlated withthe severity of tissue inflammation. Whether Treg cells exert their suppressive activity *in vivo* is not certain. Also, Treg cells can be turned into a Th17 cells in the presence of appropriate stimuli. Transcriptional factors, RORγt andFoxP3, interact and block each other's function and in the course of pSS TLRs-stimulated production of IL-6 along with IL-23 by DCs, will lead to generation of pathogenic Th17 cells (14).

IL-12 is composed of p35 and p40 subunits, and bioactive IL-12p70 in myeloid cells induces IFN-γ production and polarization of Th1 cells. Recently, a novel anti-inflammatory cytokine composed of the IL-12p35 and EBI3 subunits has been identified as IL-35. Recently, significant association between a SNP within the IL-12A locus (rs485497) and susceptibility to pSS has been shown. IL-12p70 level is elevated in sera of pSS patients and correlates with active disease and IL-12 positive cells infiltrate salivary glands of pSS patients. IL-35 exerts immunosuppressive effects on Th17 cells and induces the generation of novel regulatory cell population, iTr35 cells. pSS patients with high disease activity have low level of circulating IL-35, indicating that IL-35 could efficiently control the disease activity and possibly prevent the occurrence oflymphoma (15).

Figure 1. Current model of pSS immuno-pathogenesis



Initial event in the pathogenesis of primary Sjögren's syndrome is the activation of salivary gland epithelial cells (SGECs) and upregulation of HLA class I and II antigens, TLRs and autoantigen presentation. The subsequent activation of plasmacytoid dendritic cells (pDCs) induces the production of high levels IFN-α in individuals with the risk alleles of the susceptibility genes IRF5 and STAT4, which induces production of B-cell activating factor (BAFF) from SGECsthat stimulate B cells.

Activated SGECs and DCs secrete proinflammatory cytokines and chemokines leading to activation of Th17 cells in salivary gland infiltrates. Th17 cells differentiate towards Th17.1 cells or Th1 cells. IL-17A, IL-22, IL-21 and IFN-γ amplify ongoing inflammation, promote expression of MMPs and induce germinal center (GC) formation in salivary glands along with autoantibodies production. B cells in salivary glands produce IL-6, and further stimulate formation of Th17 cells. Tregs cells via IL-10 and TGF-β suppress proinflammatory Th1 cells, Th17 cells and the Th17.1.

The cause of pSS is not known, but it is presumed that microbial pathogens (viral antigens) may lead to activationof pDCs and production of IFN-α. B cells are adaptive immune cells that produce antibodies and B cell activating factor (BAFF) which is induced by type I and type II interferons and promotes activation, proliferation and maturation of B cells. Elevated BAFF levels in circulation and salivary gland tissues and increased expression of genes related to interferon type I or type II are linked to more severe pSS. Salivary glands in pSS contain ectopic germinal centers which further highlight the role of activated B cells in pSS. There is plethora of autoantibodies in sera of patients with pSS, among which some of them are diagnostic or associated with early disease onset, enlargement of parotid gland or extraglandular manifestations (16). Fig.1 summarizes the current concept of the pathogenesis of pSS. Research of the immune-mediated mechanisms in the pathogenesis of pSS will yield the new insights into potential new targets for biologic therapies in this disease.

AUTOANTIBODIES IN SJÖGREN'S SYNDROME

Patients with pSS have many circulating autoantibodiesdirected against self-antigens and current research aims indefining new biomarkers for early diagnosis of pSS. The most common autoantibodies in patients with pSS areshown in Table 1. Autoantibodies may be present in patients` sera years prior to the diagnosis of the pSS. Anti-Ro/SS-A and anti-La/SS-B autoantibodies are detected in two-thirds of patients with pSS and have been associated with more severe dysfunction of exocrine glands and morepronounced lymphocytic infiltrates in the minor salivary glands. Rheumatoid factor is often found in patients with pSS and is usually associated with positive anti-Ro/SS-A and anti-La/SS-B autoantibodies (17).

Table 1. Common autoantibodies in patients with pSS

Autoantibodies in pSS	Prevalence	Clinical Association
Anti-Ro/SSA	50-70%	Younger age, severe disease, extraglandular manifestations, neonatal lupus
Anti-La/SSB	25-40%	Extraglandular manifestations, neonatal lupus
RF	36-74%	Systemic disease, severe disease
Anti-CCP	3-10%	Arthritis
AMA	3-10%	Elevated liver enzymes
ACA	3-27%	Raynaud's phenomenon

Anti-Ro/SSA and anti-La/SSB antibodies were originally described as two antibodies reacting with salivary andlacrimal glands' antigens in patients with SS. Ro/SS-A antigen is a RNP complex constituted by two different Ro proteins of 52kDa, and 60 kDa that binds to small cytoplasmic RNAs known as hY-RNAs. The biological functions of Ro/SS-A complex are not known, but there is some evidence that the Ro 60-kD protein is involved in a discard pathway for defective 5S rRNA precursors. La/SS-B antigen isa 48 kDa phosphorylated protein, located in nucleus and cytoplasm which binds to many RNA molecules during thecell cycle. Functionally, La/SS-B protein is a transcription termination factor for RNA polymerase III transcripts. Unlike SS-A and SS-B antigens which are found in every cell, novel autoantibodies as biomarkers of SS are directedto proteins selectively present in the salivary and lacrimal glands. These are auto-antibodies to salivary protein-1 (SP-1), parotid secretory protein (PSP), and carbonic anhydrase VI (CA-6). SP-1, PSP, CA-6 proteins have various physiologic activities, including roles in the attenuation of microbial agents. PSP is a protein that is involved in the binding and clearance of infectious agents, while CA-6 is an enzyme in the acinar cells of submandibular and parotidglands involved in buffering of saliva (18).

Patients with pSS have autoantibodies directed against muscarinic acetylcholine type 3 receptors (M3R)in their sera and these IgG antibodies functionally inhibit salivary secretion. Namely, acetylcholine (ACh) released from parasympathetic nerves regulate water and electrolyte transport in salivary glands via M3R, a prototypical G-protein-coupled receptor (GPCR), endogenously expressed in salivary glands. M3R IgG autoantibodies may directly bind and occupy M3R receptors and their continued presence lead to M3R internalization and progressive loss of M3R function. Borda et al. (19) recently demonstrated that autoantibody to M3R and pilocarpine induced the increment of superoxide dismutase (SOD) and catalase (CAT) and the production of nitric oxide (NO) and prostaglandin E2 (PGE2) in patients with pSS. The increased activity of SOD and CAT in pSS patients maybe regarded as a defensive reaction to the increased reactive oxygen species (ROS), which may cause damage of salivary glands. Kim et al. (20) reported that downregulation of MHC I from the cell membrane is associated with the presence of anti-M3R autoantibodies which is a novel pathogenic mechanism in pSS.

The most specific autoantibodies for both the classification and the diagnosis of pSS are directed towards intracellular antigens Ro52/SS-A, Ro60/SS-A and La/SS-B. Rheumatoid factor (RF) is commonly found in the sera of patients with autoimmune rheumatic diseases including pSS. RF is associated with the presence of anti-Ro/SS-A and anti-La/SS-B antibodies as well as systemic disease and is associated with disease severity and more severe exocrine gland manifestations (keratoconjunctivitis sicca).

RF and cryoglobulins are detectable in 35-70% and 5-10%of patients, respectively and correlate with clinical featuresof pSS such as lymphomagenesis (17). Anticentromere antibodies are present in 5-15% of pSS patients and these patients have more frequent Raynaud's phenomenon, 'scleroderma-like clinical features' and primary biliary cirrhosis and more pronounced dysfunction of exocrine glands with higher risk of developing salivary gland lymphoma. Anti-mitochondrial antibodies and anti-smoothmuscle antibodies have been described in pSS patients in association with primary biliary cirrhosis and autoimmuneliver involvement. Anti cyclic-citrullinated peptides (anti-CCP) antibodies, found in low prevalence in pSS patients, are related with nonerosive arthritis and RF positivity (21).

CLINICAL MANIFESTATIONS IN SJÖGREN'S SYNDROME

Patients with pSS may present with sicca syndrome, general symptoms and systemic manifestations. Classical symptoms of pSS are combination of dryness of the eyes (xeropthalmia) and oral cavity (xerostomia). Xerostomia may predispose to oral complications such as oral candidiasis, dental caries and periodontal disease. Ocular infections, photosensitivity and destruction of the cornea are possible complications of xerophtalmia. Sicca syndrome may also present as hoarseness, non-productive cough, skin dryness and, in woman, dyspareunia. Among general symptoms, fatigue is most commonly present in the majority of patients with pSS. In addition, pSS patients experience chronic pain due to accompanying fibromyalgia and/ or polyarthralgia along with depression and anxiety whichare often seen in pSS patients. SS is a systemic disease strongly associated with organ specific and systemic autoimmunity. Numerous systemic manifestations are presentin patients with SS (22, 23) as shown in Fig. 2.

DIAGNOSIS OF SJÖGREN'S SYNDROME

The diagnosis of pSS is based on the American-European consensus group (AECG) classification criteria for Sjögren syndrome (24). These criteria include: subjective presence of ocular dryness; subjective presence of oral dryness; objective measures of ocular dryness by Schirmer's test or corneal staining; focus score > 2 in a salivary gland biopsy; salivary scintigraphy showing reduced salivary flow (1.5 mL in 15 minutes) and/or diffuse sialectasias andpositive autoantibodies against SS-A and/or SS-B. PrimarySS is diagnosed when 4 out of 6 items are present; either salivary gland pathology or the presence of autoantibodiesagainst SS-A/SS-B is mandatory. The diagnosis of secondary SS is made when patients present with underlying autoimmune diseases such as RA, systemic SLE or scleroderma which were diagnosed prior to developing their sicca symptoms.

Figure 2. Clinical manifestations in pSS

Neurological complications
peripheral neuropathy
cranial neuropathy

Dry eyes

Dry mouth

Glandular problems
parotid, submandibular and
lacrimal gland enlargement

Pulmonary involvement
bronchitis
lymphocytic interstitial pneumonitis
pleurisy

Liver complications
hepatitis
cirrhosis

Vascular Problems
vasculitis
Raynaud's phenomenon

Kidney problems
interstitial nephritis
renal tubular acidosis
hypokalemia

Arthralgia/Arthritis

Fatigue

Ultrasonography of the major salivary glands (parotid and submandibular glands) is not included in classificationcriteria, but may be helpful in diagnosis or follow-up of patients with pSS (25-28).

EULAR validated two indexes for the assessment of disease activity in primary Sjögren's syndrome. The EULAR Sjögren's Syndrome Patient Reported Index (ESSPRI) is the mean of three visual-analogue scales that measure mouth and eye dryness, fatigue, and pain in a simple patient-administered questionnaire (29). The EULAR Sjögren's Syndrome Disease Activity Index (ESSDAI) 20 assesses systemic complications of the disease in 12 domains and is used mainly in clinical trials (30). ESSPRI and ESSDAI do not have diagnostic value in pSS. 2016 ACR/EULAR criteria incorporated ESSDAI to be assessed in patients with suspected SS who complain of either ocular or oral drynessor systemic involvement. There is a need for subjective or objective determination of the level of the disease activity in clinical practice. However, ESSPRI and ESSDAI are mainly used in clinical trials. In the last years, a numberof clinical trials using biological therapy for the treatment of SS have been performed and some are still ongoing. In order to define key inclusion criteria and response criteriain clinical trials with biologic drugs, it is important to haveobjective measures of both, disease activity and disease damage which could also improve clinical research in primary SS. European League Against Rheumatism (EULAR)SS study group recommends use of ESSDAI and ESSPRIin assessment disease activity in the clinical studies. A criterion for applying systemic therapy in SS is ESSAI $\geq$ 5, whereas therapeutic response is achieved when ESSDAI isdecreased for $\geq$ 3.

THERAPY OF SJÖGREN'S SYNDROME

There is no cure for SS and the main treatment goals are relieving of symptoms, prevention of complications and selection of patients for immunosuppressive therapy. Therapy of SS requires multidisciplinary approach with the *team* including a *rheumatologist*, a *dentist* and an ophthalmologist since the occurrence of oral and ocular manifestations and complications are frequent. If systemiccomplications exist the contribution of other specialists is needed.

Therapy of SS is mainly symptomatic. At present, the data from a small number of randomized clinical studies are not conclusive regarding the use of systematic therapyin patients with SS. Management of oral manifestations includes intense oral hygiene, prevention and treatmentof oral infections, use of saliva substitutes, and local and systematic stimulation of salivary secretion. Cholinergic agents, such as pilocarpine is the cornerstone of current therapy in SS. The treatment of patients with SS includes substitution therapy with artificial tears and saliva. Patientsare advised to take fluids more frequently and sugar-free chewing gums as local active sialogouges. Smoking, use of alcohol beverages and sparkling water should be avoided. When hyposalivation exist the oral cavity is less protected and oral mucosa becomes vulnerable to various physical and microbial insults. Therefore, keeping the oral hygiene is of utmost importance in prevention of oral complications and regular visits to dentist are recommended.

Given the fact that exocrine gland dysfunction in SS is related to chronic inflammation it is surprising that treatment of patients with low dose corticosteroids or immunosuppressive agents such as methotrexate, cyclosporine and azathioprine have not been efficient in achieving increased function

of lacrimal or salivary glands (31). Hydroxychloroquine is0 efficient treatment drug for numerous extraglandular manifestations of the disease and also for increasing the lacrimal and salivary flow rate (31). Anti-TNF therapy (infliximab and etanercept) have not demonstrated any efficacy in patients with extraglandular disease (32). The anti-B-cell therapy with anti-CD20 autoantibody (rituximab) and anti-CD22 monoclonal antibody (epratuzumab) is a promising option for glandular and extraglandular manifestations of the disease, as well asfor the management of SS-associated lymphoma (33, 34). Several immune pathways exist that biological drugs couldinterfere as a target therapy such as BAFF-APRIL pathway,modulation of T cell co-stimulation, IL-17 and chemokinesblockers, and IL-6 blockers as a clinical trial aimed at assessing Tocilizumab in pSS is actually ongoing (35).

ORAL DISORDERS IN SJÖGREN'S SYNDROME

Sjögren's syndrome is characterized by dysfunction and destruction of the major salivary glands. Patients with pSS may present with parotid enlargement, but also with isolated submandibular gland enlargement. The hallmarks ofpSS are decreased secretion of saliva and xerostomia whichnegatively affect oral health in patients. Optimal saliva secretion is essential for swallowing, eating, oral cleansing, speech, digestion and taste (3).

Saliva

Saliva contains mainly water and a mixture of proteins, glycoproteins, enzymes, and many antimicrobial molecules such as defensins, proteases, lysozyme essential to maintaining oral health (36). Saliva secretion is regulated by mechanical and chemical factors, and food and chewingstimulate salivary flow. Mechanical and chemical afferent signals are transmitted from sensory receptors in the periodontal ligament and taste buds via the trigeminal, facial, and glossopharyngeal nerves to the salivary nuclei in the medulla oblongata of the brain. Efferent impulses to the salivary glands are transmitted via sympathetic and parasympathetic autonomic nerves (37).

Xerostomia, dryness of the mouth, is associated with diminished saliva secretion when unstimulated salivaryflow is below 50% of the normal. However, recent data suggest that xerostomia in SS patients is a result of the alterations of salivary mucins rather than low salivary flow. Additionally, changes of proteins that maintain cell-cell and cell-extracellular matrix interactions have been reportedin SS patients and these changes may affect the quality of saliva and may even promote chronic inflammation (38).

Dryness of the mouth is not specific symptom for SS and may be found in other diseases (sarcoidosis, diabetes) or could be related to medication use (antidepressants, antihistaminics, diuretics) or by head and neck radiation. Xerostomia Inventory (SXI) is a questionnaire which results represent, subjectively, the severity of xerostomia. 2012 ACR/EULAR criteria excluded the subjective criteria of oral dryness in order to increase the specificity of the criteria. However, 2016 ACR/EULAR criteria included the subjective evaluation of ocular or oral dryness underscoring the importance of anamnestic data and subjective assessment of disease progression. In order to pre-select patients with suspicious SS the 2016 ACR/EULAR criteria are intended to be applied to any patient with at least 1 symptomof ocular or oral dryness (based on American-European Consensus Group [AECG] questions) or suspicion of SS due to systemic features derived from the ESSDAI score.

One of the diagnostic criteria is unstimulated salivary flow (UWS), whereas stimulated salivary flow (SWS) is used for the evaluation of the residual functional tissue of salivary glands. This is important regarding the therapy with *secretagogues*, as these agents are used only if thereis functional tissue remained. Stimulated salivary secretion is used for the evaluation of parotid gland function as these glands produce saliva on stimulation, while submandibular glands continuously produce saliva. Xerostomia is diagnosed if unstimulated salivary flow is ≤1,5ml/15 min. Sialometry has sensitivity of 56% and specificity of 81% forthe diagnosis of SS (39).

Hyposalivation in patients with pSS can cause stomatitis, ulcers and atrophic changes in the oral mucosa. Low salivary flow rate, but also changed quality of salivais causes of intraoral manifestation in patients with pSS. Salivary epidermal growth factor (EGF) is considered an important cytoprotective factor that maintains mucosal integrity. Recently, it was shown that salivary EGF output in SS patients was significantly lower than that in non-SS patients, EGF output decreased with prolonged diseaseduration, and more rapidly than salivary flow rate and wasrelated with poor quality of life in SS patients. These findings suggest that topical EGF supplementation may be a novel therapeutic strategy in patients with pSS (40).

Emerging evidence suggest that patients with pSS have different oral microbiome compared to healthy subjects but whether these changes are disease specific or a consequence of reduced salivary secretion is not elucidated. The recent study demonstrated that patients with pSS hadheterogenous oral bacterial composition and the relative abundance of Haemophilus, *Neisseria* and *Lactobacillus* was related to lower stimulated salivary flow and not with pSS, indicating that hyposalivation is the reason for these changes, while lower *Streptococcus* abundance in patients with pSS appears to be a disease-specific (41).

Investigation of novel biomarkers is performed for their potential use in diagnosis and the prognosis of pSS. Salivary proteomics is a promising tool since proteins in saliva may closely reflect the underlying disease processes in the salivary glands in pSS. Namely, the decrease in manysecretory proteins has been found including α-amylases precursor, carbonic anhydrase VI, PIP, SPLUNC-2, G3P-DH, and

cystatins. The available data indicate that secretory proteins of acinar origin were reduced in pSS patients,while inflammatory phase proteins are increased in comparison with healthy subjects. The increased expressionof β2-microglobulin, Immunoglobulin κ light chain and Immunoglobulin γ light chain in pSS saliva is thought to reflect both B-cells activation and an increased immunoglobulin synthesis within salivary glands. Furthermore, an increase of proteins related to the systemic and local inflammation (α-enolase, lipocalin and S100-A7 and A9 proteins) was found. Proteomics represents a novel approach for use in diagnosis of pSS. The proteomics studies so far have described putative protein biomarkers that will eventually contribute to diagnosis of pSS and stratification of different disease subsets (42, 43).

Figure 3. Oral manifestations in pSS

Dental erosion
Dental caries
Mucosal friability
Dry cracked or peeling lips
Angular cheilitis
Dry plaque laden coarse tongue
Erythematous tongue
Mucositis
Ulcers
Oral candidiasis
Halitosis
Oral/dental infection

Oral manifestations

Oral manifestations are commonly present in patients with pSS as shown in Fig. 3. The low salivary secretion leads to less lubrication and diminished antimicrobial propertiesof saliva which results in the increased incidence of various oral disorders. Patients with pSS present with moreoften conditions such as dental erosion, dental caries, drycracked or peeling lips, angular cheilitis, dry plaque ladencoarse tongue, erythematous tongue, mucosal infection,ulcers, oral candidiasis, halitosis and oral/dental infection. The most common oral complication of Sjögren's syndrome is dental caries, specifically, root and incisal caries,which are less frequent in general population. The dentalcaries process involves dental biofilm and bacterial species that colonize tooth surfaces. Sjögren's syndrome patients have been reported to have higher numbers of cariogenic and acidophilic micro-organisms which indicatesalteration in oral microbiota. Sjögren's syndrome patientsdue to low salivary flow cannot sufficiently perform selfcleansing of oral cavity that includes buffering, lubricating and antimicrobial effects. Oral candidiasis is another common and often chronic complication of Sjögren's syndrome. Patients with SS and oral candidiasis usually present with signs such as angular cheilitis, atrophy orloss of filiform papillae on the dorsal tongue or erythema of the tongue and other mucosal surfaces (44). There are several reports describing common oral complications in patients with pSS (45-47).

Musculoskeletal symptoms occur in patients with pSS. The most frequent joints involved are hands, wrists, ankles, and feet, but also temporomandibular joint (TMJ) may be involved. In addition to oral manifestations and rheumatologic implications, Sjögren's syndrome seems to play a role in TMD especially as regards to muscular contracture (high prevalence of myalgia to muscle palpation and positive endfeel) and joint disease (greater pop-noise and deflection during mandibular opening), causing an increase in orofacial painand an altered chewing function (48).

SS is a common and underdiagnosed autoimmune, chronic inflammatory disease of the exocrine glands witha significant impact on oral health. Dentists are frequentlythe first to encounter the patients with early signs of SS. Therefore, dentists should be familiar with the manifestations of the disease and their contribution to the diagnosis,management and treatment of the oral complications of SSare essential.

Chemosensory disorders and other oral complaints in patients with Sjögren's syndrome

Taste and smell impairments have been reported in patients with pSS (49-51). There are scarce data about chemosensory disorders and other oral complaints, such as dysgeusia, burning sensations in the tongue (BST) and halitosis in patients with pSS. Chemosensory disordersinclude gustatory and olfactory dysfunctions, in which the senses of taste or smell can be reduced, distorted, or totally absent. Olfactory disorders are classified as follows: anosmia (complete loss of smell), hyposmia (reduced ability to smell), and dysosmia (distorted smell perception) (52). Gustatory disorders are similarly classified, namely ageusia (complete loss of taste), hypogeusia (partial loss of taste), and dysgeusia (distorted taste perception in the presence of normal stimuli) (53). Many patients with chemosensory disorders experience burning sensations or numbness in the mouth, especially inor on the tongue, the sensations that may originate in the gustatory nerve fibres (54). Burning mouth syndrome (BMS) is defined as the intensive burning sensation inthe tongue (BST), or burning in other mucosal membranes, lasting for at least 4-6 months (55). Patients with both SS and burning mouth syndrome (BMS) often have similar oral complaints; however, these diseases have significantly different etiopathogeneses, diagnostic criteria, and treatments. SS is a chronic

autoimmune disorder most commonly present in middle age women. BMS isa chronic disorder characterized by burning sensation of the oral mucosa in the absence of any apparent clinical abnormalities or local and/or systemic etiology. Similarto SS, BMS affects women more commonly than men and patients may report oral dryness, but the xerostomia is often perceived rather than actual. The diagnostic challenges are evidenced by the fact that both SS and BMS patients typically experience symptoms for years prior to achieving a diagnosis (56). Halitosis (or oral malodour, defined as an unpleasant breath odour of oral or extraoral origin), is another common oral complaint that canbe associated with low salivary secretion or chemosensory disorders (57).

Recent study demonstrated poorer olfactory and gustatory function, more often reported dysguesia, BST and halitosis, lower salivary secretion rates and poorer OHRQoL in 31 patients with pSS in comparison with healthy subjects(58). Interestingly, authors have not observed correlationsbetween saliva secretion rates and the presence of dysgeusia, BST, halitosis or olfactory and gustatory scores. For OHRQoL assessment the short-form Oral Health Impact Profile (OHIP-14) was used in this study. The OHRQoL was found to be associated with dysgeusia, BST and halitosis and no associations with saliva secretion rates. Thereare conflicting results whether hyposalivation causes smelland taste impairments as well as burning sensation in the mouth, since no correlation between low salivary flow andtaste performance was observed (59).

Burning sensation in the mouth, dysgeusia and halitosis are common complaints among SS patients and the important notion is that the intensity of discomfort cannot be associated to clinical symptoms (54, 57). Current findings show that pSS patients' OHRQoL was more influenced by dysgeusia, BST, and halitosis, rather than by impaired smell and taste functions. Oral malodour was present in almost half of the patients in the study, but the question remains whether they had a genuine halitosis. The presence of chemosensory disorders such as smell andtaste dysfunction in these patients may be an alternative explanation for this complaint. This study shows that there were no associations between dysgeusia, BST, halitosis, orchemosensory dysfunction with patient's age, the number of medications taken or the disease duration. Presumably, a questionnaire better designed to detect taste and smell dysfunction is needed to gain more specific insight into the patients' chemosensory experiences and oral healthrelated quality of life.

Possible underlying cause of the olfactory and gustatory dysfunctions in pSS patients could be immunopathological mechanisms operating in pSS. Systemic chronic inflammation in primary Sjögren's syndrome is associatedwith overexpression of interferon-inducible genes (60) and higher response of B cells and monocytes to stimulation with IFN-α and IFN-γ (61). Proinflammatory pathways mediated by Toll-like receptors and interferons in taste tissue may interfere with normal taste transduction and turnover of taste bud cells (62). Further studies of inflammatory pathways in the peripheral taste and smell organs at the cellular and molecular level are warranted in order to gain more knowledge regarding the pathogenesis of oral disorders in pSS patients.

The oral manifestations in pSS could present as higher number of decayed, missing and filled teeth; and higher plaque index, gingival index and papillary bleeding index. Thus, it is very common that these patients require dentalimplants to rehabilitate any extractions arising from decayor periodontal disease. In addition, such patients are often treated with immunomodulators (hydroxychloroquine, methotrexate) and sometimes with immunosuppressivedrugs that affect patient's immune response. Results ofthe studies suggest that the dental implant therapy in SS patients seems to present high implant survival rate, low marginal bone loss (MBL) and low biological complications. In addition, an increase in the quality of life of SS patients who were rehabilitated through dental implants isdocumented (63, 64).

CONCLUSION

In conclusion, patients with Sjögren's syndrome commonly experience many oral complications including impaired olfactory and gustatory functions and a higher occurrence of oral disorders such as dysgeusia, BST and halitosis. Due to their predominant oral symptomatology patients are often firstly seen by dental professionals who must recognize the signs and symptoms of xerostomia. Early diagnosis, prevention and treatment are crucial to maintaining oral health in patients with SS. Health care professionals have to work together to improve patient outcomes and quality of life for the patient diagnosed withSjögren's syndrome.

CONFLICTS OF INTEREST

The authors declare no financial or commercial conflicts of interest.

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ACYLCARNITINES' LEVEL IN THE DRIED BLOOD SPOT SAMPLES OF HEALTHY NEWBORNS IN SERBIA-THE PILOT STUDY

Andjelo Beletic[1], Aleksandra Tijanic[1], Tatjana Nikolic[2], Petr Chrastina[3], Aleksandar Stefanovic[2,4] and Sanja Stankovic[1]

[1]*Center for Medical Biochemistry, Clinical Center of Serbia, Belgrade, Serbia*
[2]*Clinic for Gynecology and Obstetrics, Clinical Centre of Serbia, Belgrade, Serbia*
[3]*Department of Pediatrics and Inherited Metabolic Disorders, First Faculty of Medicine, Charles University and General University Hospital, Prague, Czech Republic*
[4]*University of Belgrade, Faculty of Medicine, Belgrade, Serbia*

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Corresponding author:

Andjelo Beletic

Center for Medical Biochemistry, Clinical Center of Serbia, Belgrade, Serbia

Phone: +381 60 1511083

E-mail: andjelobeletic78@gmail.com

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ABSTRACT

Analysis of the acylcarnitines' (ACs) is the mainstay for screening for fatty acid oxidation disorders (FAOD). Data about the ACs profile in the dried blood spot samples of healthy newborns in Serbia are not at disposal. Therefore, we determined the ACs levels and established the cut-offs. Between August 2018 and August 2019 a total of 1771 samples had been analysed. Cut-offs, established using a non-parametric approach, were verified in comparison with the worldwide target ranges and the data for several Caucasian populations. The majority of ACs had comparable distribution in Serbian and the worldwide population. In case of discrepancy, the individual alterations had a frequency of less than 10%. Seventeen out of 25 established cut-offs were in the worldwide target range. Reliability of the cut-offs positioning out of the target ranges is not jeopardized, since alterations are negligible or similar findings were reported for other Caucasian populations. The established and verified set of cut-offs can be used in the future screening for carnitine uptake/transport defect, medium-chain acyl-CoA dehydrogenase deficiency, very long-chain acyl-CoA dehydrogenase deficiency, long-chain L-3 hydroxyacyl-CoA dehydrogenase deficiency, trifunctional protein deficiency, carnitine palmitoyltransferase deficiency Ia and II, as well as carnitine:acylcarnitine translocase deficiency.

Keywords: *Acylcarnitines, newborns, fatty acid oxidation, tandem mass spectrometry, Serbia.*

INTRODUCTION

Acylcarnitines (AC) have an essential role in the fatty acid oxidation (FAO), one of the main catabolic pathways in majority of human cells (1). Therefore, the fluctuations in the AC levels can mirror the presence of the FAO disorder (FAOD). These genetic conditions represent a heterogeneous group of more than 15 autosomal recessive traits, in which a deficiency of FAO enzymes and transporters profoundly depletes the potential for energy production (2). The FAOD are rare diseases, with the estimated joint frequency of 1: 5,000-10,000 newborns. Nevertheless, their manifestations can be extremely complex and often life-threatening. They are triggered by the prolonged fasting or/and increased energy demand; whereby clinical phenotype depends on the age of onset. The therapy includes lifelong nutritional support, avoidance of fasting and intensive therapy in situation of metabolic exacerbation (3).

Analysis of the ACs level is the mainstay of the FAOD diagnostics. Furthermore, the introduction of ACs analysis in the dried blood spot samples (DBS) from newborns, using tandem mass spectrometry, allowed a reliable, robust and cost-effective screening for FAOD. Additional positive outcomes followed-the diagnostic efficiency increased and created the path for timely diagnostic interventions to avoid metabolic crises and other highly deleterious sequels (3-5). Also, NBS results updated data on the FAOD epidemiology, what is the prerequisite for follow-up and genetic counselling (2). In line with that, the Recommended Uniform Screening Panel (RUSP), created by the American College of Medical Genetics (6), claims that every NBS should include the following five FAOD, marked as the "core": carnitine uptake defect/carnitine transport defect (CUD), medium-chain acyl-CoA dehydrogenase deficiency (MCADD), very long-chain acyl-CoA dehydrogenase deficiency (VLCADD), long-chain L-3 hydroxyacyl-CoA dehydrogenase deficiency (LCHADD) and trifunctional protein deficiency (TFPD). The decreased levels of free carnitine (C0), acetylcarnitine (C2), palmitoylcarnitine (C16), stearylcarnitine (C18) and oleylcarnitine (C18:1) indicate the presence of CUD. Screening results showing the increased levels of hexanoylcarnitine (C6) and octanoylcarnitine (C8), accompanied with the increased C8/C2 ratio are presumptive of MCADD. A raise in concentration of tetradecenoylcarnitine (C14:1), tetradecanoylcarnitine (C14), together with increased C14:1/C2 and C14:1/C16 ratios, represent the positive screening results for VLCADD. Finally, LCHADD and TFPD share the screening features: increased hydroxy stearylcarnitine (C18-OH), hydroxy palmitoylcarnitine (C16-OH) and hydroxy oleylcarnitine (C18:1-OH), supported with a raise in C18-OH/C18 and C16-OH/C16. Besides "core", these ACs and ratios allow for screening three more FAOD: carnitine palmitoyltransferase deficiency Ia and II (CPTD-I, CPTD-II) as well as carnitine:acylcarnitine translocase deficiency (CACTD). Results indicative for CPTD-I are high C0, decreased C14, C16, C18, C18:1 and (C16+ C18:1)/C2, whereby C0/(C16+C18) raises. Apart of C0, which is not significantly changed, the screening pattern, mutual for CPTD-II and CACTD, is inverse to the pattern indicative for CPTD-I (7, 8).

Assessment of the ACs level in the population of healthy newborns is the initial prerequisite for the establishment of a FAOD screening programme (8). Currently, such data for the Republic of Serbia are not available (9). Accordingly, our aim was to determine the level of abovementioned ACs and ratios, as well as to establish and validate their preliminary cut-offs for the healthy newborns.

PATIENTS AND METHODS

Newborns

We were including apparently healthy newborns from the Clinic for Gynecology and Obstetrics, Clinical Centre of Serbia, Belgrade, Serbia during the period August 2018-August 2019. The exclusion criteria were premature delivery, parenteral nutrition and transfusion (4). Ethics Committee of Clinical Center of Serbia approved the study (Permission No. 747/33/19.07.2018) and mother of each newborn signed the informed consent.

Samples

DBS samples were collected from heel, following the approved standard recommendations (10). Time of collection was between 48 and 72 hours after the delivery (4).

Laboratory methods

After punching a 3.2 mm DBS disk, ACs were extracted and derivatized with the commercial kit [Amino Acids and Acylcarnitines from Dried Blood (Chromsystems, Grüfelfing, Germany)], according to the manufacturer's instructions. ACs were analysed on the modular line consisting of Nexera XR LC-20 AD pump with the associated devices (Shimadzu, Kyoto, Japan) and 3200 Q TRAP LC-MS/MS System (AB Sciex, Framingham, MA, USA). For quality assurance purposes, we used commercial control material [MassCheck® Amino Acids, Acylcarnitines, Succinylacetone Dried Blood Spot Controls Level I and II (Chromsystems, Grüfelfing, Germany)].

Statistical analysis

The normality of ACs and ratios distribution was analysed with the Kolmogorov-Smirnov test. The Tukey procedure identified outliers i.e. results below lower quartile minus 3 times the interquartile range, or above the upper quartile plus 3 times the interquartile range. Distribution of the ACs values was graphically compared with the 1%, 50% and 99%ile for the worldwide population of healthy newborns obtained in the Region 4 Stork (R4S) Study and Centers for Disease Control project (R4S) (7). Recommendations from the CLSI guidelines (8) were the basis for establishing the cut-off values. Following the

statistical approach lower cut-offs were set at the 0.1%ile and higher at 99.9%ile value. The calculation-based approach was applied for the higher cut-offs only. They remained at the 99%ile of the tested population when it was higher than the worldwide 99%ile. Otherwise, they were calculated as 50%ile plus two times the range between the 99%ile and 50%ile, if no outliers were present, i.e. plus three times the mentioned range in case of the outliers' presence.

RESULTS

The study included a total of 1771 newborns. None of the ACs and ratios had the normal distribution (Table 1-3). Comparison with the distribution in worldwide normal population (7) showed that in 1.30% of the tested newborns C0 was below 11 μmol/L (range: 10.03-10.95 μmol/L). For C2, 0.51% results were below 10 μmol/L (range: 8.93-9.99 μmol/L) and one result (58.26 μmol/L) was above the world 99%-ile. Just two samples had C6 concentration reduced under 0.02 μmol/L (0.017 and 0.019 μmol/L). In case of C8, 0.68% of values were lower than 0.02 μmol/L (range: 0.013-0.019 μmol/L), while distribution of C14 and C14:1 results fitted between the 1% and 99%-ile of the worldwide normal population (7). The C16 was lower than 0.8 μmol/L in only one sample (0.65 μmol/L). Also, 6.32% of the C16-OH results were below 0.01 μmol/L (range: 0.001-0.009 μmol/L). There were no C18 lower than 0.31 μmol/L and four results exceeded 1.70 μmol/L (range: 1.71-1.78 μmol/L). The C18-OH concentrations also corresponded to the range between 1% and 99%-ile esteemed on the worldwide level (7). However, 2.65% of the tested newborns had C18:1 higher than 2.5 μmol/L (range: 2.51-2.81 μmol/L) and 8.19% of the C18:1-OH results remained under 0.01 μmol/L (range: 0.001-0.009 μmol/L).

The cut-offs established for ACs and ratios, together with the target ranges from the R4S project (7) are quoted in Table 1. The lower cut-offs for C0, C2, C0/(C16+C18) and (C16+C18:1)/C2 were in the agreement with the ranges, while in case of C16 (6.2%), C18 (6.4%) and C18:1(14.3%) the cut-offs were above the range. Among the higher cut-offs, when they were set using statistical approach, only the cut-off for C18 fitted into the target range. Much better concordance was present for the calculated cut-offs. Twelve of them was in the range: C0, C14:1, C16, C16-OH, C18-OH, C18:1, C0/(C16+C18), C8/C2, C14:1/C2, C14:1/C16, (C16+C18:1)/C2 and C18-OH/C18. Three cut-offs positioned above [C6 (16.7%), C18 (16.3%) and C18:1-OH (25.0%)] and the same number remained below the range [C8 (9.5%), C14 (8.0%) and C16-OH/C16 (3.0%)].

DISCUSSION

This paper reports the pioneer data for the Republic of Serbia, about the levels of ACs measured using tandem mass spectrometry in DBS of healthy newborns. In general, there is an acceptable concordance between the ACs distribution on the worldwide level (7) and in Serbian population. For the half of the ACs the levels fitted between the 1%ile and 99%ile of the worldwide population (7) or only individual deviations were present. Furthermore, in cases with more prevailing discrepancies between the distributions, the alterations' frequency of the individual ACs did not exceed 10%. Also, the intervals for the worldwide 1%ile i.e. 99%ile, calculated on the basis of the corresponding coefficient of variations (7), include the "extreme" values of all measured ACs except C16-OH, C18:1 and C18:1-OH. In the recent article about NBS experience in Turkey, the 1%-ile of normal population for C16-OH was 0 μmol/L [11], which is similar as in our study. For the purpose of screening for FAODs only increase in C18:1-OH levels represent a positive result (7), so the values that remained out of the worldwide distribution pattern, do not seem to merit a substantial attention. Nevertheless, the interpretation of C18:1 levels deserved additional caution, because it is the only AC with the distribution skewed to exceed the 99%-ile of the world population (7). In addition, the range of the "extreme" C18:1 results overlapped with the lower quartile of the values encountered in CACTD and CPTD-II (4, 7, 11). Since the results of other screening markers in the newborns with the "extreme" C18:1 results were not indicative for CACT or CPT-II (7), we attributed this finding to specificities of the tested population and set the higher cut-off for C18:1 at the 99%ile of the investigated population.

The applicability of the traditional statistical approach of setting the cut-offs at the 0.1% and 99.9%ile (8) was questionable. For the lower cut-offs the concordance with the worldwide target range (7) was rather acceptable. Nevertheless, that was not the case for almost all higher cut-offs, which were all below the range, thus raising the probability of false-positive results. Therefore, we referred to the recommendations for calculative adjustment of the cut-offs (7), similar to other authors (11), and achieved that more than a half of the calculated cut-offs were in the target range, established in the R4S project (7). This outcome was satisfactory, since McHugh et al. reported the overall agreement of 42% between the participants' individual cut-offs and corresponding range (7). Also, the differences in the structure and quantity of data between R4S project and our study, together with the consequential methodological specificities, could have an impact on the agreement rate. In R4S project the total amount of data, comprising almost 30 million of healthy and more than 10 000 newborns confirmed with certain metabolic disorders, allowed to calculate the target interval between the 99%ile of population with disorder and 1%ile of normal population in case of the low cut-offs or *vice versa* for the high cut-offs (7). However, we analyzed only samples from healthy newborns, so the additional calculative adjustments (7)were necessary to establish the cut-offs.

Even in cases when the calculated cut-offs remained out of the worldwide target, their applicability is not substantially jeopardized. Considering the low cut-offs for C16, C18 and C18:1, it is noteworthy that their decreased levels are not primary markers for any of the "core" FAOD (2, 4, 7,11-13). Also, almost all ratios including these ACs are within the

target ranges, thus additionally indicating the negligible significance of the discordance. Similar to our results, the cut-off for C6 in Italian population was 0.25 μmol/L (4), thus exceeding the R4S Project range (7). Also, the discrepancy with the target range was noted for C8 cut-off, likewise the population of the Republic of Slovenia, one of the countries neighboring the Republic of Serbia (14). The position of cut-off for C14 slightly below the target range may be attributed to the additional adjustments of the worldwide target range to improve specificity and minimize the probability of false positive results (7). In context of interpretation, the cut-off of C16-OH/C16, positioned below the target range, seems to be successfully "counterbalanced" with the cut-off of C16, placed within the corresponding range. C18 is the only of the investigated AC for which the preliminary high cut-off should be set at 99.9%ile. Finally, the cut-off for C18:1-OH slightly exceeding the rather narrow target range may not be surprising, specially taking into the account that the same cut-off is also used for NBS in Czech Republic (13) and Turkey (11).

The utmost outcome of our study are the preliminary interpretative patterns, necessary for successful future implementation of NBS for FAOD in Serbia. In case of MCADD, the most frequently FAOD (15), the pattern consists of C6>0.28 μmol/L, C8>0.19 μmol/L and C8/C2>0.011. For CUD the primary screening marker will be C0<10.10 μmol/L, supported with C2<9.28 μmol/L, C16<0.85 μmol/L, C18<0.33 μmol/L and C18:1<0.56 μmol/L. Presence of VLCADD can be suspected when the results show C14:1>0.54 μmol/L, C14>0.46 μmol/L, C14:1/C2>0.024 and C14:1/C16>0.147. Confirmatory investigation of the remaining two "core" FAOD, LCHADD or TFPD, will be necessary in newborn with C18-OH>0.08 μmol/L, C16-OH>0.11 μmol/L, C18:1-OH>0.10 μmol/L, C18-OH/C18>0.108 and C16-OH/C16>0.032. The results showing C0>64.43 μmol/L, C0/(C16+C18)>27.55 and (C16+C18:1)/C2<0.1 enable differentiation of CPTD-I from CUD. CPTD-II and CACTD can be distinguished from VLCAD by the finding of C16>6.37 μmol/L, C18>1.75 μmol/L and C18:1>2.64 μmol/L. The C0/(C16+C18) <2.82 and (C16+C18:1)/C2>0.393 are the features providing the distinction of CPTD-II and CACTD from CPTD-I.

Significantly lower number of participating newborns in comparison with the R4S project (7) and several individual studies in Caucasian populations (11-13) may represent a limitation of our study. Nevertheless, number of analyzed samples fulfills the CLSI recommendations, quoting "hundreds or even thousands" as necessary (8). Also, the R4S project (7) and the abovementioned studies collate experiences of comprehensive NBS implementation lasting at least two years. On the contrary, only the population of healthy newborns was included in our pilot study, ACs and ratios were analyzed and their cut-offs established, thus paving the possibility for the future introduction of NBS for FAOD in Serbia. Their application in further, high volume testing will provide benefits for several healthcare areas (e.g. epidemiology of FAOD in the Republic of Serbia, treatment policies, genetic counseling, etc) (16).

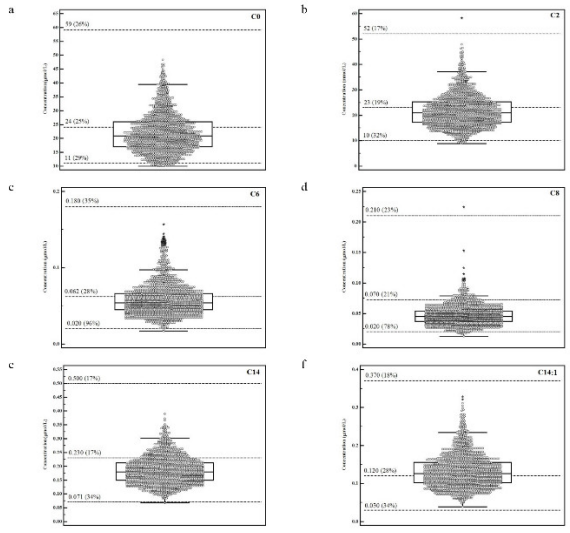

Figure 1. The concentration of (a) free carnitine (C0), (b) acetylcarnitine (C2), (c) hexanoylcarnitine (C6), (d) octanoylcarnitine (C8), (e) tetradecanoylcarnitine (C14) and (f) tetradecenoylcarnitine (C14:1). Box represents the values from the lower to upper quartile. The middle line in the box represents the median. A line extends from the minimum to the maximum value. Circles outside the minimum and maximum value are outside values i.e. below the lower quartile minus 1.5 times the interquartile range, or above than the upper quartile plus 1.5 times the interquartile range. Asterisks represent outliers. The dashed and dotted line gives 1%, 50%, and 99%ile, estimated for the worldwide population of healthy newborns, with the CV in parenthesis [7].

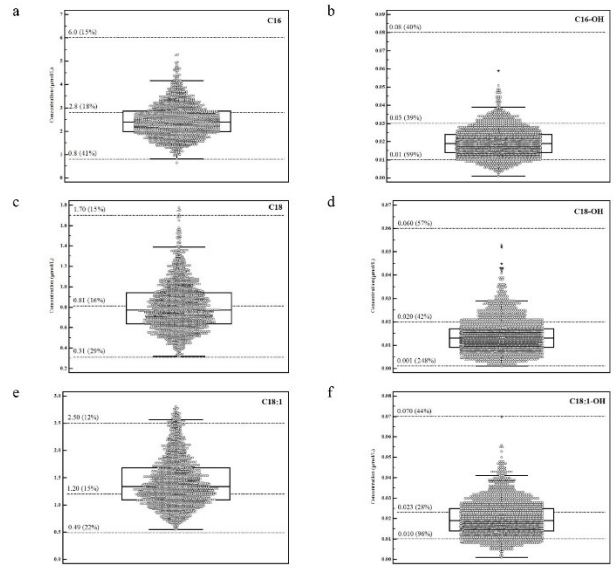

Figure 2. The concentration of (a) palmitoylcarnitine (C16), (b) hydroxy palmitoylcarnitine (C16-OH), (c) stearylcarnitine (C18), (d) hydroxy stearylcarnitine (C18-OH), (e) oleylcarnitine (C18:1) and (f) hydroxy oleylcarnitine (C18:1-OH). Box represents the values from the lower to upper quartile. The middle line in the box represents the median. A line extends from the minimum to the maximum value. Circles outside the minimum and maximum value are outside values i.e. below the lower quartile minus 1.5 times the interquartile range, or above than the upper quartile plus 1.5 times the interquartile range. Asterisks represent outliers. The dashed and dotted line gives 1%, 50%, and 99%ile, estimated for the worldwide population of healthy newborns, with the CV in parentheses [7].

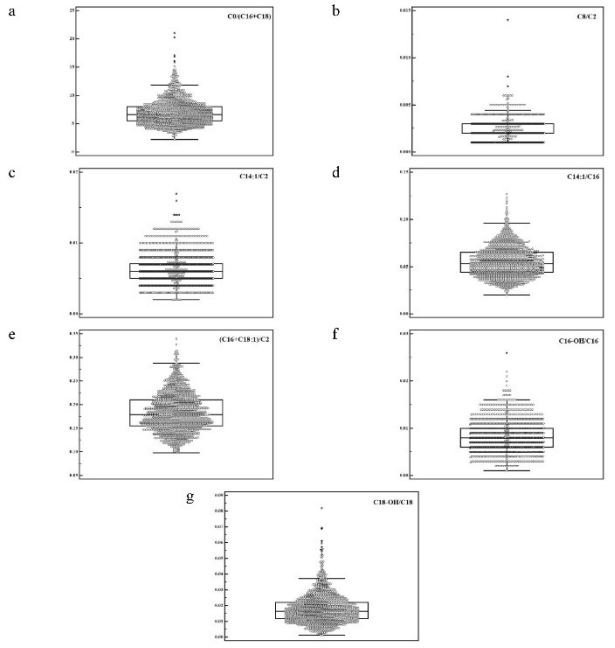

Figure 3. Acylcarnitines' ratios - (a) C0/(C16+C18), (b) C8/C2, (c) C14:1/C2, (d) C14:1/C16, (e) (C16+C18:1)/C2, (f) C16–OH/C16 and (g) C18–OH/C18. Box represents the values from the lower to upper quartile. The middle line in the box represents the median. A line extends from the minimum to the maximum value. Circles outside the minimum and maximum value are outside values i.e. below the lower quartile minus 1.5 times the interquartile range, or above than the upper quartile plus 1.5 times the interquartile range. Asterisks represent outliers.

CONCLUSION

The distribution of the ACs levels, measured using tandem mass spectrometry, in DBS from healthy newborns in the Republic of Serbia substantially matches the worldwide estimates. Also, the established cut-off values were successfully verified in comparison with the worldwide ranges and data from individual studies in several Caucasian populations.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

The study was conducted in accordance with the Declaration of Helsinki and Ethics Committee of Clinical Center of Serbia approved the study (Permission No. 747/33/19.07.2018). Mother of each newborn signed the informed consent.

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None.

CONFLICT OF INTEREST

The all authors declare that there no conflict of interests.

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PROGNOSTIC VALUE OF D-DIMER IN YOUNGER PATIENTS WITH PULMONARY EMBOLISM

Ljiljana Jovanovic[1], Vesna Subota[1], Milena Rajkovic[1], Bojana Subotic[2], Boris Dzudovic[2], Natasa Novcic[2], Jovan Matijasevic[3], Milica Miric[3], Sonja Salinger[4], Natasa Markovic Nikolic[5], Maja Nikolic[6], Vladimir Miloradovic[6], Ljiljana Kos[7], Tamara Kovacevic-Preradovic[7] and Slobodan Obradovic[2,8]

[1] *Military Medical Academy, Institute of Biochemistry, Belgrade, Serbia*
[2] *Clinic of Cardiology and Emergency Internal Medicine, Military Medical Academy, Belgrade, Serbia*
[3] *Institute for Pulmonary Diseases of Vojvodina, School of Medicine, University of Novi Sad, Sremska Kamenica, Serbia*
[4] *Clinic of Cardiology, Clinical Center Nis, University of Nis, Serbia*
[5] *University Clinical Center Zvezdara, School of Medicine, University of Belgrade, Serbia*
[6] *University of Kragujevac, Faculty of Medical Sciences, Clinical Center Kragujevac, Clinic of Cardiology, Serbia*
[7] *Clinic of Cardiology, Clinical Center Banja Luka, School of Medicine, University of Banja Luka, Bosnia and Herzegovina*
[8] *University of Defense, School of Medicine, Belgrade, Serbia*

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Corresponding author:

Ljiljana Jovanovic

Military Medical Academy, Institute of Biochemistry, Belgrade, Serbia

E-mail: biohemicar@ymail.com

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ABSTRACT

In patients with pulmonary embolism (PE), the D-dimer assay is commonly utilized as part of the diagnostic workup, but data on D-dimer for early risk stratification and short-term mortality prediction are limited. The purpose of this study was to determine D-dimer levels as a predictive biomarker of PE outcomes in younger (<50 years of age) compared to older patients. We conducted retrospective analysis for 930 patients diagnosed with PE between 2015 and 2019 as part of the Serbian University Multicenter Pulmonary Embolism Registry (SUPER).All patients had D-dimer levels measured within 24 hours of hospital admission. The primary outcome was mortality at 30 days or during hospitalization.Patients were categorized into two groups based on age (≤ 50 and >50 years of age). Younger patients constituted 20.5% of the study cohort. Regarding all-cause mortality, 5.2% (10/191)of patients died in group under the 50 years of age; the short-term all-causemortality was 12.4% (92/739) in older group.We have found that there was significant difference in plasma D-dimer level between patients ≤ 50 years of age and older group (>50), p= 0.006.D-dimer plasma level had good predictive value for the primary outcome in younger patients (c-statistics 0.710; 95% CI, 0.640-0.773; p<0.031). The optimal cutoff level for D-dimer to predict PE-cause death in patients aged > 50 years was found to be 8.8 mg/l FEU(c-statistics 0,580; 95% CI 0.544-0.616; p=0.049). In younger PE patients, D-dimer levels have good prognostic performance for 30-day all-cause mortalityand concentrations above 6.3 mg/l FEU are associated with increased risk of death. D-dimer in patients aged over 50 years does not have predictive ability for all-caused short-term mortality. The relationship between D-dimer and age in patients with PE may need further evaluation.

Keywords: *D-dimer, pulmonary embolism, younger population, mortality.*

INTRODUCTION

Pulmonary embolism (PE) is a common life-threatening condition which contributes to the global burden of cardiovascular diseases in terms of morbidity and mortality(1-5). The 30-day mortality rate due to PE exceeds 15% (6). Prompt diagnosis, risk assessment and adequate treatment can improve the outcome (7). A reliable risk stratification of PE severity based on the 2019 ESC Guidelines, pulmonary embolism severity index (PESI) or simplified PESI (sPESI) indicates risk of early (inhospital or 30-day) death(8-11). Adding biomarkers data may help in improving prognosis and categorization of patients with acute PE into risk classes (11-12). The use of elevated D-dimer levels to evaluate severity of PE remains limited (6). D-dimer age adapted cutoff level (D-dimer cut off = age x 10 μg/l FEU,age > 50) defined categories of patients ≤50 and >50years of age (13,14). Although it is well recognized that PE has been shown to be major cause of mortality in younger population, there are relatively few studies looking particularly at PE in this age group (15).The aim of this study is to determine age-specifically role of D-dimer in both age groups in patients with PE.

MATERIALS AND METHODS

The present report is a retrospective analysis of the Serbian University Multicenter Pulmonary Embolism Registry (SUPER). The PE patients diagnosed by multidetector computed pulmonary angiography (MDCT-PA) in university cardiology or pulmonology clinics (Military Medical Academy Belgrade, Institute for Pulmonary Diseases of Vojvodina Sremska Kamenica, Clinical Center Nis, University Clinic Zvezdara and Clinical Center Kragujevac) from January 2015 to September 2019, were selected. The study end point was defined as thirty day mortality in patients who died within 30 days of hospital admission. In 930 patients who were aged ≥18 years, D-dimer tests were performed within 24 hours of hospitalization as a part of diagnostic workup. TheD-dimer levels in citrated plasma were measured via Siemens (Marburg, Germany) BCS analyzer and the immunoturbidimetric assay (Siemens Innovance D-dimer test kit). Patients were categorized into two groups based on age (≤50, >50 years).

STATISTICAL ANALYSIS

Descriptive statistics are presented as frequency and percentages for categorical data and their distribution was further analyzed with χ^2 test. D-dimer was given as median and 25^{th}-75^{th} percentile. Comparison in D-dimer between two independent groups was analyzed with Mann-Whitney test. All tests were performed using SPSS 21.0 (SPSS Inc., Chicago, IL, USA) and p values of < 0.05 were considered significant. Receiver operating curve (ROC) analysis was utilized to determine area under the curve (AUC) and cutoff level for D-dimer in both age groups using MedCalc for Windows version 18.11.3 (MedCalc Software, Acacialaan, Belgium).

RESULTS

The final analysis enrolled 930 patients with PE, mean age 62.96 ± 15.46 and significant difference between deceased patients (69.44 ± 14.74) and survivors (62.16 ± 15.37), p<0.001. There were 102 short-term (within 30-days)all-causedeaths of which 60.78 % caused by PE.

Table 1. shows the baseline characteristics of the studied patients. Among the patients aged > 50 years,BMI higher than 25 (p=0.032), chronic diseases such as diabetes mellitus (p=0.043) and coronary heart failure (p=0.015), history of stroke (p=0.001) and thrombosis (p=0.028), were the major factors that would cause death. Beside kidney failure as the independent predictor of all-cause mortality for all PE patients (p<0.001), malignancy (p=0.018) was the most common risk factor in younger patients (age ≤ 50 years). Younger patients may often have investigation for possible thrombophilia and pregnancy. Of the 36 patients who underwent a thrombophilia screen, 20 of them were positive and 3 patients were pregnant.

There was significant difference in plasma D-dimer level between patients ≤ 50 years of age and older group (>50), p= 0.006. D-dimer concentration below group specific cutoff were in 1.6% of patients age ≤50 years (cut off= 0.5 mg/l FEU) and 2.7% of older population (cut off = age x 0.01 mg/l FEU). D-dimer values between deceased and survivors was significantly different in patients aged ≤ 50 years (p=0.026). In older groupno significant difference was observed with respect to all-cause mortality, but mean D-dimer level was significantly higher in patients who died of acute PEwithin 30-days than survivors (p=0.005).

Among patients classified as being at low risk by the sPESI, mortality was 2.7% (9/330). The sPESI classified 600 patients (64.5%) in the high risk group (≥1 point) and mortality of high risk patients was 15.5% (93/600) (Table 2). sPESI and PESI score stratified patients as it was presumed, and 30-day mortality rate were higher in patients with sPESI ≥1 and increased significantly from the first to the fifth PESI risk class in both age groups.The median D-dimer level in the sPESI ≥1 group was significantly higher than in the sPESI= 0 group, 5.0 mg/l FEU (2.6-9.5 mg/l FEU) versus 4.4 mg/l FEU (2.3-8.0 mg/l FEU), respectively, p=0.029, as shown in Figure 1. According to sPESI criteria in patients ≤ 50 years of age,high-risk patients had significantly higher D-dimer levels than low-risk group (4.7 mg/l FEU, 3.6 mg/l FEU, p=0.005). On the other hand,PESIclassificationin patients aged over 50 years showed slightly significant difference between five risk groups (p=0.05).

PE patients were evaluated according to the algorithm proposed by the 2019 ESC guidelines for prediction risk of early mortality. The mortality was 2.2% (7/319) in the low risk class, 5.3% (11/206) in the intermediate-low, 14.8% (41/277) in the intermediate-high and 33.6% (43/128) in the high risk class.

To estimate the predictive capability of the D-dimer in different age groups (≤50 vs >50), a ROC analysis was conducted (Table 3). D-dimer concentrations above 6.3 mg/l FEU was good prognostic biomarker for 30-day all-cause mortality in younger patients (c-statistics 0.710; 95% CI, 0.640-0.773; p=0.031). In population over 50 years of age, D-dimer showed modest discriminative power for 30-day PE-cause mortality with 8.8 mg/l FEU cutoff and an c-statistics 0,580; 95% CI 0.544-0.616; p=0.049.

DISCUSSION

D-dimer levels are used in diagnostic workup of suspected PE, and its elevated values are predictive in detecting and recurrence of PE (16-18). There was a relationship between increased D-dimer concentrations and clot burden, thrombus localization, major bleeding and RV dysfunction (18-20). Studies of D-dimer in patients with PE have demonstrated various data regarding the severity of PE and early risk stratification (22-23).

In this study, high risk patients stratified according to newrevised ESC Guidelines were 18.4% of all PE patients and up to one third of them died within the first 30-days. Since risk of development of PE doubles with each decade after forties (24), 90.7% of deceased in high risk group were older than 50 years.But, D-dimer levels were not significantly associated with short-term all-cause mortality in older patients.The reason might be as followings: D-dimer as a fibrin degradation product can be increased in cancer, infections and wide spectrum of inflammatory, vascular and systemic diseases. Also, comorbidities increase with age and may affect the outcome.

Thus comorbidity burden may explain some of limitations of D-dimer prognostic performance (25-26). Consequently, D-dimer was slightly significant predictor of PE-cause mortality.

Prognostic ability of D-dimer to predict short-term mortality in younger population with PE was observed. Our data showed that every fifth patient with PE was under 50 years of age, similar to theother findings (27-28). In this study was found a difference for the AUCs of D-dimer in patients <50 and ≥50 years of age, showing much better discrimination in the younger group (AUC of 0.710 vs. 0.541, respectively).There were no deaths in patients (<50 years of age) with sPESI=0 and in higher risk classes of PESI (4th and 5th). It appears that lower mortality, better general health status and lower comorbidity rate in younger patients (27) were reflected by behavior of D-dimer.

The main limitation of this study is the relatively low number of deaths in younger population and its retrospective nature. Moreover, D-dimer values below age-adjusted cutoff were present in 2.5% of patients. These results were considered as false negative because of increased age of thrombus, collagenized poorly degradable fibrin and prior treatment with anticoagulation (29).

CONCLUSION

In conclusion, our findings suggest that high levels of D-dimer (6.3 mg/l FEU), measured on hospital admission, identify patients under 50 years of age at increased risk of death during the first 30-days of PE diagnosis. The use of D-dimer in patients aged over 50 years does not have prognostic power for all-cause short-term mortality.

Table 1. Baseline characteristic between PE subgroups

Variables		All patients			≤50 years			>50 years		
		30-day mortality								
		No N=828	Yes N=102	p value	No N=181	Yes N=10	p value	No N=647	Yes N=92	p value
Gender, N (%)	Male	408 (49.3)	43 (42.2)	0.208	114 (63.0)	7 (70.0)	0.749	294 (45.4)	36 (39.1)	0.265
	Female	420 (50.7)	59 (57.8)		67 (37.0)	3 (30.0)		353 (54.6)	56 (60.9)	
Comorbidities (N, %)										
Smoking		141 (18.2)	11 (12.1)	0.078	60 (34.9)	1 (11.1)	0.215	81 (13.5)	10 (12.2)	0.273
Oral contraceptives		12 (1.5)	0 (0)	0.625	6 (3.4)	0 (0)	1.000	6 (1)	0 (0)	1.000
BMI 25-29.99, Overweight		247 (35.8)	20 (24.4)	0.032	44 (28.0)	2 (22.2)	0.462	203 (38.2)	18 (24.7)	0.032
BMI ≥30, Obese		165 (23.9)	17 (20.7)		40 (25.5)	1 (11.1)		125 (23.5)	16 (21.9)	
COPD		89 (10.7)	17 (16.7)	0.097	5 (2.8)	0 (0.0)	1.000	84 (13.0)	17 (18.5)	0.148
CHF		102 (12.3)	24 (23.5)	0.003	7 (3.9)	1 (10.0)	0.355	95 (14.7)	23 (25.0)	0.015

CAD	90 (10.9)	19 (19.0)	0.056	1 (0.6)	0 (0.0)	0.948	89 (13.8)	19 (21.1)	0.079
History of DVT/PE	119 (14.5)	5 (5.0)	0.005	30 (16.7)	0 (0.0)	0.368	89 (13.9)	5 (5.5)	0.028
Hypertension	473 (57.3)	62 (60.8)	0.525	29 (16.0)	1 (10.0)	1.000	444 (68.8)	61 (66.3)	0.632
MI+Stroke+ PAD	127 (15.4)	23 (23.0)	0.061	9 (5.0)	0 (0.0)	1.000	118 (18.3)	23 (25.6)	<0.115
DM	147 (17.8)	28 (27.5)	0.022	13 (7.2)	0 (0.0)	1.000	134 (20.7)	28 (30.4)	0.043
History of stroke	49 (5.9)	17 (16.7)	<0.001	5 (2.8)	0 (0.0)	1.000	44 (6.8)	17 (18.5)	0.001
Malignancy	98 (11.8)	21 (20.6)	0.018	10 (5.5)	4 (40.0)	0.003	88 (13.6)	17 (18.5)	0.205
Major surgery	126 (15.2)	15 (14.7)	1.000	23(12.7)	2 (20.0)	0.623	103 (15.9)	13 (14.1)	0.760
Creatinine Clearance <60	223 (27.9)	71 (70.3)	<0.001	8 (4.6)	6 (60.0)	<0.001	215 (34.3)	65 (71.4)	<0.001
Creatinine Clearance <30	45 (5.7)	29 (28.7)	<0,001	1 (0.6)	3 (30.0)	<0.001	44 (7.1)	26 (28.6)	<0.001

Baseline characteristic between PE subgroups.*p value < 0.05 significant; (%)- percentage of PE patients with (Yes) or without (No) adverse events.Abbreviations: M, male; F, female; BMI, body mass index; COPD, chronic obstructive pulmonary disease; CHF, coronary heart failure; DVT, deep vein thrombosis; PE, pulmonary embolism; CAD, coronary artery disease; MI, myocardial infarction; PAD, pulmonary artery disease; DM, diabetes mellitus;Data were missing for smoking (7.1%), BMI (17.1%), oral contraceptives (3,4%), CAD (0.3%), DVT/PE (0.8%), hypertension (0.2%), MI+Stroke+PAD (0.4%), history of stroke (0.1%), creatinine clearance <60 (3.1%), creatinine clearance <30 (3.8%).

Table 2. Risk assessment in PE subgroups

Variables		**All patients**			**≤ 50 years**			**>50 years**		
		30-day mortality								
		No N=828	Yes N=102	p value	No N=181	Yes N=10	p value	No N=647	Yes N=92	p value
D-dimer (mg/l FEU)		4.6 (2.5-8.7)	5.5 (3.0-10.0)	0.028	4.0 (2.0-7.0)	7.8 (4.1-17.4)	0.026	4.9 (2.7-9.0)	5.2 (3.0-10.0	0.201
Risk factors (N, %)										
sPESI	0	321 (38.8)	9 (8.8)	<0.001	96 (53.0)	0 (0)	0.002	225 (34.8)	9 (9.8)	<0.001
	≥1	507 (61.2)	93 (91.2)		85 (47.0)	10 (100.0)		422 (65.2)	83 (90.2)	
PESI	0	321 (38.8)	9 (8.8)	<0.001	96 (53.0)	0 (0)	<0.001	225 (34.8)	9 (9.8)	<0.001
	1	266 (32.1)	22 (21.6)		53 (29.3)	3 (3.0)		213 (32.9)	19 (20.7)	
	2	162 (19.6)	27 (26.5)		26 (14.4)	4 (40.0)		136 (21.0)	23 (25.0)	
	3	58 (7.0)	32 (31.4)		4 (2.2)	3 (30.0)		54 (8.3)	29 (31.5)	
	4	19 (2.3)	10 (9.8)		2 (1.1)	0 (0)		17 (2.6)	10 (10.9)	
	5	2 (0.2)	2 (2.0)		0 (0)	0 (0)		2 (0.3)	2 (2.2)	
Mortality risk	Low	312 (37.7)	7 (6.9)	<0.001	91 (50.3)	1 (10.0)	0.001	221 (34.2)	6 (6.5)	<0.001

Variables		All patients			≤ 50 years			>50 years		
		30-day mortality								
		No N=828	Yes N=102	p value	No N=181	Yes N=10	p value	No N=647	Yes N=92	p value
D-dimer (mg/l FEU)		4.6 (2.5-8.7)	5.5 (3.0-10.0)	0.028	4.0 (2.0-7.0)	7.8 (4.1-17.4)	0.026	4.9 (2.7-9.0)	5.2 3.0-10.0	0.201
Risk factors (N, %)										
N (%)	Intermediate-low	195 (23.6)	11 (10.8)		38 (21.0)	0 (0)		157 (24.3)	11 (12.0)	
	Intermediate-high	236 (288.5)	41 (40.2)		32 (17.7)	5 (50.0)		204 (31.5)	36 (39.1)	
	High	85 (10.3)	43 (42.2)		20 (11.0)	4 (40.0)		65 (10.0)	39 (42.4)	

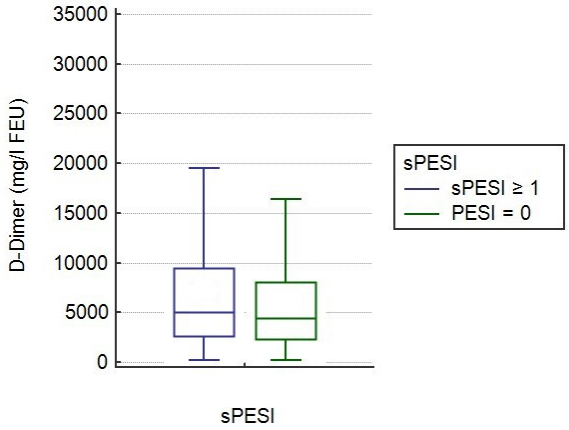
Data for D-dimer are presented as a median, 25th-75th percentile. Baseline characteristic between PE subgroups.*p value < 0.05 significant; (%) - percentage of PE patients with (Yes) or without (No) adverse events.Abbreviations: M, male; F, female; PE, pulmonary embolism; sPESI, simplified pulmonary embolism severity index.

Table 3. ROC Analysis for D-dimer predicting adverse outcomes in PE subgroups

	N	AUC	95% CI	p	Cut-off	Sensitivity	Specificity
All patients	930	0.567	0.534-0.599	0.031	7.6	41.18	70.89
≤50 years	191	0.710	0.640-0.773	0.031	6.3	70.00	72.93
> 50 years	739	0.541	0.504-0.578	0.212	8.8	35.87	74.19

AUC indicates area under the curve.

Figure 1. Median D-dimer levels (mg/l FEU) in sPESI=0 and sPESI ≥1 (sPESI>0) patients



Data reported as median values, interquartile ranges (boxes), and 2.5th and 97.5th percentiles (whiskers).

CONFLICT OF INTEREST

None.

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THE CORRELATIONS AMONG SEVERITY OF DEPRESSION, LEVEL OF OBSERVED EXPRESSED EMOTIONS, PERCEIVED SOCIAL SUPPORT AND PSYCHOSOCIAL FUNCTIONING IN PATIENTS WITH UNIPOLAR DEPRESSION

Nevena Igrutinovic[1,2], Borjanka Batinic[3,4] and Goran Mihajlovic[5,6]

[1]*Clinical Centre of Kragujevac, Clinic of Pediatrics, Kragujevac, Serbia*
[2]*University of Kragujevac, Faculty of Medical Sciences, Kragujevac, Serbia*
[3]*Clinical Centre of Serbia, Clinic of Psychiatry, Belgrade, Serbia*
[4]*University of Belgrade, Faculty of Philosophy, Department of Psychology, Belgrade, Serbia*
[5]*Clinical Centre of Kragujevac, Clinic of Psychiatry, Kragujevac, Serbia*
[6]*University of Kragujevac, Faculty of Medical Sciences, Department of Psychiatry, Kragujevac, Serbia*

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Corresponding author:

Nevena Igrutinovic

University of Kragujevac, Faculty of Medical Sciences, Kragujevac, Serbia

Phone: + 381 69 4698005

E-mail: niblackpearl@gmail.com

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ABSTRACT

The aims of the study were to examine the correlations among severity of depression, level of psychosocial functioning, level of observed expressed emotions and perceived social support in patients diagnosed with unipolar depression. The sample included 61 patients. The following study instruments were applied: the Beck Depression Inventory II, the Functioning Assessment Short Test, the Level of Expressed Emotion scale and the Multidimensional Scale of Perceived Social Support. Descriptive statistics of the sample have shown the mean age of 42.39years (SD=13.387), moderate severity of depression (AS=22.7, SD=11.984), moderate level of observed expressed emotions (AS=74.70, SD=17.959), moderate level of psychosocial difficulties (AS=26.33, SD=17.207) and high level of perceived social support (AS=5.24, SD=1.395). There were significant positive correlations between severity of depression and psychosocial difficulties (r=0.69) and perceived social support (r=-0.42), as well as between psychosocial difficulties and both levels of observed expressed emotions (r=0.31) and perceived social support (r=-0.53). Hierarchical multiple regression analysis showed that 47.1% of the psychosocial functioning variance can be predicted by severity of depressive symptoms, and perceived social support explained the additional 7% of variance. The level of observed expressed emotion didn't show an additional explanation of the psychosocial functioning variance. Our research showed that the severity of depressive symptoms and perceived social support predict difficulties in psychosocial functioning, while the level of observed expressed emotion was not an important predictor.

Keywords: *Unipolar depression, psychosocial functioning, level of observed expressed emotions, perceived social support.*

INTRODUCTION

Depressive disorders are considered to be one of the most important health problems, due to their prevalence, recurrent or chronic course, negative impact on the quality of life, risk of suicide and frequent comorbidity with other mental disorders and physical illnesses (1). Depressive disorders are manifested through depressed mood, a decrease in hedonia, problems with sleep and appetite, concentration problems, feelings of guilt, low self-esteem and lack of energy and will(1).

Many studies have examined the relationship between depression and recognition of facial emotion, and some of them have concluded that depressed people have difficulties in identifying all the basic emotions except sorrow (2). However, few studies have investigated the relationship between depression and level of observed expressed emotions, which represents persons' assessment of the emotional environment in their most important relationships (3). One of those is a research by Hooley et al. that investigated the correlation between depressive symptoms and lack of emotional support, intrusiveness, irritability and criticism, as the components of perceived expression of emotions (3). This study showed that during a nine-month follow-up period, 59% of depressed patients with a high level of observed expressed emotions (based on the spousal observation) relapsed, whereas there was no recurrence of depression among those with a lower level of observed expressed emotions (3). Some studies showed that patients diagnosed with a depressive disorder had an increased neural activity when exposed to affectively negative stimuli, and a reduced activity when exposed to positive stimuli (4). Worsening of the depressive symptoms is usually caused by criticism from the family members and other relatives (5, 6). Difficulties in perception and recognition of emotions lead to miscommunication and may be involved in the development and maintenance of emotional disorders or complicated depressive symptoms (7).

Patients diagnosed with depression often display some form of difficulties in psychosocial functioning. This is usually manifested through a reduced social activity and may lead to problems in initiating and maintaining relationships with others (8). Numerous studies have indicated that social inadequacy and unsatisfying relationships with others play an important role in exacerbating vulnerability in depressed patients (9). Moreover, Evans et al. showed that reduced interest in leisure activities and work assignments is not confined to the early phases of the disorder, but is also present later on, as depression evolves (10).

The adequate social support reduces depressive symptoms, however, depressed individuals do not often receive enough support from their environment because their behaviour sometimes may have a negative influence on others (11). Patients with depression reported less social support, greater marital dissatisfaction, and more dysfunctional relationships than a control group in the study of Zaidi (11). Furthermore, other studies have shown that the family support is correlated with the history of suicidal behaviour in depressed patients (12).

Although some studies investigated the correlation between depression and specific problems in psychosocial functioning, level of observed expressed emotions and perceived social support, there is still a scarcity of research examining the correlation between all previously mentioned variables through one prediction model. Therefore, our research was conducted with the aim of improving understanding of the nature of depressive disorder and factors that can influence its course and outcome through investigating the potential correlations of depressive symptoms and psychosocial functioning, level of observed expressed emotions and social support available to patients diagnosed with depression.

PATIENTS AND METHODS

The research was conducted at the Clinic of Psychiatry, the Clinical Centre of Kragujevac, from January 2015 to September 2015. The study sample comprised patients diagnosed with unipolar depression: major depressive episode (F32.2) and recurrent depressive disorder (F33.2), without psychotic features. The diagnoses were made by attending psychiatrists, in accordance with the International Classification of Diseases, the 10th Revision (ICD-10) (1). Patients with manic or hypomanic episodes were excluded from the research.

The study was approved by the Ethical Committee of the Clinical Centre of Kragujevac and conducted in accordance with the Declaration of Helsinki. The goal and nature of the research were explained to the participants, who provided written informed consent. The participants completed the study measures in a single assessment session.

Sample

The study sample included 61 patients (19 men and 42 women) diagnosed with unipolar depression. The participants were between 21 to 61 years old (M = 42.39, SD = 13.387). The educational level of participants varied (primary school: n = 8; high school: n = 39; college or university: n = 14). The occupational status was also variable (27 participants were employed, 29 were unemployed and 5 were retired).

Instruments

The following study instruments were applied:

A short structured interview was used to obtain the data on socio-demographic characteristics (sex, age, educational level and employment), duration of disorder and the number of hospital admissions.

The Beck Depression Inventory II (BDI-II) was used to assess the presence and severity of depressive symptoms over the previous two weeks. The BDI-II is a self-report questionnaire consisting of 21 items aligned with the criteria for depression specified in the Diagnostic and Statistical Manual of Mental Disorders, the 4th Edition (DSM-IV) (13). Respondents estimate the presence and severity of their depressive symptoms using a four-point scale (from 0 to 3). The total score (0 to 63) is obtained by summing the scores for individual items; higher scores indicate greater depressive symptoms. The reliability coefficient of the questionnaire (Cronbach's alpha coefficient) ranges from 0.73 to 0.95 (14) and in our sample it was 0.91.

The Functioning Assessment Short Test (FAST) is a self-report questionnaire, designed to assess the main functional problems experienced by psychiatric patients, consisting of 24 items covering autonomy, working functioning, cognitive functioning, financial problems, interpersonal relations and free time (15). Respondents indicate the severity of the problems described in each item using a four-point Likert scale (from 0 to 3). The total score (0 to 72) is obtained by summing the scores for individual items; higher scores indicate greater difficulties in functioning. The reliability coefficient (Cronbach's alpha coefficient) of the whole questionnaire in this sample of patients was 0.94.

The Level of Expressed Emotion (LEE) is a self-report questionnaire consisting of 38 items organised into four subscales which measure patients' perceptions of the level of expressed emotion in family interactions: the lack of emotional support, intrusiveness, irritability and criticism (16). Respondents used a four-point Likert scale (1 to 4) to indicate their level of agreement with each statement. The total score (ranging from 38 to 152) and four subscale scores were obtained by summing the scores for individual items, after recoding reverse statements. In our sample, the whole questionnaire had the reliability coefficient (Cronbach's alpha coefficient) of 0.91.

The Multidimensional Scale of Perceived Social Support (MSPSS) is a 12-item, self-report questionnaire in which responses are given using a seven-point Likert scale (1 to 7) (17). The statements are grouped into three subscales, representing different sources of social support: family, friends, and significant others. The overall score is the average score for all 12 statements and subscale scores are the average scores for the items making up the relevant subscale. In our sample, the reliability coefficient (Cronbach's alpha coefficient) of the whole questionnaire was 0.93.

Statistical analysis

The data were analysed using SPSS v. 20.0. Descriptive statistics and reliability coefficients were calculated for all questionnaires. The correlations and hierarchical multiple regression analysis were used to verify the hypothesis of predictive values of the depressive symptoms severity, level of observed expressed emotions and perceived social support on the level of psychosocial functioning difficulties.

RESULTS

The patients reported moderate depressive symptoms and BDI-II scores were grouped around lower values (Table 1). The patients also reported a medium level of psychosocial difficulties, and moderate level of observed expressed emotions, with both scores grouped around lower values. The sample reported a high level of perceived social support, with scores largely grouped around higher values, especially for the support perceived from significant others.

Table 1. Descriptive statistics for main variables

	Mean	SD	Min/Max.	skewness
BDI-II	27.30	11.98	8/59	0.558
FAST	26.33	17.21	4/67	0.871
LEE	74.70	17.96	42/131	0.763
emotional support	32.61	10.70	19/68	1.221
intrusiveness	20.20	5.41	8/31	-0.024
irritability	11.82	3.93	6/21	0.404
criticism	10.08	3.30	5/17	0.419
MSPSS	5.24	1.40	1/7	-0.812
significant others	5.42	1.56	1/7	-1.115
friends	5.17	1.78	1/7	-1.013
family	5.12	1.75	1/7	1.753

The average duration of disorder was 4.64 years (SD = 5.128), and 36.1% of the participants reported that they had experienced depressive symptoms for a less than a year. The average number of hospital admissions was 2.56 and 28 patients had never been hospitalised. Women reported more severe depressive symptoms than men did ($F(1, 55) = 5.429$; $p < 0.05$) and were more likely to report that they had difficulty in everyday functioning ($MD = 12.706$; $F(1, 55) = 4.928$; $p < 0.05$).

The duration of disorder and the number of hospital admissions were also in a moderate to a high positive correlation with BDI-II scores ($r = 0.46$, $p < 0.05$; $r = 0.51$, $p < 0.01$ respectively) and psychosocial functioning difficulties ($r = 0.57$, $p < 0.01$; $r = 0.52$, $p<0.01$ respectively).

The educational level was also systematically related to the level of observed expressed emotions, with college-educated patients reporting higher levels of observed expressed emotions ($MD = 18.585$; $F (2, 55) = 4.861$; $p < 0.01$).

The correlation between clinical and psychosocial aspects of depressive disorder

The correlations between depressive symptoms, psychosocial functioning, level of observed expressed emotion and perceived social support are shown in Table 2. All correlations were significant, except the one between the BDI-II scores and the level of observed expressed emotions. However, the patients who reported more depressive symptoms also reported higher levels of observed intrusiveness ($r = 0.29$, $p < 0.01$) and irritability ($r = 0.28$, $p < 0.01$). Similarly, the levels of perceived social support were lower when the patients perceived the lack of emotional support ($r = 0.533$, $p < 0.01$), higher intrusiveness ($r = 0.387$, $p < 0.05$) and higher criticism ($r = 0.243$, $p < 0.05$).

Table 2. Intercorrelations between the BDI-II scores, difficulties in psychosocial functioning, level of observed expressed emotion and perceived social support

	FAST	LEE	MSPSS
BDI-II	.69**	.13	-.42**
FAST		.31*	-.53**
LEE	.31*		-.52**

** $p < .01$, * $p < .05$

In the next section, we conducted the hierarchical multiple regression analyses in order to determine the predictive values of the depressive symptoms severity, level of observed expressed emotions and perceived social support on the psychosocial functioning difficulties. The first model includes the depressive symptoms severity, which showed the highest correlation with psychosocial functioning, afterwards perceived social support that also showed a high correlation, and finally, level of observed expressed emotions that showed a moderate correlation with the scores on FAST.

The results of linear regression analysis showed that 47.1% of the psychosocial functioning variance can be predicted by the severity of depressive symptoms (ß = .686, R2 = 47.1, F (1, 59) = 52.58, $p < .001$) as shown in Table 3 and Table 4. Including perceived social support into the model explained the additional 7% of variance, that is 54% in total of the psychosocial functioning variance (ß = .736, R2 = 54.1, F (2, 58) = 34.20, $p < .01$).

Table 3. Hierarchical multiple regression analysis - model summary

Model	R	R^2	Adjusted R^2	Change Statistics				
				R^2Change	F Change	df1	df2	Sig. F Change
1	.686[a]	.471	.462	.471	52.576	1	59	.000
2	.736[b]	.541	.525	.070	8.839	1	58	.004
3	.743[c]	.552	.528	.011	1.384	1	57	.244

a. Predictors: (Constant), depression
b. Predictors: (Constant), depression, social support
c. Predictors: (Constant), depression, social support, level ofobserved expressed emotions
d. Dependent variable: psychosocial functioning

Including level of observed expressed emotions into the model didn't lead to an additional explanation of the psychosocial functioning variance. The results are shown in Table 4.

Table 4. Hierarchical multiple regression analysis coefficients

Model		Unstandardized Coefficients		Standardized Coefficients	t	Sig.
		B	Std. Error	Beta		
1	(Constant)	-.576	4.047		-.142	.887
	depression	.986	.136	.686	7.251	.000
2	(Constant)	23.136	8.836		2.618	.011
	depression	.809	.141	.563	5.739	.000
	soc. support	-3.600	1.211	-.292	-2.973	.004
3	(Constant)	9.366	14.647		.639	.525
	depression	.827	.141	.576	5.854	.000
	soc. support	-2.747	1.408	-.223	-1.952	.056
	obs.emotions	.118	.100	.123	1.177	.244

a. Dependent variable: psychosocial functioning

DISCUSSION

Our results showed that the patients with more severe depressive symptoms had more problems in everyday functioning and perceived lower levels of social support. The patients with more difficulties in everyday functioning reported a higher level of perceived expressed emotions and lower levels of social support.

Depressive symptoms were shown to predict the majority of the psychosocial functioning variance (47.1%). The previous study also found that reduced interest in leisure activities and lower task performance were not confined to the initial stages of depression, implying that depression could be a chronic disorder with much more complex consequences for patients' functioning in later phases (10). A study of over 3000 depressed subjects reported that depressive symptoms, particularly sad mood and problems with concentration, had a negative effect on psychosocial functioning (18). Persons diagnosed with depression who experience failure in performance of everyday activities become less satisfied with themselves and less prepared to face difficulties (10, 18). The correlation between depression and psychosocial functioning has also been confirmed by the meta-analysis of 31 clinical studies, which showed that psychotherapy improves social functioning of patients with depression (19).

Additionally, perceived social support explained 7% of the psychosocial functioning variance (54% in total) in our study. We found that perceived social support was negatively related to difficulties in everyday functioning and that perceived social support was an important predictor of functioning levels, which is in accordance with the results of some earlier studies (20). The lack of social support can corroborate a person's poor self-image and thus lead to impaired functioning. In our study sample, perceived social support was also in a moderate correlation with severity of depressive symptoms, which is in accordance with a large number of other studies (21, 22). Depressed patients report they receive less support from their social environment than persons without depressive symptoms, as well as they experience greater marital disagreement and less satisfactory relationships with people in their environment (11). Dour et al. reported that perceived social support was a risk factor for suicide attempts (23), and Bell et al. showed that 11% of the change in depressive symptoms over an 18-month period could be attributed to changes in perceived social support (24).

Although we found a moderate correlation between the level of observed expressed emotions and psychosocial functioning, LEE scores didn't lead to an additional explanation of the psychosocial functioning variance. This result might be due to a high correlation (r = .52) between these scores and perceived social support, since the patients who perceived a higher level of observed expressed emotions in their surroundings reported lower levels of social support too. This is also in accordance with the previous reports (25). A greater level of expressed emotions (particularly lack of emotional support, but also high intrusiveness, irritability and criticism), can lead to a greater level of social anxiety or feeling of inadequacy that may lead to poor social relations and consequently lower level of perceived social support.

Limitations of the study

Since our study showed a high positive correlation between perceived social support and level of observed expressed emotion, it's recommended to conduct the research on a larger sample using the *Structural equation modeling* (SEM analysis) that will assume indirect influence of level of observed expressed emotions on psychosocial functioning mediated by its correlation with perceived social support. Besides having a relative small study sample, it should be considered that we conducted the correlation study and that the conclusions about causal relations aren't possible. Finally, we used the self-reports measures that may impact the objectivity of results.

CONCLUSION

This research showed that the severity of depressive symptoms and perceived social support can predict difficulties in psychosocial functioning, while the level of observed expressed emotion was not an important predictor of psychosocial functioning. The study indicated the importance of early psychosocial interventions, psychoeducation of the patient's environment and empowering personal social skills in treating depression, in addition to the application of pharmacotherapy and individual psychotherapy.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

The study was approved by the Ethical Committee of the Clinical Centre of Kragujevac and conducted in accordance-with the Declaration of Helsinki. The goal and nature of the research were explained to the participants, who provided written informed consent.

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None.

CONFLICT OF INTEREST

None.

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COMPARING THE EFFECTIVENESS OF LASER THERAPY, MEDICAMENTS AND SURGICAL TREATMENT IN PATIENTS WITH ANGLE CLOSED GLAUCOMA

Svetlana Paunovic[1], Mirjana A. Janicijevic Petrovic[2], Suncica Sreckovic[2], Milan Paunovic[3], Katarina Janicijevic[4] and Zora Stankovic[5]

[1] *University Clinical Centre Kragujevac in Kragujevac, Clinic of Ophthalmology*
[2] *Faculty of Medical Sciences University of Kragujevac, Clinic of Ophthalmology*
[3] *Faculty of Medical Sciences University of Kragujevac, Clinic of Pediatric Surgery*
[4] *Faculty of Medical Sciences University of Kragujevac, Department of Social Medicine*
[5] *Clinical Centre of Serbia, Belgrade, Clinic of Ophthalmology*

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Corresponding author:

Svetlana Paunovic

University Clinical Centre Kragujevac in Kragujevac, Clinic of Ophthalmology, Kragujevac, Serbia

E-mail: cecaana.paunovic@gmail.com

ABSTRACT

The study goal was to compare the efficiency of the medicaments, laser and surgical treatment in the patients with primary angle closure glaucoma (PACG) in order to achieve: normalization of intraocular pressure (IOP), maintaining the useful visual acuity and stabilization of visual field loss. All patients were treated at Clinic of Ophthalmology of Clinical Centre Kragujevac in Kragujevac, Serbia, in the period from June 15, 2010 to June 15, 2014. There were 116 patients in this study. They diagnosed with PACG, and they had been adequate selected for this study. They were treated with: medicaments; Nd:YAG (Neodymium:Yittrium Aluminum Garnet) laser iridotomy, and glaucoma surgery. Clinical ophthalmology controls have been introduced once or twice a month, and the vision field loss was tested three times every year. During the monitoring period of one year: no statistically significant difference occurred in terms of changes of the visual acuity among the three study-groups. The best IOP regulation was achieved after the laser treatment (53.4%), followed by the surgical treatment (28.5%), while the weakest was recorded in patients treated with the medicaments (18.1%). The percentage of the visual field loss was the biggest of patients treated with the medicaments (57%), and then the patients treated with the laser iridotomy (35%), while the least one occurred of the patients with the surgical treatment (23%). In the laser-treated group of 62 patients, the frequency of complications was 17.8%; while the out of 33 patients who had been treated (surgical), the frequency of complications was 19%. Laser iridotomy was shown to be effective of 89.5% of study-patients with PACG, while non-reactive study-patients underwent to the trabeculectomy. In addition to the high efficacy of Nd:YAG laser iridotomy in regulating of IOP values of the patients with PACG, the advantage of laser-method was in: the maneuvering of outpatients, it was easy to do, had a short lead time and was used in local anesthesia.

Keywords: *Glaucoma, primary angle closure glaucoma, patients, therapy, iridotomy Nd:YAG laser.*

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INTRODUCTION

Glaucoma is an optic neuropathy characterized by loss of retinal ganglion cells and atrophy of the optic nerve, with an increase of intraocular pressure (IOP). The angle closure, in primary angle close glaucoma is cause of 50% of all blindness in the world. It is the most destructive form of the progressive glaucoma disease (1).

The primary angle closure glaucoma occurs of a positive family anamnesis, in later life (years), more often in women and the people of Asian than European areas (origin) (2). Primary angle closure glaucoma is one of the rarest diseases in ophthalmology whose outcome must be largely predicted in relation to the anatomical predisposition of the eye (1, 2). The mechanism of IOP increase during PACG is: pupillary block, anterior chamber angle block in the root part of the iris, ciliary body edema and its forward positioning, etc (2).

The neodymium YAG laser is a photo-disruptor which emits radiation near the infrared part of the electromagnetic spectrum, and whose wavelength is 1065 nm. Indications for Nd:YAG laser iridotomy are: primary angle closure glaucoma, acute, intermittent and chronic glaucoma, the paired eye in the patients suffering from acute glaucoma, narrow angles suitable for closure, secondary angle closure with papillary block, and the glaucoma formed by combined mechanisms (2).

With our laser treatment, our patients were painless and pain-free; easily tolerate intervention, which does not take too much time; it is also suitable for the ambulant patients, and without possible risks in general anesthesia (3).

The rare complications of Nd:YAG laser iridotomy of the clinical treatment are: corneal burns, macular burns, opalescence of lens, damages of blood vessels, dispersion of iris pigment, retinal detachment (ablation of chorioretina), etc. Patients sometimes have some pain, some blurred of the vision, blinding sensations and diplopic (4).

PATIENTS AND METHODS

This study was cohort, interventional and with "before and after" analysis. All study-patients were treated at Clinic of Ophthalmology of Clinical Center Kragujevac in Kragujevac, Serbia, in the period from June 15, 2010 to June 15, 2014. There were of 116 patients, aged 41-80 (years), gender equality, with diagnosis of PACG. All study-patients were adequately selected (included or excluded) in this study.

The detailed ophthalmological examination was performed, which included: measurement functional visual acuity (Snellen-tables), measurement IOP (Goldmann's applanation tonometry), bio-microscope examination (slit lamp), gonioscopy (Goldmann's glass with three mirrors), ophthalmoscopy with three mirrors, and the static perimetry. Depending on the clinical stage of glaucoma disease, study-patients are treated with: medicaments, laser (Nd:YAG laser iridotomy by *Carl Zeiss*a's slit lamp) and by the surgical method.

Ophthalmological examinations were performed once or twice a month. Visual field was inspected and analysis a three times per year. There were the total of 33 patients, who were surgically treated in this study. The average age of patients was 64 years old, while the age interval was from 51 to 77 years.

Conditions and indications for performing iridotomy with Nd:YAG laser were: transparent all ocular media in front of the target tissue, the existence of the anterior chamber of the eye with minimal depth, etc.

The statistical method included: comparison of mean values of numerical variables between the three examined groups using Hi-quarry (χ2-test; $p>0.05$).

For the statistical data processing, the authors used statistical program SPSS, version 20.

The results of the study are presented in tables and graphs (figures).

The consent of the regional Ethics Committee of the Clinical Center of Kragujevac in Kragujevac, Serbia, was obtained for this study.

RESULTS

During one-year study of follow-up and analysis, the authors did not notice statistically significant differences in changes of visual acuity between the three study-groups of treatment (Nd:YAG laser iridotomy, surgical therapy, medicaments therapy) ($p>0.05$), Table 1, Table 2.

Table 1. Functional visual acuity (Snellen-tables) before Nd:YAG laser iridotomy, surgical therapy and medicaments

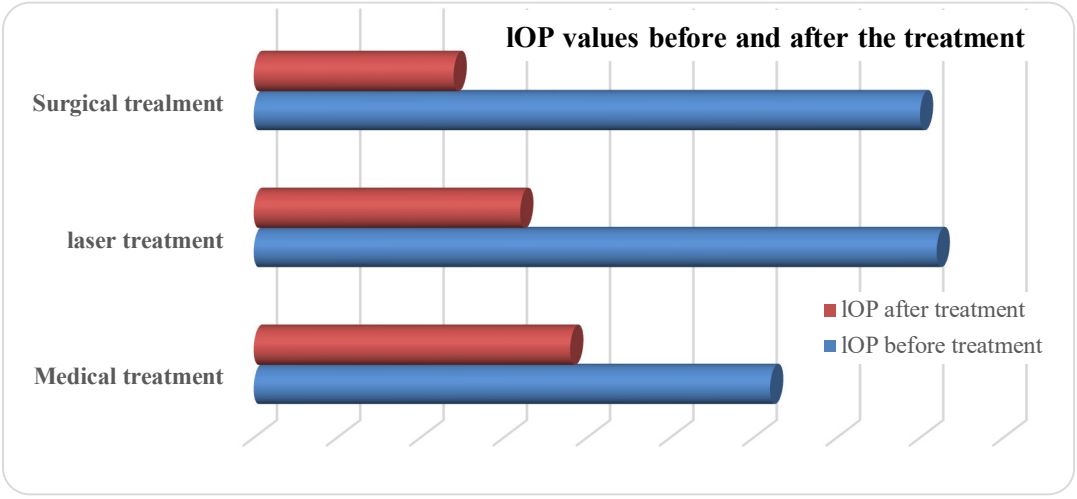
Visual Acuity	L+	0,01	0,05	0,06	0,1	0,2	0,3	0,4	0,7	0,8	0,9	1,0
Surgical		1	1		6	5	6	4	1	9		
Laser		2	3	5	9	7	8	9	9	10		
Medicaments					2	1	2	5	5		6	

Table 2. Functional visual acuity (Snellen-tables) after Nd:YAG laser iridotomy, surgical therapy and medicaments

Visual Acuity	L+	0,01	0,05	0,06	0,1	0,2	0,3	0,4	0,7	0,8	0,9	1,0
Surgical	1	2	2	3	4	3	4	4	5	5		
Laser		4	5	6	9	6	8	8	7	9		
Medicaments				1	2	2	3	4	4	5		

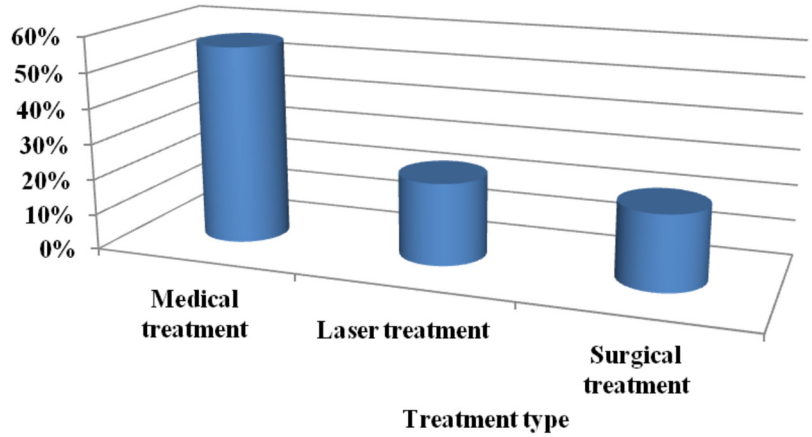
The best regulation of IOP was achieved after laser treatment (53.4%), i.e. there was a very significant difference in IOP after treatment between laser treatment ($p<0.01$) and surgery (31%); there was also a significant difference in IOP after surgery compared to preoperative IOP values ($p<0.01$). The worst effect was observed in patients who were on medicaments treatment (19%); however, there was still a significant difference compared to IOP values before the medicaments ($p<0.05$), Figure 1.

Figure 1. IOP values (mmHg) before and after Nd:YAG laser iridotomy, surgical treatment and medicaments



Visual field loss was most pronounced in the patients treated with medicaments (56%), followed by patients treated with laser (23%), while it was lowest in patients treated surgery (21%), ($p<0.05$), **Figure 2.**

Figure 2. Percentage of visual field loss of relative type of the therapy



The significant decrease of IOP, the changes in functional visual acuity and the stability of changes in visual field (after laser treatment of iridotomia, ($p>0.05$)) was shown, as prevention of the development of primary angle closure glaucoma, and stopping and/or slowing the progression of glaucoma disease.

In the group of 62 patients who were laser treated, 8 of them experienced increase of IOP; 5 of them experienced the iridotomy closure, while 3 patients experienced to the iritis. The frequency of complications was 17.8%, while of 33 operated patients the frequency of complications was slightly higher (19%).

DISCUSSION

Nd:YAG laser iridotomy has the great importance in the treatment of PACG, especially in acute glaucoma, as well in preventing the onset of acute attack in both eyes (responsible potential risk factors for acute angle closure glaucoma) (4).

Our results were: regulation of IOP was achieved by laser treatment (53.4%), surgical treatment (28.5%), while the least regulated was observed in the patients treated with medicaments (18.1%). The percentage of visual field loss was highest in the patients treated with medicaments (57%), followed by those treated with laser (35%), while the lowest loss occurred in the patients treated with surgery (23%). The incidence of laser-treated complications was 17.8%. In 33 patients, who were treated surgically, the frequency of complications was 19%. Laser iridotomy has been shown to be effective in 89.5% of our patients with PACG.

In many studies, laser treatment has been shown in 80% effective in the eyes with PACG, but it should be noted that the angle remains open after laser iridotomy and/or when the IOP is normal (5). In our study, the best regulation of IOP was achieved after laser treatment in 53.4% of patients.

Other authors have shown that laser iridotomy is responsible for the slow progression of visual field loss in angle closure glaucoma (6, 7). In our study, the loss of visual field in the patients treated with laser treatment was 35%.

In manuscript published by Singh P et al, about the efficacy of peripheral laser iridotomy, the authors demonstrated in 78% of the eyes with normal IOP after the Nd:YAG laser iridotomy (8), but in our study the best regulation of IOP was achieved after laser treatment of 53.4%.

Many studies indicate that surgical treatment of acute primary angle closure glaucoma (treatment strategies) has been reported, not only with risk and complication (our complications - 17.8%), but also with the consequential and final economic costs, evidences and economical considerations (9). Our research also confirms the facts presented by the mentioned authors, but we did not rely directly on the economic status of options in the treatment of angle closure glaucoma.

Many studies have shown that the indications for performing Nd:YAG peripheral laser iridotomy are: clinically significant or suspected pupillary block, as well as prevention of acute and/or chronic angular block, and bilateral localization (10), and have shown potential risk factors for acute glaucoma (11), and which opinions we share. Our results showed that laser peripheral iridotomy reduces the occurrence of primary angle closure glaucoma, and prevents the development of acute glaucoma of the other eye.

Chung HJ and co-authors indicated that complications of laser iridotomy were rare both yesterday and today (12), as shown by our clinical study.

A comparison of the laser iridotomy with the short duration of 532 nm Nd:YAG laser (Pascal) of a conventional laser in dark irises indicates better results (12), as some as in our experience.

Mingguang H et al have shown that laser effects and prophylaxis are better if laser iridotomy is performed in the lower quadrants, and in bilateral treatment (13), but our better experience has been associated with localization in the upper quadrants, contrary to the above recommendation, but the same of laser bilateral treatment.

Many authors presented the long term results of laser intervention after acute angle closure (in contrast to the lateral eye), after 6 years of follow-up, when glaucoma progression was present in 2.5% of patients, and 80% of patients maintained good functional visual acuity (14, 15), which is also indicated by our experience.

Flores-Sanchez et al have shown that laser prophylaxis of the patients with an acute attack was safe and effective in preventing angle closure glaucoma, and preventing long term increase IOP (16), which largely coincides with our study.

Jong VL et al pointed to prognostic factors for the success of laser iridotomy in acute angle closure glaucoma (17), and which are the subject of our future research, which will continue on this topic.

Lingam Vijaya et al suggested that glaucoma surgery, i.e. that the trepanotrabeculectomy was effective, but the operation was associated with an increased risk for postoperative cataract development, which will lead to the shallower anterior chamber and the other complications in glaucoma surgery (18), and which to indicated of the monitoring of our patients in postoperative course, too.

Laser peripheral iridotomy (LPI) in primary and acute angle closure glaucoma increased the angle width in all stages of primary angle closure, and had a good safety profile. There were limited data on the comparative efficacy of LPI versus the other treatments for the various stages of angle closure glaucoma, as a different efficacy of laser treatment (19, 20, 21), but which will be the subject in our future research.

CONCLUSION

The use of the laser therapy in the patients with acute closure glaucoma does not cause significant side effects, and leads to the prevention of primary angle closure glaucoma: significant reduction of IOP, minimal changes of functional visual acuity and stability the changes in visual field. The best regulation of IOP is achieved after laser treatment compared to surgical and medicaments treatments. This is one of the reasons why authors tend to choose Yag-laser method, when it is possible. The patients with the low risk of glaucoma progression should be treated with medicaments and laser iridotomy was also acceptable. The patients with high risk of glaucoma progression should be treated with the laser iridotomy and/or surgically. Apart from the great efficiency of Yag-laser iridotomy of IOP regulation in eyes with PACG: advantages of this method lay in possibility of outpatient treatment, the local anesthesia instead of general one, the comfort ability and the efficiency.

This original manuscript contains data that could be used to the clinical practice of ophthalmology. The topic covered of our study is always actual and applicable in every day in the clinical practice.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

The study was conducted in accordance with the Declaration of Helsinki and was approved by Ethics Committee of the Clinical Center of Kragujevac in Kragujevac, Serbia. All patients gave their informed consent for inclusion before they participated in the study.

CONFLICTS OF INTEREST

The authors declare no conflicts of interest.

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MORPHOLOGICAL AND FUNCTIONAL CHANGES OF PITUITARY GH AND PRL CELLS FOLLOWING PROLONGED EXPOSURE OF FEMALE RATS TO CONSTANT LIGHT

Natasa Nestorovic, Natasa Ristic, Vladimir Ajdzanovic, Svetlana Trifunovic and Verica Milosevic

University of Belgrade, Institute for Biological Research "Sinisa Stankovic" - National Institute of Republic of Serbia, Department for Cytology, Belgrade, Serbia

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Corresponding author:

Verica Milosevic, Ph.D.

Department for Cytology, Institute for Biological Research "Sinisa Stankovic"
National Institute of Republic of Serbia
142 Despot Stefan Blvd. Steet, 11060 Belgrade, Serbia

E-mail: dimi@ibiss.bg.ac.rs

ABSTRACT

Light regulates numerous physiological functions including secretion of different hormones. Our aim was to determine morphological and functional changes of the pituitary growth hormone (GH) and prolactin (PRL) producing cells in female rats exposed to constant light regime from the peripubertal to adult period of life. Starting from the thirtieth postnatal day, female Wistar rats were exposed to constant light (600 lx) for the following 95 days. Controls were maintained under the regular laboratory lighting conditions. The GH and PRL cells were immunohistochemically visualized. Changes in cell volumes and volume densities were evaluated by stereology. Concentrations of PRL and GH in circulation were also determined. We detected significant decrease of the GH cell volume and volume density, followed by reduced the GH blood concentration in comparison to the controls. In contrast, PRL cells were larger in size and their volume density was significantly increased when compared to the controls. Accordingly, PRL concentration was elevated. It can be concluded that exposure of female rats to constant light regime, from peripubertal to adult period of life, causes inhibition of the pituitary GH and stimulation of PRL cells.

Keywords: *Female rat, pituitary, GH cells, PRL cells, immohistochemistry, stereology, constant light.*

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INTRODUCTION

Numerous biological activities, including energy metabolism, body temperature, feeding, locomotion, reproduction and other types of behavior are rhythmic in nature and synchronized with 24h light-dark cycle. Among the others, endocrine factors are highly rhythmical. Their rhythms are mostly endogenously generated, persist without environmental cues and are driven by intrinsic timekeeping mechanism known as the master circadian clock, which in mammals is located within the suprachiasmatic nucleus (SCN) of the hypothalamus (1). Although endogenous, circadian rhythms are synchronized, or entrained, with external environmental stimuli of which the light is most predictable synchronizing agent. The SCN receives direct retinal input through the retinohypothalamic tract that maintains entrainment of the endogenously rhythmic cells with the light-dark cycle (2). The SCN directly or *via* pineal nocturnal hormone melatonin coordinates the timing of circadian rhythms, including daily control of hormone secretion. However, some of endocrine rhythms are diurnal and are driven by alternating cycles of light and darkness, rather than being internally organized. In contrast to circadian rhythms, diurnal rhythms cease when environmental conditions are constant and no longer provide time cues (1, 2). The hypothalamo-pituitary-adrenal and -gonadal axes, prolactian (PRL) and melatonin secretion are under circadian regulation, while the growth hormone (GH) secretion is diurnal and essentially sleep-dependent, *i.e.* GH secretion is not rhythmic in the absence of sleep (3). However, it has been suggested that GH secretion relies on an interactive effect of the exogenous and endogenous components, which both drive its 24-h rhythm (4).

Growth hormone and PRL are synthetized and secreted from somatoropic (GH) and lactotropic (PRL) cells of anterior pituitary. Somatotropic and lactotropic cells are considered to be "sister cells" since they are differentiated from the same, Pit1 cell lineage (5). Both hormones are controlled by stimulating and inhibitory influences from the hypothalamus, modulated by sex steroids and have important role in pubertal maturation. Growth hormone promotes growth and development of the body directly affecting muscle and bone growth, lipid metabolism, milk production, and the release of several hormones. Indirectly, it affects several processes in the body by stimulating the production and release of insulin-like growth factor from the liver (6, 7). The release of GH from anterior pituitary is regulated predominantly by two hypothalamic peptide hormones; the GH-inhibiting hormone, somatostatin and the GH-releasing hormone, (GHRH) (6). The GH release follows diurnal rhythm in humans, with the highest mean plasma GH levels and pulse frequency during sleep onset (8). Interestingly, in male rats the GH secretion patterns are independent of the time of day, but in females the GH release seems to follow a diurnal rhythm, with pulse frequency and amplitude found to be increased during the dark period (9).

Prolactin in the mammals affects reproductive, sexual, metabolic, immune, and other functions. Synthesis and secretion of the PRL is controlled by prolactin-releasing factors such as the thyrotropin-releasing-hormone, oxytocin, neurotensin, vasoactive intestinal polypeptide and vasopressin; prolactin-inhibiting factors such as dopamine, somatostatin, or gama-aminobutyric acid (10). Its secretion has a circadian pattern that persists in constant conditions in humans and rats (10) and is abolished after lesions of the SCN (11). Experimental data strongly suggest that the daily rhythm exhibited by neuroendocrine dopaminergic neurons is endogenous in nature and entrained by light (1, 10, 12).

Modern world society is subjected to the disturbances of circadian rhythms by shift work, sleep deprivation, and environmental light pollution. Lighting during nighttime can have detrimental effect on human health (13). Cardiovascular diseases (14), metabolic syndrome (15), depression (16), and even cancer (17) are associated with disturbances of circadian rhythms.

Since the light is one of the major factors entraining circadian rhythms with the environment, valuable insight into the effects of their disturbance comes from studies of animals exposed to constant light, known as functional pinealectomy (18). It causes melatonin and serotonin deprivation associated with changes in behavior (19) including sexual behavior (20); structural and functional changes in the pituitary-thyroid (21) and -adrenal axes (22, 23); alteration in metabolism (24); promotion of aging and tumorigenessis (25) as well as changes in blood pressure (26). Additionally, continual exposure of female rats to an environment of constant illumination is used for developing of the polycystic ovary syndrome in the rodent model (27).

Having in mind that the PRL and GH secretion patterns are sex- and age-dependent, and their importance in pubertal maturation, we aimed to examine the effects of functional pinealectomy on morphofunctional features of the GH and PRL cells in female rats chronically exposed to constant light, from peripubertal to adult period of life.

MATERIAL AND METHODS

Animals and Experimental Design

Female Wistar rats were housed in the unit for experimental animals at the Institute for Biological Research "Sinisa Stankovic", Belgrade, Serbia. The first group of rats (n=6) was placed into constant ambient light conditions (24h/day, light intensity of 600 lx) on the 30th day of life (CL). Animals were maintained in these conditions for the following 95 days. The second group was kept under regular laboratory lighting conditions (12h light–12h dark) during the same period and served as a control group (n=6; C). All animals had free access to food and water and were maintained at constant temperature conditions (21±2°C). All animal procedures were in compliance with the European

Communities Council Directive (86/609/EEC) and were approved by the Ethical Committee for the Use of Laboratory Animals of the Institute for Biological Research "Sinisa Stankovic", the University of Belgrade (No 2–12/13).

Tissue Preparation and Immunohistochemistry

Pituitary glands were excised, weighted, fixed in the Bouin's solution for 48 h and dehydrated in increasing concentrations of the ethanol and xylene. After embedding in Histowax (Histolab Product Ab, Göteborg, Sweden), each tissue block was serially sectioned at 5 μm thickness on a rotary microtome (RM 2125RT Leica Microsystems, Wetzlar, Germany). Series of sections cut through three tissue levels (dorsal, middle and ventral portions) of the pars distalis were used for the immunohistochemical and immunofluorescence localization of the GH and PRL containing cells. After rehydration, the sections were stained immunohistochemically. Somatotropic and PRL cells were visualized using the peroxidase enzymatic method as previously described (28). Briefly, sections were incubated with polyclonal hGH or hPRL antibodies (1:300 v/v (DAKO A/S, Glostrup, Denmark) for 24 h at 4°C. After a 5-min rinse in the PBS, sections were incubated for 45 min with secondary antibody (1:500 v/v porcine-anti-rabbit IgG (obtained from DAKO A/S, Glostrup, Denmark), rinsed for 5 min in the PBS, incubated for 45 min with rabbit-antiperoxidase serum (1:100) (DAKO A/S, Glostrup, Denmark) and again rinsed in the PBS. The antigen–antibody complex was visualized using a 0.05% DAB liquid substrate chromogen system (Dako A/S, Glostrup, Denmark). Sections were thoroughly washed under running tap water and counterstained with hematoxylin. Antibody localization was visualized using a 0.05% DAB liquid substrate chromogen system (Dako A/S, Glostrup, Denmark). Sections were thoroughly washed under running tap water and counterstained with hematoxylin.

Stereological measurements

Immunohistochemically stained 5 μm thick sections of the pars distalis were used for morphometric examinations of the GH and LH cells that possessed visible nuclei. The cell volumes of the examined cells (Vc) and their volume densities (V_V) were estimated by light microscopy at 1000x magnification using the M_{42} multipurpose test system (29). The volumes of cells were expressed in μm^3, while their volume densities were given as percentages of total pituitary cells in mm^3.

Determination of serum hormone concentrations

Serum concentrations of the GH and PRL in control and experimental rats were measured by the Delfia method (hGH and hPRL Delfia kits; LKB, Turku, Finland).

Statistical analysis

All results were expressed as means for six animals per group ± SD. The data were tested for normality of distribution by the Kolmogorov–Smirnov test, whereas the homogeneity of variances was evaluated by the Leven's test. Student's t-test was used to compare the mean values. The minimum level of statistical significance was set at $p<0.05$.

RESULTS

Body mass and pituitary weights

Results on bodymass, absolute and relative pituitary weights are summarized in Table 1. Exposure of female rats to CL, from peripubertal period of life to adulthood, resulted in significant decrease of the weight by 22.9% ($p<0.05$) comparing to the control group. In absolute and relative pituitary weights, no significant changes were detected (Table 1).

Table 1. Effects of prolonged exposure to constant light (CL) on body mass and pituitary weight in adult female rats

(g)	Body mass	Absolute pituitary weight (mg)	Relative pituitary weight (mg x 100)/ body mass
C	304.2 ± 19.6	15.3 ± 0.8	5.1 ± 0.5
CL	234.4 ± 25.6*	14.3 ± 1.6	5.3 ± 0.9

All values are given as mean ± SD (n=6); *p<0.05 CL vs. Control

Immunohistochemical analysis

Somatotropic cells (GH) in the pituitary pars distalis of control females were stained intensely and homogenously. They were ovoid to pyramidal in shape, usually present in groups and in close contact with blood capillaries (Fig. 1a). After chronic exposure to constant light, the immunolabeled GH cells were ovoid to polyhedral in shape, but smaller in size and more frequently present alone than in groups (Fig. 1b) comparing to the controls.

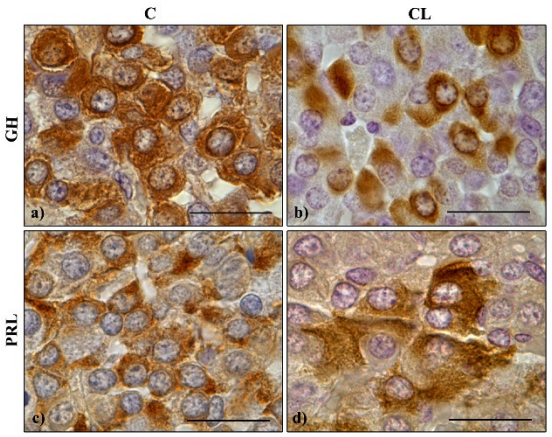

Figure 1. Representative micrographs of the GH (a, b) and PRL cells (c, d) in the pituitary of control (C; a, c) and females exposed to constant light (CL; b, d). Bar scale – 20 m.

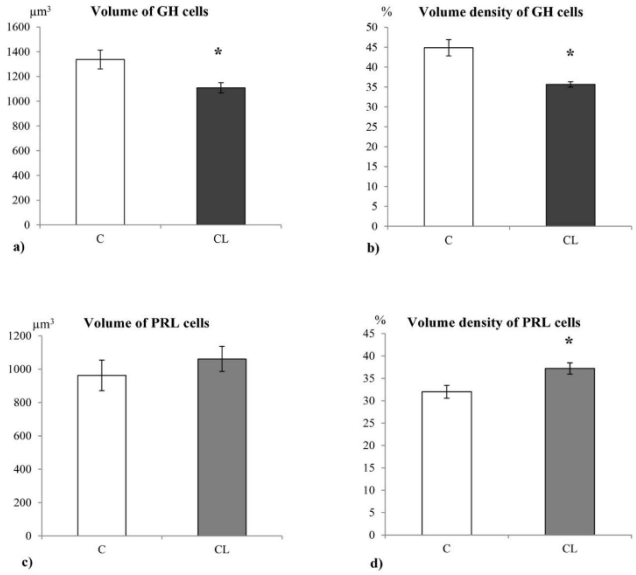

Figure 2. Stereological parameters of the GH (a, b) and PRL cells (c, d) in the pituitaries of control (C) and females exposed to constant light (CL). All values are provided as the mean ± SD; n = 6. *p < 0.05, CL *vs.* C.

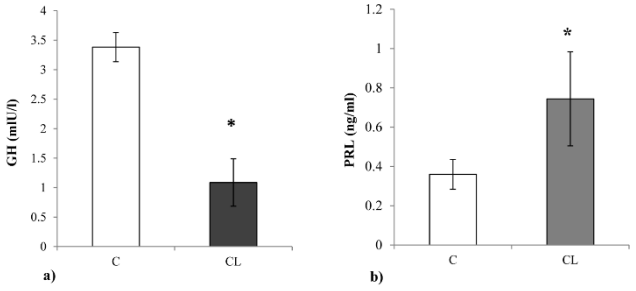

Figure 3. Serum levels of the GH (a) and PRL (b) in control (C) and females exposed to constant light (CL). All values are presented as the mean ± SD; n = 6. *p < 0.05, CL *vs.* C.

Lactotropic (PRL) cells of control females were polygonal in shape with the spherical nuclei, surrounded by relatively small portion of the pronounced immunolabeled cytoplasm (Fig. 1c). In the pituitaries of females exposed to constant light, PRL cells were larger; their shape was irregular, with more pronounced prolactin content within their cytoplasm (Fig. 1d).

Stereological analisys

Stereological analysis confirmed findings of the histological examination. Namely, volume of the GH cells was significantly decreased after constant light exposure, by 17.1% ($p<0.05$), comparing to the control values (Fig. 2a). Consequently, the percentage of somatotropic cells within the pituitaries of females exposed to constant light, i.e. their volume density, was decreased by 20.5% ($p<0.05$) (Fig. 2b). Volume of the PRL cells was increased by 10.0% compared to the control values (Fig. 2c), however, this increase did not reach statistical significance. On the other hand, volume density of the immunoreactive PRL cells was increased by 16.3% ($p<0.05$), comparing to the controls (Fig. 2d).

Serum GH and PRL concentrations

After exposure to the constant light, serum GH concentration decreased by 67.0% ($p<0.05$) (Fig. 3a). On the other hand, the PRL concentration increased ($p<0.05$) two-fold (Fig. 3b) compared to the controls.

DISCUSSION

In an effort to elucidate whether the functional pinealectomy influence pituitary GH and PRL cells, their immunohistochemical and morphofunctional properties were assessed in adult female rats exposed to the constant light regime from peripubertal period of life. Our results provide evidence that constant light conditions induce structural and functional changes of pituitary GH and PRL cells.

In the pituitary *pars distalis* of light-treated females, the GH cells were smaller in size than in control females which was confirmed by stereological analysis, *i.e.* their volume and volume density were decreased. On the other hand PRL cells were enlarged, with more intensely stained cytoplasm and increased volume density. Francou et al. (30), however, did not observe changes in average percentage of the GH or PRL cells in females that were exposed to continuous illumination in adulthood. This discrepancy may originate from the different experimental settings. Namely, in our study females were exposed to constant light conditions throughout sensitive pubertal maturation, which elicit more stronger changes in PRL and GH cells, since it is also a period of maturation of Gh1 and Prl genes expression (31).

Smaller GH cell volume and decreased overall percentage in *pars distalis* resulted in decreased GH blood concentration in our study. Changes of environmental lighting conditions cause lower amplitudes of GH peaks in adult male rats (32) and decreased growth rates, judged by the decreased body weight and tail length (33) exposed to continuous light in adult or juvenile period of life, respectively. We have also observed the reduced body weight in light-treated females in the present and in our previous study (21).

Alternation of circadian PRL rhythms and elevated PRL blood level in females exposed to long periods of constant light (60-120 days) (34) or from birth till adulthood (35) were previously reported. Accordingly, in our study we observed increased volume density of PRL cells in light-treated females that resulted in the increased PRL concentration. Rhythmic urine PRL, but with elevated average value, was also observed in males under longer (three weeks) (36) or shorter (one week) (37) continuous light regime.

The effects of melatonin on the GH and PRL cells, *i.e.* its lowered concentration in animals exposed to the constant light (36) must be taken into account. It has been reported that the GH rhythm was suppressed in rats with removed pineal gland, while after melatonin administration a distinct increase of GH concentration during the day was observed (38). Melatonin plays facilitatory role in the GH secretion at the hypothalamic level by inhibiting endogenous somatostatin release in men (39). Additionally, there is evidence that melatonin treatment decreases rat levels of ghrelin (40), an endogenous ligand for the growth hormone secretagogue receptors (41).

It is generally accepted that melatonin modulates the PRL secretion in the rat. Its inhibitory effect on the hypothalamic dopamin release, thus decreasing inhibitory influence of dopamin on the secretion of PRL (42) has been shown *in vitro*. However, there are reports that melatonin reduced the PRL production from rat pituitary cells in culture (43). Presence of melatonin receptors was not confirmed in the PRL cells of pituitary *pars distalis* (44), in contrast to pituitary *pars tuberalis*, which contains a high density of receptors for the melatonin and it is implicated in the expression of both circadian and seasonal endocrine cycles (45). This structure also expresses clock genes (46). Hence, in melatonin mt1 receptor knockout mice amounts of circulating prolactin are significantly higher than in wild-type littermates (47).

Alterations in light cycles are typically considered as stressful. Continuous light exposure caused a significant increase in serum adrenocorticotropic hormone and serum corticosterone concentration in female rats (22, 23). Stimulated pituitary-adrenal axis and elevated glucocorticoid levels may affect the GH and PRL cells since exposure to different stressors induces the decrease of GH and increase of PRL levels (48). Growth hormone concentration can be modulated by elevated glucocorticoids by different actions on the hypothalamus, pituitary gland and liver (49). There is also evidence that corticotropin-releasing hormone serves as the mediator of the stress-induced suppression of GH *via* direct synaptic connections between somatostatin neurons (6). Many hypothalamic substances are implicated in the prolactin-secretory stress response, and besides dopamine they include serotonin, histamine, N-methyl-D,L-aspartic acid, atrial natriuretic peptide, b-endorphin and dynorphin, oxytocin, vasopressin (10) and melanocortins (50).

Chronic exposure of female rats to constant light regime is used for developing of the polycystic ovary syndrome in the rodent model, in which levels of estradiol and estrone are elevated (27). It has been show that estradiol inhibits the GH (51) and stimulates the PRL secretion (10).

In conclusion we have demonstrated that exposure of female rats to constant light regime from peripubertal to adult period of life, cause inhibition of pituitary GH and stimulation of PRL cells, judged by their immunohistochemical and morphometric features, and physiological endpoint, *i.e.* concentration of these hormones in circulation.

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SERBIAN TRANSLATION AND VALIDATION OF THE SF-36 FOR THE ASSESSMENT OF QUALITY OF LIFE IN PATIENTS WITH DIAGNOSED ARTERIAL HYPERTENSION

Aleksandra Nikolic[1], Vladimir Biocanin[2], Nemanja Rancic[3], Mirjana Duspara[4] and Dusan Djuric[5,6]

[1]*University Clinical Center Kragujevac, Kragujevac*
[2]*Faculty of Stomatology, Pancevo, University of Business Academy in Novi Sad, Serbia*
[3]*Centre for Clinical Pharmacology, Military Medical Academy;Faculty of Medicine of the Military Medical Academy, University of Defence, Belgrade, Serbia*
[4]*Public health institution Health center Tuzla ,,Dr Mustafa Šehović", Tuzla, Bosnia and Hercegovina*
[5]*University of Kragujevac, Faculty of Medical Sciences, Department of Pharmacy, Kragujevac, Serbia*
[6]*Institute for rehabilitation, Belgrade, Serbia*

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Corresponding author:

Dusan Djuric, PhD

University of Kragujevac, Serbia, Faculty of Medical Sciences; Department of Pharmacy, Department of Clinical Pharmacy, Institute for rehabilitation, Belgrade, Serbia

Phone: +381 653110073

E-mail: duca1duca@gmail.com

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ABSTRACT

Precise estimation of life quality is of special importance in patients with chronic diseases, such as arterial hypertension. There are many questionnaires for that purpose. Short-form 36 (SF-36) with 8 domains has been proved as one of the most appropriate. To date, there was no translated and validated SF 36 in Serbian language for hypertensive patients. The aim of this study was to test validity and reliability of SF-36 in Serbian patients with diagnosed arterial hypertension. Cronbach's alpha coefficient was calculated to assess the internal consistency of the Serbian version of the SF-36. After deducting the overlap between each of the 36 items and its related domain, the collective validity was considered to be good if the correlation coefficient remains > 0.4. Only 2.54% answers on the questions were missing. Values of all the 8 domains were higher in men than in women. Cronbach alpha coefficient was high for SF-36, 0.897, and it suggesting that the SF-36 had good internal reliability. All 8 domains showed high values non-rotating factorial weights (>0.300) (range from 0.742-0.856), and all measure the same thing. It means that all components in this questionnaire measure the things they are assigned to.

Keywords: *SF-36, Serbian population, quality of life, hypertension.*

INTRODUCTION

There is increasing demand by doctors and other medical professionals to measure the quality of life in different disease groups. It is very important to assess quality of life especially in patients with chronic diseases, having in mind their longevity and associate problems (1, 2). In Serbian population, there is growing number of hypertensive patients. For that reason, it is important to analyze the quality of life in among these patients. Patients`perception of their life quality may be affected by disease and medication, especially important in patients with arterial hypertension. Namely, patients with moderate and mild hypertension usually do not have any symptoms (3). On the other hand, many antihypertensive drugs may show some side effects that may impact quality of life (4, 5). It was already shown that hypertensive patients had poorer quality of life in comparison to healthy individuals (2).

Many researchers have found wide range of questionnaires to measure patient's quality of life. A short form health survey questionnaire (SF-36) has been proved as one of the most appropriateand has been translated and validated into variety of languages (6-10).

SF -36 consists of 36 questions grouped in 8 domains (11, 12). Scientific evidences showed that SF-36 is reliable questionnaire for the assessment quality of life in different populations (13).

There is an increasing need among health professionals to use validated quality of life questionnaires in hypertensive patients (14). At present, there is no validated form of Serbian version of SF-36. The aim of this study was to test validity and reliability of SF-36 in Serbian patients with diagnosed arterial hypertension.

MATERIAL AND METHOD

The 169 patients with diagnosed arterial hypertension were recruited from September 2019- January 2020 from the Pharmacy department in Kragujevac. Each patient gave informed consent to participate in the study, according to ethical standards and Declaration of Helsinki. The study was anonymous, and the results remain confidential. The completed questionnaires did not contain any identifying information about the individual participant. Enrollment in the study was voluntary-based.

The SF-36 Health Survey is composed of 36 questions and standardized response choices, organized into eight multi-item scales:

- physical functioning (PF);
- role limitations due to physical health problems (RP);
- bodily pain (BP), general health perceptions (GH);
- vitality (VT), social functioning (SF);
- role limitations due to emotional problems (RE);
- general mental health (MH).

In this study, translated Serbian form of SF-36 was used (**Appendix 1**).

Statistical analysis – Reliability.

Cronbach's alpha coefficient was calculated to assess the internal consistency of the Serbian version of the SF-36. Internal consistency estimates of a magnitude of >0.70 were considered acceptable for group comparisons (15). Test-retest reliability of the SF-36 was assessed using interclass correlations (ICC) between baseline and retest. A correlation of >0.80 was considered 'good' (16).

Statistical analysis – Validity.

The complete statistical analysis was performed using the computer program IBM SPSS Statistics 19.0. All continuous variables are shown in the form of the mean ± standard deviation with 95% confidence interval or median with interquartile range (25-75th percentiles) (IQR), while the categorical variables are shown with the percentage of certain category frequency. Chi-square test and independent t-test were used for the analysis of socio-demographic characteristics of patients. Kolmogorov-Smirnov test (tests of normality) was used for testing normality of SF 36 domains. Mann-Whitney U test was done to compare SF 36 domains according to gender.The correlation between the two continuous variables was examined by Pearson linear correlation.

Cronbach's alpha, split-half and test-retest methods of reliability were used. For the evaluation of the validity of this scale, the Principal Component Analysiswas applied and the criterion for the number of the extracted components was Eigenvalue > 1. The factor loading of 0.4 or greater was considered.

In the present study, factor analysis for the eight domains was used to evaluate the structural validity of the SF-36 questionnaire. The Kaiser–Meyer–Olkin (KMO) statistic and Bartlett's spherical check were carried out to check for sample suitability for the factor analysis. Factor analysis is a statistical method used to test the structural validity of a scale and describes variability among observed variables in terms of fewer unobserved variables – called factors.

RESULTS

Characteristics of patients

The SF-36 was completed by 169 adult patients with diagnosed arterial hypertension. This sample represented 76 males and 93 females with a mean age of 65.04 ± 11.18 years (age range 35-88 years) (Table 1).

Missing data

Out of all patients (169) and all questions (6084), 2.54% or 157 answers on the questions were missing. About 97.5% SF-36 questions were completed.

Descriptive statistics of SF-36

Descriptive statistics of all 8 domains of SF 36 was shown in Table 2. When the check of normality was done, we could see that only in one domain (General health) the condition for normality of data was fulfilled, but with other domains no. Meridian range of all 8 domains was from 50.00 (Role limitations due to physical health) to 66.67 (Role limitations due to emotional problems).

Gender differences

SF-36 domains for the responder sample were further analysed to determine any differences between males and females. Table 3 displays the descriptive characteristics as a function of gender (mean, standard deviation). Analysis revealed many differences between males and females for SF-36 domains (Physical functioning, Role limitations due to physical health, Energy/fatigue, Emotional well-being, Social functioning, and Pain).Values of all the 8 domains were higher in men than in women.

Reliability Analysis

Cronbach alpha coefficient was high for SF-36, 0.897, and it suggesting that the SF-36 had good internal reliability. If we look at the correlation matrix, we will see excellent correlation among all SF 36 domains (r>0.300) (Table 4). The lowest correlation was 0.469 and the highest 0.729. Average inter-item correlation was 0.598.

The summary statistics of all SF 36 domains was displayed in Table 5. If any domain removes from analysis, there is no growing ofCronbach's Alpha. It means that all domains should be analysed and they are valid for our population (Cronbach's Alpha if Item Deleted range from 0.880-0.894). Likewise, in Table 5 we could see good correlation of every domain with overall sum of SF-36 score (Corrected Item-Total Correlation range from 0.688-0.774).

Validity Analysis

Structural validity was evaluated by means of factor analysis. Results showed the KMO measure to be 0.904 and the Bartlett's spherical check to be $\chi 2 = 888.231$ and $p < 0.001$, which taken together, indicated that the samples in this study were suitable for factor analysis.

Factor analysis results indicated that when one component summary score, were included the eight domains whose characteristic roots were > 1 or approaching 1, the accumulative contribution rate was up to 64.93% (Figure 1 and Table 6).

In the end, Component Matrix was gained with factorial analysis, where all 8 domains showed high values non-rotating factorial weights (>0.300) (range from 0.742-0.856), and all measure the same thing. It means that all components in this questionnaire measure the things they are assigned to (Table 7).

Using a Pearson product moment correlation analysis, the results are presented in Table 8. The outcome of the correlation analysis indicated that the vast majority of items correlated more strongly with their related domain by comparison to an unrelated domain.

The retest of the correlation between the items showed that $r > 0.900$ could be achieved for all eight domains ($p < 0.001$) (Table 9), demonstrating good stability for the SF-36 questionnaire. This analyze was performed in 19 patients two weeks after first test. The difference between the mean values for each domain after two rounds of measurements was not statistically significant (p>0.05 for all domens).

Figure 1. Scree plot

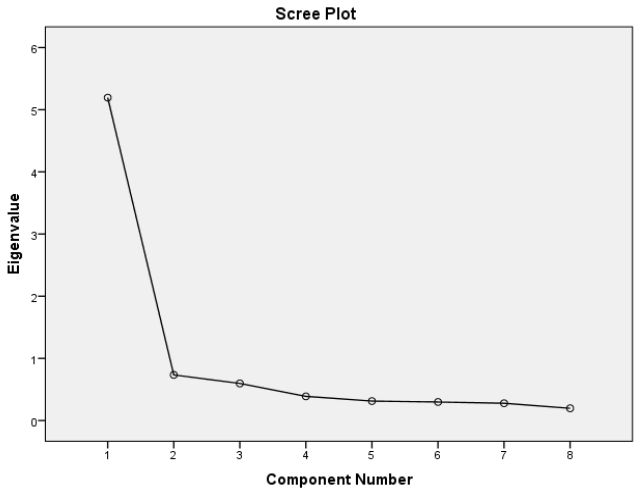

Table 1. Socio-demographic characteristics of patients with diagnosed hypertension

	Male	Female	p value
Gender; n (%)	76 (45%)	93 (55%)	
Age; M ± SD	65.32 ± 11.27	64.83 ± 11.17	0.789*
Level of education; n (%)			
primary school	13 (17%)	22 (25%)	0.050**
secondary school	31 (42%)	48 (54%)	
higher school	10 (13%)	7 (8%)	
University	21 (28%)	12 (13%)	

n (%)- number (%); M ± SD- mean ± standard deviation; *- Independent samples t test; **- Chi-square test

Table 2. Parametric and non-parametric descriptive statistics of SF-36 domains

SF-36 domains	Mean	SD	95% CI	p value*	Median	IQR
Physical functioning	62.02	29.71	57.50-66.53	<0.001	65.00	44.72-89.44
Role limitations due to physical health	46.94	43.83	40.29-54.00	<0.001	50.00	0.00-100.00
Role limitations due to emotional problems	53.55	44.51	46.79-60.31	<0.001	66.67	0.00-100.00
Energy/fatigue	54.21	20.55	51.09-57.33	0.030	52.00	44.00-68.00
Emotional well-being	65.59	19.09	62.69-68.48	<0.001	63.33	52.00-83.33
Social functioning	64.79	23.88	61.17-68.42	<0.001	62.50	50.00-87.50
Pain	62.97	23.84	59.35-66.59	<0.001	57.50	45.00-78.75
General health	51.36	20.98	48.17-54.55	0.200	52.27	38.64-65.91

*- Kolmogorov-Smirnov test (Tests of normality); SD- standard deviation; 95% CI- 95% confidence interval; IQR- interquartile range (25-75th percentiles)

Table 3. Descriptive statistics of SF-36 domains according to gender

SF-36 domains	Male	Female	p value*
Physical functioning	75.00 (51.25-90.00)	60.00 (35.00-86.94)	0.033
Role limitations due to physical health	75.00 (0.00-100.00)	25.00 (0.00-100.00)	0.007
Role limitations due to emotional problems	83.33 (0.00-100.00)	66.67 (0.00-100.00)	0.184
Energy/fatigue	56.00 (49.00-76.00)	52.00 (36.00-64.00)	0.001
Emotional well-being	68.33 (53.33-86.67)	63.33 (50.00-73.33)	0.025
Social functioning	75.00 (53.12-87.50)	50.00 (50.00-75.00)	<0.001
Pain	67.50 (45.00-90.00)	55.00 (45.00-77.50)	0.002
General health	52.27 (38.64-65.91)	47.73 (31.82-68.18)	0.168

*- Mann-Whitney U test; Median (IQR- interquartile range (25-75th percentiles))

Table 4. Inter-item correlation matrix of SF-36 domains*

	Physical functioning	Role limi-tations due to physical health	Role limita-tions due to emotional problems	Energy/ fatigue	Emotional well-being	Social function-ing	Pain	Gen-eral health
Physical functioning	1.000							
Role limitations due to physical health	0.618	1.000						
Role limitations due to emotional problems	0.502	0.699	1.000					
Energy/fatigue	0.595	0.605	0.545	1.000				

	Physical functioning	Role limitations due to physical health	Role limitations due to emotional problems	Energy/ fatigue	Emotional well-being	Social functioning	Pain	General health
Emotional well-being	0.478	0.485	0.531	0.729	1.000			
Social functioning	0.592	0.601	0.527	0.684	0.708	1.000		
Pain	0.673	0.647	0.547	0.619	0.548	0.634	1.000	
General health	0.630	0.522	0.469	0.715	0.601	0.634	0.606	1.000

*- Pearson linear correlation

Table 5. Item-Total Statistics of SF-36 domains

	Scale Mean if Item Deleted	Scale Variance if Item Deleted	Corrected Item-Total Correlation	Squared Multiple Correlation	Cronbach's Alpha if Item Deleted
Physical functioning	399.412	25586.534	0.714	0.572	0.881
Role limitations due to physical health	414.487	21526.545	0.763	0.638	0.883
Role limitations due to emotional problems	407.879	22161.700	0.688	0.540	0.894
Energy/fatigue	407.223	27552.407	0.774	0.692	0.882
Emotional well-being	395.844	28435.334	0.692	0.636	0.888
Social functioning	396.637	26795.792	0.754	0.639	0.880
Pain	398.457	26787.682	0.756	0.597	0.880
General health	410.070	27853.907	0.709	0.600	0.886

Table 6. Total Variance Explained

Component	Initial Eigenvalues			Extraction Sums of Squared Loadings			Rotation Sums of Squared Loadings*
	Total	% of Variance	Cumulative %	Total	% of Variance	Cumulative %	Total
1	5.19	64.93	64.93	5.19	64.93	64.93	4.66
2	0.73	9.18	74.11	0.73	9.18	74.11	4.28
3	0.60	7.46	81.57				
4	0.39	4.86	86.42				
5	0.31	3.91	90.34				
6	0.30	3.72	94.06				
7	0.28	3.48	97.54				
8	0.20	2.46	100.00				

Extraction Method: Principal Component Analysis; *- When components are correlated, sums of squared loadings cannot be added to obtain a total variance.

Table 7. Component Matrix

	Component
	1
Energy/fatigue	0.856
Social functioning	0.838
Pain	0.819
General health	0.806
Role limitations due to physical health	0.802
Emotional well-being	0.790
Physical functioning	0.789
Role limitations due to emotional problems	0.742

Extraction Method: Principal Component Analysis; One component extracted.

Table 8. Item to domain correlations for the SF-36 data (for all correlations p<0.001)*

	Physical functioning	Role limitations due to physical health	Role limitations due to emotional problems	Energy/fatigue	Emotional well-being	Social functioning	Pain	General health
SF1	-0.507	-0.384	-0.328	-0.490	-0.408	-0.465	-0.495	**-0.663**
SF2	-0.427	-0.319	-0.271	-0.378	-0.250	-0.333	-0.455	**-0.515**
SF3	**0.755**	0.523	0.367	0.509	0.332	0.383	0.624	0.553
SF4	**0.822**	0.537	0.426	0.536	0.415	0.535	0.557	0.559
SF5	**0.863**	0.578	0.478	0.567	0.433	0.547	0.538	0.581
SF6	**0.837**	0.536	0.424	0.490	0.325	0.417	0.552	0.477
SF7	**0.822**	0.509	0.339	0.514	0.419	0.545	0.544	0.546
SF8	**0.823**	0.519	0.431	0.422	0.252	0.393	0.583	0.506
SF9	**0.852**	0.536	0.439	0.572	0.469	0.556	0.604	0.602
SF10	**0.856**	0.506	0.427	0.498	0.449	0.516	0.562	0.538
SF11	**0.786**	0.377	0.316	0.407	0.357	0.467	0.438	0.446
SF12	**0.711**	0.392	0.376	0.393	0.401	0.503	0.418	0.460
SF13	0.570	**0.856**	0.629	0.535	0.491	0.535	0.558	0.476
SF14	0.471	**0.877**	0.603	0.465	0.393	0.515	0.543	0.385
SF15	0.510	**0.869**	0.607	0.532	0.390	0.545	0.559	0.433
SF16	0.600	**0.896**	0.568	0.607	0.451	0.532	0.623	0.540
SF17	0.511	0.669	**0.885**	0.549	0.484	0.526	0.551	0.461
SF18	0.424	0.656	**0.911**	0.484	0.475	0.437	0.487	0.405
SF19	0.385	0.506	**0.865**	0.448	0.464	0.423	0.440	0.377
SF20	-0.527	-0.553	-0.487	-0.554	-0.598	**-0.890**	-0.536	-0.513
SF21	-0.604	-0.578	-0.445	-0.510	-0.418	-0.502	**-0.934**	-0.507
SF22	-0.659	-0.625	-0.550	-0.572	-0.501	-0.620	**-0.945**	-0.583
SF23	-0.623	-0.556	-0.468	**-0.793**	-0.526	-0.595	-0.580	-0.676
SF24	0.337	0.322	0.319	0.461	**0.665**	0.418	0.276	0.359
SF25	0.382	0.363	0.425	0.566	**0.834**	0.558	0.379	0.452
SF26	-0.352	-0.373	-0.418	-0.556	**-0.732**	-0.561	-0.392	-0.465
SF27	-0.559	-0.560	-0.520	**-0.772**	-0.659	-0.617	-0.620	-0.663
SF28	0.347	0.388	0.393	0.542	**0.737**	0.473	0.350	0.413
SF29	0.384	0.455	0.458	**0.777**	0.638	0.466	0.368	0.525
SF30	-0.351	-0.367	-0.390	-0.466	**-0.712**	-0.474	-0.432	-0.471
SF31	0.411	0.426	0.358	**0.863**	0.516	0.483	0.392	0.482
SF32	0.534	0.523	0.437	0.619	0.612	**0.902**	0.548	0.575
SF33	0.522	0.539	0.455	0.607	0.554	0.599	0.550	**0.788**
SF34	-0.472	-0.358	-0.336	-0.427	-0.336	-0.436	-0.398	**-0.726**
SF35	0.412	0.309	0.255	0.456	0.376	0.404	0.349	**0.736**
SF36	-0.534	-0.386	-0.373	-0.644	-0.512	-0.474	-0.452	**-0.878**

*- Pearson linear correlation

Table 9. Test-retest reliability

SF-36 domains	Mean test	Mean retest	Test-retest reliability *	p value**
Physical functioning	65.22	64.96	0.990	0.749
Role limitations due to physical health	53.51	54.03	0.987	0.742
Role limitations due to emotional problems	52.63	52.81	0.995	0.875
Energy/fatigue	52.74	53.58	0.970	0.130
Emotional well-being	63.75	65.86	0.945	0.131
Social functioning	63.82	63.82	0.987	1.000
Pain	60.92	61.45	0.978	0.630
General health	49.40	49.04	0.972	0.723

*- Pearson Correlation test; **- Paired samples t test

DISCUSSION

The SF 36 is widely used as a reliable, concise and valid questionnaire to measure quality of life in patients with various diseases (17, 18). This study evaluated validity and reliability of SF 36 in Serbian hypertensive patients. To the best of our knowledge, this is the first study to validate SF 36 in Serbian patients with hypertension.

Arterial hypertension is great risk factor for many cardiovascular diseases (19). Hypertensive patients have poorer quality of life and may develop anxiety and depression (20, 21). It was interesting to find out quality of life in Serbian hypertensive patients. Before that, in order to assess quality of life in Serbian hypertensive patients, SF 36 have to be validated.In general, the findings of our study showed that the Serbian version of SF 36 had good agreement with English version of the questionnaire. This is in accordance with previous similar studies (22, 23).

The number of missing items in our study was low (2.54%). This fact indicated good acceptance of SF 36 in Serbian hypertensive patients. Low missing items obtained in our study was similar to other studies in healthy individuals and patients with other diseases (24, 25).

It was an interesting finding that SF-36 domains in our study (Physical functioning, Role limitations due to physical health, Energy/fatigue, Emotional well-being, Social functioning, and Pain) were higher in men than in women. This is similar to the study of de Carvahloet al (2) whoassessed the quality of life in hypertensive and normotensive patients and found higher values of all areas of SF in males compared to females.

The Cronbach α was >0.8 for all eight domains of SF 36, indicating very good reliability. In addition, it indicates good internal uniformity. This is in accordance with the study of Zhang et al. [26,] where the overall Cronbach α was 0.794.

In our study the SF 36 demonstrated good stability due to the result of retest of correlation between the items ($r > 0.900$) achieved for all eight domains ($p < 0.001$).

The limitation of this study was relatively small number of patients. For that reason, the results of this study should be interpreted with caution to whole population.

CONCLUSION

The findings of our study confirmed cross-cultural validity of SF 36 for hypertensive patients, with good reliability and validity. According to that, we believe the Serbian version of SF 36 is suitable for measurement the quality of life in hypertensive patients.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

The study was conducted in accordance with the Declaration of Helsinki. The protocol of the study was approved by the Ethical committee from the Faculty of Medical Science in Kragujevac (No 01/18-4834). Each patient gave informed consent to participate in the study.

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CONFLICTS OF INTEREST

The authors declare no conflict of interest.

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MOLECULAR DOCKING ANALYSIS OF NOVEL THIOUREA DERIVATIVES OF NAPROXEN WITH POTENTIAL ANTITUMOR ACTIVITY

Nikola Nedeljkovic[1], Vladimir Dobricic[2], Marina Mijajlovic[1], Zorica Vujic[2] and Milos Nikolic[1]

[1]*University of Kragujevac, Faculty of Medical Sciences, Department of Pharmacy, Kragujevac, Serbia*
[2]*Department of Pharmaceutical Chemistry, University of Belgrade – Faculty of Pharmacy, Belgrade, Serbia*

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Corresponding author:

Milos Nikolic

University of Kragujevac, Faculty of Medical Sciences, Kragujevac, Serbia, 69 Svetozara Markovica Street 34000 Kragujevac, Serbia

E-mail: milos.nikolic@medf.kg.ac.rs

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ABSTRACT

Naproxen, as a propionic acid derivative, causes serious gastrointestinal side effects due to the presence of free carboxylic group. In that sense, masking of carboxylic group with other pharmacophores may be a promising strategy to decrease gastrointestinal toxicity. Thiourea derivatives have been intensively investigated as potential antitumor drugs, whereby their activity is based on potential inhibition of protein kinases, topoisomerases, carbonic anhydrase and sirtuins. In addition, it was shown that inhibition of certain protein kinases might reverse resistance to chemotherapeutic drugs by enhancing the cell death in the presence of low concentrations of drug. Twenty new thiourea derivatives of naproxen were designed and their binding to four selected protein kinases involved in tumor multidrug resistance (AKT2, mTOR, EGFR and VEGFR1) was estimated using two molecular docking programs (AutoDock Vina and OEDocking). According to OEDocking, the highest potential to inhibit AKT2 and mTor has derivative 1, while derivative 20 demonstrates the highest potential towards EGFR and VEGFR1. According to AutoDock Vina, the highest potential for inhibition of EGFR, AKT2 and VEGFR1 have derivatives 16 and 17. Therefore, derivatives 1, 16, 17 and 20 are potentially the most potent protein kinase inhibitors that could be further synthesized and tested for anticancer activity.

Keywords: *Antitumor activity, AutoDock Vina, molecular docking, naproxen, thiourea derivatives.*

INTRODUCTION

Naproxen is a non-steroidal anti-inflammatory drug which prevents conversion of arachidonic acid to eicosanoids through competitive inhibition of both cyclooxygenase isoenzymes (COX-1 and COX-2), resulting in analgesic and anti-inflammatory effects (1). As a propionic acid derivative, naproxen causes serious gastrointestinal side effects due to the presence of free carboxylic group (2). Therefore, masking carboxylic group with other moieties may be a promising strategy in order to decrease gastrointestinal toxicity (3). Piffar and coworkers showed that naproxen was able to reduce tumor growth in rats by 58% (4). Hydroxamic acid derivative of naproxen demonstrated histone deacetylase inhibition (5), while propanamide and urea derivatives showed cytotoxic effect against the cancer cell line HCT–116 (6).

Thiourea and other structure-related derivatives, such as thiosemicarbazones, have attracted great attention of scientists in terms of evaluation of their biological activity (7-9). The thiourea moiety has been described as an important pharmacophore in a variety of pharmacologically active compounds, including anti-inflammatory (10), antiviral (11), anticancer (12,13), hypoglycemic (14) and antimicrobial agents (15). In the past few decades, thiourea derivatives have been intensively investigated as potential anticancer drugs. This class of compounds has been recognized as agents with promising inhibitory activity towards human lung adenocarcinoma cell lines, human breast cancer cells and human colorectal carcinoma (16-18). Antitumor activity of thiourea derivatives is based on potential inhibition of protein kinases (19), topoisomerases (20), carbonic anhydrase (21) and sirtuins (22).

Protein kinases are widely studied targets in the drug design studies due to their pivotal role in regulation of cell functions (23). The activation of protein kinases in cell signaling pathways is associated with cancer cell survival, tumor invasiveness and drug resistance (24). Therefore, compounds targeting protein kinases have become one of the most studied classes of cytotoxic agents (25). Multidrug resistance (MDR) is a predominant cause of cancer chemotherapy failure, which is responsible for over 90% mortality of cancer (26,27). MDR can be associated with elevated metabolism and increased efflux of xenobiotics, growth factors, increased DNA (Deoxyribonucleic acid) repair capacity, and various genetic factors (28,29). It was also demonstrated that inhibition of certain protein kinases not only decreases the proliferation and growth of carcinoma cells, but may reverse resistance to chemotherapeutic drugs by enhancing the cell death in the presence of low concentrations of drug, thereby reducing drug side effects (30-33). Today, a number of biomedical studies are focused on design of antitumor drugs that are able to reverse MDR.

The aims of this study were to design new thiourea derivatives of naproxen and identify the most promising candidates that could be used for the therapy of MDR tumors. For this purpose, molecular docking analysis was carried out towards selected protein kinases involved in multidrug resistance in order to identify designed derivatives with the highest enzyme inhibitory activity.

MATERIALS AND METHODS

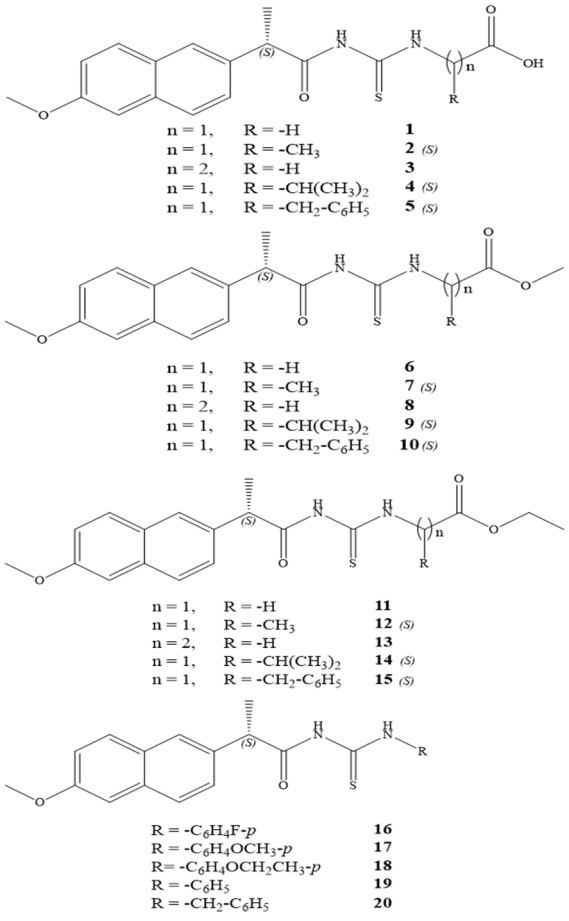
2.1. Software

Geometry of designed compounds was optimized using Chem3D Ultra 7.0 (34). Preparation of ligands for the molecular docking calculations was carried out in AutoDockTools 1.5.6 (35) and OMEGA 2.5.1.4 (36,37). Protein molecules (selected protein kinases) were prepared for molecular docking in BIOVIA Discovery Studio Visualizer 17.2.0.16349 (38), AutoDockTools 1.5.6 and MAKE Receptor 3.2.0.2 software (39). AutoDock Vina (40) and OEDocking 3.2.0.2 software (41-43) were used for the analysis of binding poses and ligand-receptor interactions.

2.2. Designed compounds

Compounds designed and tested in this study (**1-20,** Figure 1) are thiourea derivatives of naproxen, containing amino acids glycine, L-alanine, β-alanine, L-valine and L-phenylalanine (**1-5**), their methyl (**6-10**) and ethyl (**11-15**) esters, as well as aromatic amines (**16-20**).

Figure 1. The structures of the tested compounds (1-20)



2.3. Ligand preparation

2.3.1. AutoDock Vina

Geometry of tested molecules was optimized using AM1 semi-empirical quantum chemical methods in Chem3D Ultra 7.0 software. Furthermore, these molecules were imported into the Mercury 3.10.2 (44) and converted into the mol2 format. In order to prepare selected compounds for the docking calculations, AutoDockTools 1.5.6. was used to add Gasteiger charges, set rotatable bonds and save selected molecules in pdbqt format.

2.3.2. OEDocking

Prior to the molecular docking in OEDocking 3.2.0.2, ligand preparation was performed in OMEGA 2.5.1.4 and files containing 200 conformers for each ligand were generated.

2.4. Selection and preparation of receptors

Crystal structures of four protein kinases involved in multidrug resistance were taken from the Protein Data Bank (45): 1M17 (Epidermal Growth Factor Receptor - EGFR), 3E87 (RAC-beta serine/threonine-protein kinase - AKT2), 3HNG (Vascular endothelial growth factor receptor 1 - VEGFR1) and 4JSV (Serine/threonine-protein kinase - mTOR). Details of selected enzymes were presented in Table 1.

2.4.1. AutoDock Vina

BIOVIA Discovery Studio Visualizer v. 17.2.0.16349 was used to remove the co-crystalized ligands, water molecules and unnecessary receptor chains. Prior to docking, AutoDockTools 1.5.6. was used to prepare the proteins for AutoDock Vina by assigning hydrogens and converting protein structures from pdb to pdbqt format.

Table 1. Protein kinases selected for this study

Target	Selected PDB (resolution)	Co-crystalized ligand	Chains	Selected chain
EGFR	1M17 (2.60 Å)	[6,7-*bis*(2-methoxy-ethoxy)quinazoline-4-yl]-(3-ethynylphenyl)amine (Ligand ID: **AQ4**)	A	A
AKT2	3E87 (2.30 Å)	*N*-[(1*S*)-2-amino-1-phenylethyl]-5-(1*H*-pyrrolo[2,3-*b*]pyridin-4-yl)thiophene-2-carboxamide (Ligand ID: **G95**)	A, B	A
VEGFR1	3HNG (2.70 Å)	*N*-(4-chlorophenyl)-2-[(pyridin-4-ylmethyl)amino]benzamide (Ligand ID: **8ST**)	A	A
mTOR	4JSV (3.50 Å)	adenosine-5'-diphosphate (Ligand ID: **ADP**)	A, B	A

Based on the location coordinates of the co-crystalized ligand AQ4 in the 1M17 crystal structure, which were set to x = 21.697, y = 0.303 and z = 52.093, a grid box of 42, 16, and 20 points in x-, y-, and z-direction, respectively, with grid spacing of 0.375 Å was built and centered on the co-crystalized ligand. The location coordinates of native G95 ligand in the 3E87 crystal structure were x = 33.914, y = -14.631 and z = 7.695 and based on that, a grid box of 26, 32, and 24 points was built and centered on the co-crystalized ligand. The coordinates of the co-crystalized ligand 8ST in the 3HNG crystal structure were x = 3.911, y = 17.995 and z = 32.857, while grid box size was set to x = 22, y = 32 and z = 30. Finally, the location coordinates of ADP ligand in the 4JSV crystal structure were x = 50.115, y = -1.981 and z = -45.385, while grid box size was set to x = 30, y = 18 and z = 32.

2.4.2. OEDocking

The receptor sites for molecular docking in OpenEye were prepared using MAKE Receptor 3.2.0.2 software (39). The outer contour sizes were 1017 $Å^3$ (EGFR), 598 $Å^3$ (AKT2), 493 $Å^3$ (VEGFR1) and 2150 $Å^3$ (mTOR), while the grid box sizes were 6416 $Å^3$ (EGFR), 5974 $Å^3$ (AKT2), 6314 $Å^3$ (VEGFR1) and 5190 $Å^3$ (mTOR). The setup of contours was set as "Balanced" and for the docking of ligands into AKT2 following constraints were added: Glu230 as a hydrogen bond donor and Ala232 as a hydrogen bond acceptor.

2.5. Molecular docking

2.5.1. AutoDock Vina

Molecular docking calculations were performed in AutoDock Vina software (40) with the default scoring function. In this docking simulation, semi-flexible docking protocols in which the target protein was kept as rigid were used. Maximum of nine poses were generated for the each tested compound.

2.5.2. OEDocking

The OEDocking 3.2.0.2 software (41-43), which employs FRED (fast exhaustive docking) tool, was also used for the analysis of ligand binding poses into the defined receptor sites of tested enzymes. Exhaustive scoring was performed using Chemgauss4 scoring function. Further optimization was done using OEChemscore scoring function. Scoring and consensus pose selection were performed using Chemgauss4 scoring function. Other settings were set as default.

2.5.3. Validation of docking methodology

For the docking validation, the co-crystalized ligands were extracted and re-docked into the active sites of the target enzymes. Binding poses were compared with the conformations of co-crystalized ligands and root-mean-square deviation (RMSD) values were calculated. In the molecular docking study, *in silico* prediction is considered successful if the RMSD value is less than 2.0 Å for the best scored conformation (46).

RESULTS

3.1. Validation of Molecular Docking

For the evaluation of molecular docking results validity, the co-crystalized ligand has to be re-docked into the active site. RMSD value was calculated by superimposition of native and re-docked co-crystalized ligand conformations using BIOVIA Discovery Studio Visualizer. Calculated RMSD values were < 2 Å in all docking experiments.

3.2. Molecular docking analysis

Tables 2 and 3 summarize the binding parameters of the molecules with highest binding potential against selected protein kinases in AutoDock Vina and OEDocking. Binding parameters include docking score, as well as the type and number of the key binding interactions. Only those interactions that both co-crystalized ligands and tested molecules form with receptors are listed in these tables.

Table 2. An overview of the key binding interactions and docking scores of the top scored compounds in AutoDock Vina

Designed ligand number	PDB code	Hydrogen bonds	Other interactions	Docking score (kcal/mol)	
				Designed ligand	Co-crystalized ligand
16	1M17	-	Leu694, Leu820, Asp831, Ala719	-8.7	-7.2
	3E87	Asp293	Ala179, Leu158, Val166, Phe163, Met282	-9.0	-8.9
	3HNG	Glu878, Asp1040	Val841, Val892, Ala859, Leu1029, Lys861, Val909, Cys912, Phe1041	-10.9	-10.5
17	1M17	-	Ala719, Thr766, Leu820, Asp831	-8.7	-7.4
	3E87	Asp293	Ala179, Val166, Lys181, Phe163, Met282	-8.5	-8.8
	3HNG	Glu878, Asp1040	Val841, Val892, Ala859, Leu1029, Lys861, Val909, Cys912, Phe1041	-10.7	-10.2

Table 3. An overview of the key binding interactions and docking scores of the top scored compounds in OEDocking

Designed ligand number	PDB code	Hydrogen bonds	Other interactions	Docking score (kcal/mol)	
				Designed ligand	Co-crystalized ligand
1	3E87	Glu230, Ala232	Val166, Leu158, Glu279, Asp293, Phe439	-9.48	-15.20
	4JSV	Lys2187	Glu2190, Pro2169, Leu2185, Ile2356	-7.52	-9.43
20	1M17	Met769	Thr766, Met742, Ala719, Lys721, Leu694, Val702, Cys773, Asp831	-9,63	-10.49
	3HNG	Glu878, Asp1040	Cys1018, Leu1013, Leu882, Val891, Val909, Val892, Val841, Phe1041, Ala859, Leu1029, Ile885	-11.8	-14.60

Molecular docking analysis in AutoDock Vina software revealed that derivatives **16** and **17** bound to EGFR, AKT2 and VEGFR1 similarly to the corresponding co-crystalized ligands. On the other hand, in the OEDocking software the best docking scores and presence of the key binding interactions were observed for derivatives **1** (AKT2 and mTOR) and **20** (EGFR and VEGFR1).

DISCUSSION

Multidrug resistance (MDR) is one of the major challenges in cancer treatment and may result in cross-resistance to many other structurally different chemotherapeutics. Antitumor activity of thiourea derivatives has been established earlier in numerous studies (12,13). Preliminary *in silico* studies can facilitate the rapid discovery of novel antitumor drugs which are able to reverse MDR. To identify suitable antitumor agents from the designed compounds, the molecular docking study was carried out towards selected protein kinases involved in MDR.

Co-crystalized molecules AQ4, G95, 8ST and ADP (Adenosine diphosphate) are ligand molecules isolated from the crystal structures 1M17, 3E87, 3HNG and 4JSV (co-crystalized ligands). Interactions between co-crystalized ligands and corresponding enzymes are considered key binding interactions. Type and number of key binding interactions, as well as docking scores were main parameters for assessing the potential of designed compounds to inhibit selected protein kinases.

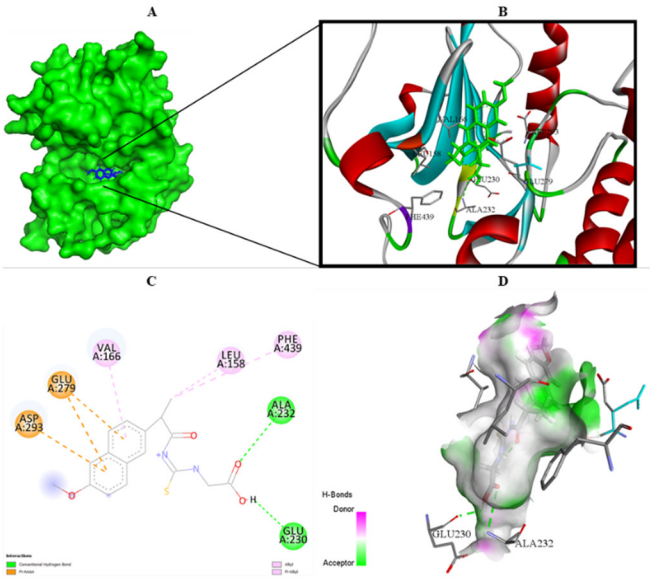
Docking visualization is presented as 2D and 3D view of the key binding interactions. In order to achieve visibility of the docked ligand into the protein structure, ligands were shown as blue color sticks in the binding pocket of the protein, shown as green surface.

The OEDocking and AutoDock Vina software were used for the binding mode analysis of tested compounds into the active sites of selected protein kinases. According to OEDocking, top scored derivatives were 1 (towards AKT2 and mTOR) and 20 (towards EGFR and VEGFR1). On the other hand, in AutoDock Vina top scored derivatives were 16 and 17 (towards EGFR, AKT2 and VEGFR1). The binding modes of these four derivatives will be further discussed in details.

Derivatives 16, 17 and 20 bound to EGFR with lower binding energies compared to the co-crystalized ligand. Nitrogen atoms of erlotinib quinazoline ring formed two hydrogen bonds with Met769 and Gln767, while the phenyl moiety of quinazoline formed π-sigma interaction with Leu694. The ethynylphenyl moiety formed π-cation interaction with Lys721 and hydrophobic interaction with Ile720, Ala719, Ile765, Lue764, Thr766 and Thr830 (47). Derivatives with the best docking results in AutoDock Vina did not accomplish any key hydrogen bond interactions, but formed four hydrophobic interactions each. On the other hand, derivative 20 formed an identical hydrogen bond with Met769 residue as erlotinib. Similar hydrophobic interactions with residues Thr766 and Ala719 were observed during the binding of *N*-allylthiourea derivatives into the EGFR active site (48).

In the active site of AKT2, co-crystalized nitrogen atoms of 1*H*-pyrrolo[2,3-*b*]pyridin-4-yl moiety formed two hydrogen bonds with Glu230 and Ala232, while phenylethyl moiety formed one weak carbon-hydrogen bond with Asp293 residue (49). Despite significantly higher binding energy of derivative 1 in comparison to the co-crystalized ligand, this derivative formed two hydrogen bonds with Glu230 and Ala232 (Figure 2). In contrast, derivatives 16 and 17 formed only one conventional hydrogen bond with residue Asp293, although they were bound with very similar binding energy in comparison to the co-crystalized ligand.

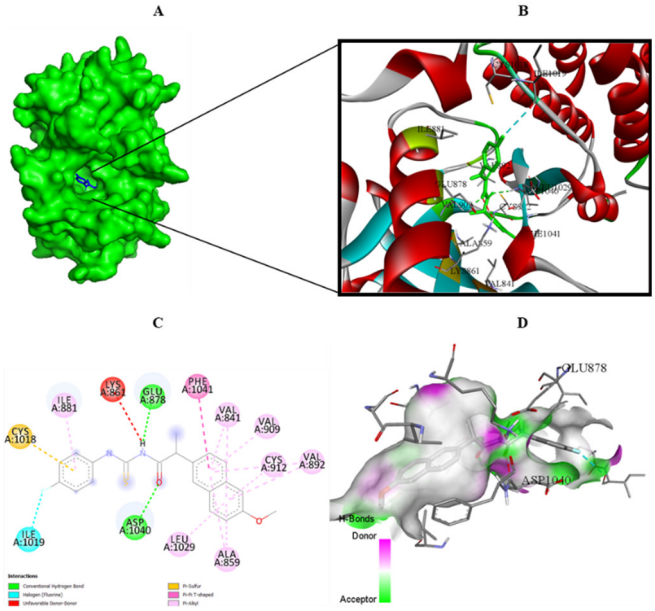
Figure 2. Compound 1 docked into the active site of AKT2.



A) Best ligand conformation in the binding pocket of the enzyme. B) and C) 2D and 3D summary views of all binding interactions achieved by compound **1** into the active site of AKT2 (hydrogen bonds were presented as green dash lines). D) 3D visualization of hydrogen bond donors and acceptors distribution of this compound.

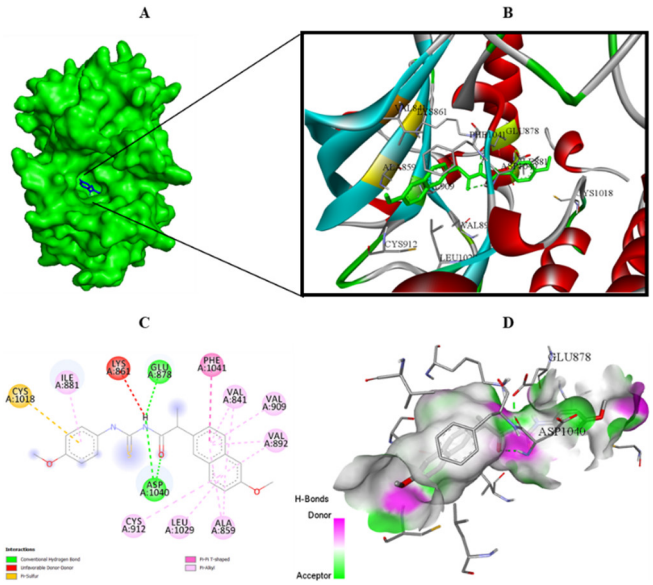
Binding score values indicate that derivatives **16**, **17** and **20** bound to the VEGFR1 with highest affinity compared to other target proteins, achieving the largest number of key binding interactions. Above-mentioned compounds formed two identical hydrogen bonds with residues Glu878 and Asp1040. On the other hand, derivative **1** formed the same hydrogen bond with Asp1040 and one different key hydrogen bond interaction with Cys912. The amino acid residues involved in the formation of key binding interactions between the best docked derivatives and active site of VEGFR1 are illustrated in the Figures 3, 4 and 5.

Figure 3. Compound 16 docked into the active site of VEGFR1.



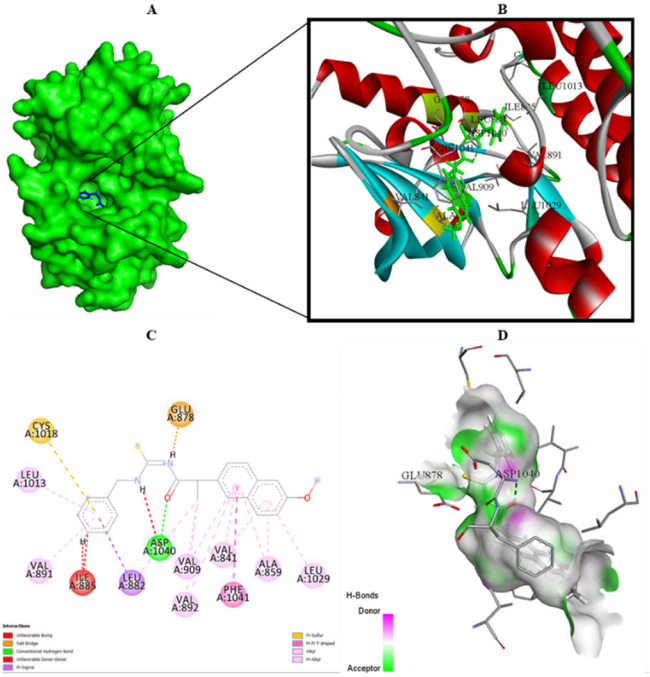
A) Best ligand conformation in the binding pocket of the enzyme. B) and C) 2D and 3D summary views of all binding interactions achieved by compound **16** into the active site of VEGFR1 (hydrogen bonds were presented as green dash lines). D) 3D visualization of hydrogen bond donors and acceptors distribution of this compound.

Figure 4. Compound **17** docked into the active site of VEGFR1.



A) Best ligand conformation in the binding pocket of the enzyme. B) and C) 2D and 3D summary views of all binding interactions achieved by compound **17** into the active site of VEGFR1 (hydrogen bonds were presented as green dash lines). D) 3D visualization of hydrogen bond donors and acceptors distribution of this compound.

Figure 5. Compound 20 docked into the active site of VEGFR1.



A) Best ligand conformation in the binding pocket of the enzyme. B) and C) 2D and 3D summary views of all binding interactions achieved by compound **20** into the active site of VEGFR1 (hydrogen bonds were presented as green dash lines). D) 3D visualization of hydrogen bond donors and acceptors distribution of this compound.

Designed compound **1** demonstrated lower binding score towards mTOR compared to the co-crystalized ADP ligand. ADP in mTOR formed three hydrogen bonds with residues Lys2187, Val2240 and Asp2357 and also seven Van der Waals interactions (50). In the active site of mTOR, derivative **1** accomplished a hydrogen bond with Lys2187 and four significant hydrophobic interactions.

Two the most critical components for a docking program are sampling algorithm and scoring function, which determine its sampling and scoring power. AutoDock Vina generates different ligands conformers using a quasi-Newton Broyden-Fletcher-Goldfarb-Shanno (BFGS) search algorithm. BFGS uses scoring function with respect to the position, orientation and torsions of the ligand. The Vina scoring function is fully empirical including Gaussian steric interactions, repulsion, hydrogen bonds, hydrophobic and torsion terms. On the other hand, the OEDocking software employs fast exhaustive docking that docks molecules using an exhaustive search algorithm. During the exhaustive search, unrealistic poses are filtered and retained ones are scored. The best scoring pose is used to rank the ligand against other ligands in the screening database. Chemgauss4 is default scoring function used by FRED that employs Gaussian-smoothed potentials to measure the complementarity of ligand poses within the active site.

AutoDock Vina and OEDocking estimated various binding affinity of the same ligands towards selected protein kinases. Due to different scoring functions and search algorithms of these two docking programs, the obtained binding parameters may be diverse even for the same protein-ligand complex. Although top scored derivatives in these two docking programs are different (**1** and **20** in OEDocking, **16** and **17** in AutoDock Vina), it can be noticed that according to OEDocking derivative **17** had lower binding score than derivatives **1** and **20,** but formed some of the key binding interactions with mTOR and EGFR. Therefore, derivative **17** also has potential to inhibit these enzymes. Similarly, according to AutoDock Vina, derivative **1** had binding scores similar to binding scores of **16** and **17** and formed some of the key binding interactions with AKT2 and VEGFR1, which gives this derivative potential to inhibit listed enzymes. According to the results obtained in both docking programs, derivatives **1**, **16**, **17** and **20** could be underlined as the most promising candidates that could be used as anticancer drugs for the therapy of MDR tumors.

CONCLUSION

Two docking programs (AutoDock Vina and OEDocking) were used for the estimation of binding of twenty designed thiourea derivatives of naproxen to four selected protein kinases involved in tumor multidrug resistance (MDR). According to OEDocking, the highest potential to inhibit these enzymes have derivatives **1** (inhibition of AKT2 and mTOR) and **20** (inhibition of EGFR and VEGFR1). According to AutoDock Vina, the highest potential to inhibit EGFR, AKT2 and VEGFR1 have derivatives **16** and **17**. Derivatives **1**, **16**, **17** and **20** are the most promising candidates that could be used for the therapy of MDR tumors.

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CONFLICTS OF INTEREST

Authors declare that there are no conflicts of interest regarding the publication of this article.

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ANALYSIS OF INJURIES AND CAUSE OF DEATH IN FATAL ACCIDENTS WITH FARM TRACTORS

Zivana Slovic[1], Katarina Vitosevic[1], Danijela Todorovic[2] and Milos Todorovic[1]

[1]*University of Kragujevac, Faculty of Medical Sciences, Department of Forensic Medicine, Kragujevac, Serbia*
[2]*University of Kragujevac, Faculty of Medical Sciences, Department of Genetics, Kragujevac, Serbia*

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Corresponding author:

Zivana Slovic

University of Kragujevac, Faculty of Medical Sciences, Department of Forensic Medicine, 69 Svetozara Markovica Street, 34000 Kragujevac, Serbia

Phone: +381 34 306800

E-mail: zivanaminic@yahoo.com

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ABSTRACT

Farm tractor accidents are a frequent cause of death for people in agricultural field of work. The aim of this study was to evaluate the characteristics of the fatal farm tractor accidents, as well as the most significant types of injuries and causes of death. Research is based on epidemiological criteria of subjects, traffic participation, cause of death, distribution of injury, outliving period and toxicological details. The study included 22 (4.2%) farm tractor related fatalities out of all 525 subjects who died from sustained injuries or complications of the injuries sustained in road traffic accidents. The highest number of deaths was reported in men in 95.5%. The highest percentage died of bleeding out (41%), while in 18% of cases, complications that occurred during the outliving period were the cause of death. Analyzing injuries through organ systems, it is observed that the highest number of farm tractor drivers had a chest injury (90%), and the most common chest injury was a rib fracture (82%). When determining the outliving period, the highest number of fatal farm tractor drivers died at the scene of accidents (68.2%). Of all of the subjects, 32% were under the influence of alcohol, while 23% had blood alcohol concentration level greater than 0.5 ‰. The high mortality of farm tractor drivers can be reduced by introducing a mandatory rollover protective structures system and the use of a seat belt in farm tractors with cabs, which would reduce the possibility of the driver falling out of the farm tractor seat and additional injuries due to rollover.

Keywords: *Farm tractor, accidents, autopsy, cause of death.*

INTRODUCTION

World Health Organization estimated that in 2030 road traffic accidents (RTA) might be the fifth leading cause of death in the world (1). More than 3000 people die in RTA every day, which adds up to about 1.25 million people a year (1). Farm tractor drivers are the type of participants in the RTA who are less represented in the overall sample, but are not negligible. Farm tractor accidents are a frequent cause of death for people in agricultural field of work (2, 3). The most used agricultural equipment on farm are farm tractors, and they are used for work purposes in agriculture, forestry (4) and as transportation vehicle in rural parts of Serbia. Among machines that are being used in agriculture, farm tractors are one of the most lethal. Farm tractors are among most lethal of agricultural machines that are being used performing agricultural activities. More farmers are injured in farm tractors related accidents per year compared to injuries related to any other piece of farm equipment. About half of all deaths associated with injuries in the agricultural industry involve farm tractors (5). In numerous studies farm tractors were reported as the most common causative agents of fatal accidents (6-8). Also, about 50% to 60% of all fatal farm tractor accidents deaths are due to rollovers (9, 10). Accidents usually occur on farmland, tracks, or roads when the vehicle suddenly overturns and the driver or passenger gets thrown to the ground and crushed. Different vector forces may cause the farm tractor to overturn backwards, forward or to one side, crushing the victim's chest, head or limbs. Medico legal autopsies are demanded in all cases of violent deaths, i.e. in all traffic accidents, including those involving farm tractors.

The aim of this study was to evaluate the characteristics of the fatal farm tractor accidents in Kragujevac and the surrounding area, as well as the most significant types of injuries and causes of death.

MATERIALS AND METHODS

This study used epidemiological, cross-sectional autopsy examining of farm tractor related fatalities between 2001 and 2016 in Kragujevac, Serbia. A retrospective review of 1,366 medico legal autopsy reports by Clinical Centre of Kragujevac, Department of Forensic Medicine and Toxicology included 525 road traffic accidents. This study included a total of 22 farm tractor related fatalities. The data on the sustained injuries were obtained by analyzing the autopsy records, available medical documentation as well as the retrospectively acquired hetero-anamnestic information. The study did not include children younger than 14 years of age.

Research is based on epidemiological criteria (age, gender, year, month and day distribution) of subjects, cause of death, distribution of injury, outliving period and toxicological details. Causes of death were classified into the following groups: bleeding out, breathing disorder, head injuries, breathing disorder and bleeding out and complications of injury like pneumonia or sepsis. The presence of injuries by systems was also analyzed: head, chest and abdomen. According to the time distribution of death after injury the subjects were divided into two categories: ones who died at the scene of the accident and others who outlived the accident for a certain period of time. Femoral blood and urine samples were collected at the autopsy from subjects who died at the scene of the accident or who outlived injuries up until 24 hours after the accident and analyzed by „headspace" gas chromatography (Shimadzu GC-2010 plus).

Statistical analysis for variances was performed using statistical Package for Social Sciences-SPSS for Windows, Version 20 (SPSS Inc Chicago, IL). All numerical variables were tested with the Kolmogorov-Smirnov and Shapiro-Wilks tests for normal distribution and appropriate descriptive statistics were employed (mean values with standard deviation). In variables that showed a nonparametric distribution, the Pearson's chi-square test (with Yates correction) was applied. The p value of 0.05 has been considered to be significant.

This study was conducted with the approval of the Ethical Committee of the Clinical Centre of Kragujevac (18/10/2016, No 01/13221).

RESULTS

Number of cases and gender distribution

At the Department of Forensic Medicine and Toxicology, Clinical Centre of Kragujevac, a total of 1,366 medico legal autopsies were performed between 2001 and 2016. The study included 22 (4.2%) farm tractor related fatalities out of all 525 road traffic accidents subjects who died from sustained injuries or complications of the injuries. The highest number of deaths was reported in men in 95.5% of cases (21 of 22), while women were represented in 4.5% of the cases (1 of 22).

Year, month and day distribution

The year 2010 had the highest number of farm tractor accidents (7, i.e. 31.8%), which are provided in Figure 1. July, August and September had the highest number of fatal accidents (4 i.e. 18.2%), while February and December are months when there weren't any farm tractor accidents (Figure 2). Depending on the days of the week, the highest number of farm tractor accidents happened on Tuesdays (7, i.e. 31.8%), while there were no accidents on Saturdays which is presented in Figure 3.

Age distribution

The study included 22 subjects, whose average age was 61.2±13.6 years (ranging from 25 to 83 years). The distribution of subjects by age ranges are presented in Table 1. Almost half of all deceased farm tractor drivers were over 65 years of age, which is statistically significant (χ^2=8.182; df=3; p=0.042).

Figure 1. Farm tractor accident distribution by year.

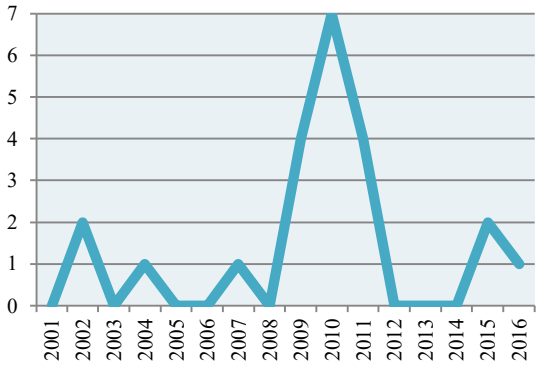


Figure 2. Farm tractor accident distribution by month.

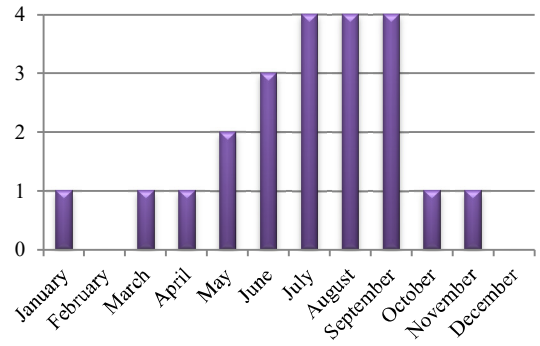
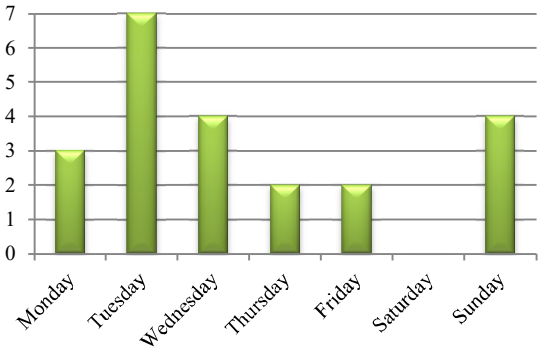


Figure 3. Farm tractor accident distribution by week.



Cause of death and *distribution of injury*

Out of all farm tractor drivers, the highest percentage died of bleeding out (9 out of 22, i.e. 41%), which is statistically significant (χ^2=26,068; df=10; p=0,004). Other causes of death such as head injuries, breathing disorder, breathing disorder and bleeding out are represented by a smaller percentage (Table 2). In 18% (4 out of 22) of cases, complications that occurred during the outliving period were the cause of death (two cases of pneumonia and sepsis). Analyzing injuries through organ systems, it is observed that the highest number of farm tractor drivers had chest injuries (20 out of 22, i.e. 90%), while abdomen injuries (10 out of 22, i.e. 45%) and head injuries (5 out of 22, i.e. 23%) were much less common. The most common chest injury was a rib fracture (18 out of 22, i.e. 82%), with fractures of ribs on both sides present in as many as 16 cases, which is statistically significant (χ^2=8.909; df=1; p=0.003). Lung injuries, i.e. lung contusions and lacerations, were also present in a large proportion (13 out of 22, i.e. 59%). Hemothorax was present in almost half of all the farm tractor drivers (10 out of 22, i.e. 45%).

Outliving period

When determining the outliving period, we concluded that only 4 (18.2%) of all 22 subjects had an outliving period of over 6 hours (Table 3). The highest number of farm tractor drivers died at the scene of accidents (15, i.e. 68.2%), which is statistically significant (χ^2=32.545; df=4; p=0.000). In the group of drivers who died within a certain period of time after the accident (7, i.e. 31.8%) the following injuries were noted: bilateral rib fractures (5, i.e. 23%), lung contusions (2, i.e. 10%), liver (3, i.e. 14%), spleen (3, i.e. 14%) and intestines injury (3, i.e. 14%).

Toxicological report

Blood alcohol concentration was measured in all subjects who died at the scene of accidents, and subjects who outlived the injuries for more than 24 hours were excluded from this study. Of the 22 subjects, 7 (32%) were under the influence of alcohol, 5 (23%) had BAC greater than 0.5 ‰, while 9 (41%) were negative, and in 6 (27%) it was not measured because they outlived the injuries longer than 24 hours. Blood alcohol concentration values in deceased farm tractor drivers ranged from 0.22 ‰ to 1.9 ‰, with mean value being 0.96±0.61‰.

Table 1. The distribution of subjects by age ranges.

Age ranges	Number	%
15-35	1	4.5
36-50	4	18.2
51-65	7	31.8
> 65	10	45.5
Total	**22**	**100**

Table 2. The distribution of cause of death.

Cause of death	Farm tractor drivers	
	Number	%
Bleeding out	9	41
Breathing disorder	3	14
Bleeding out and breathing disorder	3	14
Fatal head injury	3	14
Complication of injuries	4	17
Total	**22**	**100**

Table 3. The distribution of subjects by outliving period and injuries.

Outliving period	Farm tractor drivers	
	Number	%
Died at the scene	15	68.3
Outliving 6 hours	3	13.7
Outliving 24 hours	/	/
Outliving 72 hours	/	/
Outliving 7 days	2	9
Outliving 14 days	1	4.5
Outliving 30 days	1	4.5
Total	**22**	**100**

DISCUSSION

The agriculture industry is the sector of the economy in which occupational injuries are quite common, most frequent being farm tractor- related. According to data provided by Statistical Office of the Republic of Serbia, machines used in agriculture are outdated and old, especially farm tractors (86% of farm tractors and 82% of combine harvesters are over 20 years old. Also, there is an increase in the proportion of older people living in rural areas and a decline in the proportion of younger people.

This study presents fatalities with farm tractors over a sixteen year period in the territory of the city of Kragujevac with the surrounding area. In our study, of the total number of RTAs, approximately 4% were accidents involving farm tractors. Similarly, about 5% of deaths with farm tractors have been reported in other studies (6,7). Over 95% of farm tractor drivers were male, which is similar to the results of other studies in Turkey (6) about 86%, Croatia (4) and Poland (9) about 91%. The small share of women is explained by the fact that women are less represented in agricultural work requiring the operating of farm tractors and similar machines.

According to the data from our study, the most common farm tractor related accidents have occurred during the summer months when the most intensive agricultural and forestry work was taking place. Similar results have been reported in other studies, which point out that the occurrence of such accidents is highest in the summer months and early fall (4,6,7,9,12). In the winter months, the activity in agriculture decreased, so the minimum number of injuries and deaths was expected in these months. Although in a similar study it was shown that Monday and Friday are the days when farm tractor accidents are the most common (12), in our study most farm tractor accidents happened on Tuesdays, so some regularity cannot be established.

According to our study, farm tractor drivers are among the oldest participants in road accidents, with an average age of over 61, with the highest number being over 65 years old. In our country, the elderly population is most often engaged in agriculture (11), which explains the high proportion of older fatal farm tractor drivers. In a similar autopsy study in Croatia (4), it is emphasized that the most of RTA participants involving farm tractors who succumbed to their injuries were older than 65 years, which also corresponds to the results of our study.

According to the results of our study most frequently injured part of the body was chest (in 90%), while the injuries of other body parts were represented in much smaller numbers. The high incidence of chest injuries in farm tractor drivers can be explained by the farm tractor's technical characteristics and mechanism of injury. According to our heteroanamnestic data, these kinds of accidents mostly involve farm tractors without cabs, which leads to drivers falling out of the driver seat or farm tractor, some farm tractor implements or trailers overturning over the driver, inevitably resulting in chest injuries. In a similar study conducted in Turkey (6), head injuries and thoracoabdominal injuries are equally represented, but the majority of studies point out that in farm tractor drivers with fatal outcomes head injuries are the most common (7,13-16). When interpreting these results, it should be borne in mind that almost all agricultural machines in our country, including farm tractors, are over twenty years old and that they do not have rollover protective structure system, which is present in almost all farm tractors used in developed countries. Also, in our country, farm tractor drivers are usually elderly people, in whom all injuries, including bone fractures (and most commonly rib fractures) are produced with much less force, compared to other age groups. Rib fractures are among the most common lung injuries in traffic participants and are often associated with lung tissue injuries (20). The most common cause of death for farm tractor drivers in our study was bleeding out, which occurred after aortic rupture. In situations of farm tractor overturning and pinning the driver to the ground and causing pressure in the chest area, injuries such as serial rib fractures and aortic rupture are inevitable. The occurrence of aortic rupture is explained by several mechanisms: due to a sudden increase in blood pressure, water-hammer effect, stretching of the aortic wall, or pressure on the aorta due to compression of the chest between the sternum and the spinal column (21). In our study, the mechanism of aortic rupture was thoracoabdominal compression during rollover, due to the pressure of farm tractor parts or implements on the body of the farm tractor driver. This mechanism explains the occurrence of rib fractures and lung tissue damage in the form of contusions and lacerations.

Most of the farm tractor drivers in our study died on the spot and within the first few hours after the accident (82%), while only a small number survived injuries for some period of time (18%). Thus, a large percentage of participants who had died on the scene can be explained by the presence of thoracic injuries such as: bleeding from damaged intercostal arteries and the aorta, the breathing disorder due to fractures of the ribs and/or compression of the thorax. An additional factor that accelerates the onset of death and aggravates all of the aforementioned injuries is the age of the participants (19,22), as evidenced in our study. It should also be noted that farm tractor drivers are often isolated on fields or in

forests, sometimes in inaccessible places, which makes it impossible to provide first aid in a timely manner and transport them to the appropriate health care facility. In other studies, similar results were reported for those who died on the spot and in the first 24 hours; in the study in Portugal (18) about 65%, in Turkey (6) about 79%, in Croatia (4) about 81%, and in Poland (10) about 85%. In participants who outlived the injuries for a certain period of time, complications such as pneumonia and sepsis were the immediate cause of fatal outcome.

About one third of the farm tractor drivers in our study were under the influence of alcohol. In a study conducted in Portugal (18), one in four fatally injured farm tractor drivers was under the influence of alcohol. Many studies confirm that alcohol is a leading risk factor for work-related injuries (23,24) as slightly intoxicated people and people under the influence of alcohol tend to become more daring, bolder and inclined towards making poor decisions. The higher frequency of drunk farm tractor drivers in our country can be culturally explained, given that drinking alcoholic beverages in our region has been traditionally accepted and culturally approved.

CONCLUSION

Farm tractor drivers are among the least represented fatalities in road traffic accidents in the Kragujevac area with its surroundings. They belong to the group of oldest participants with an average age of about 61 years and are mostly male. They most often die in road accidents from May to September, during the season of the most intensive agricultural work. In farm tractor drivers the most common chest injuries are the following: rib fractures and lung injuries, and the most common causes of death are bleeding and breathing disorders as a result of farm tractor overturning. They were more likely to die on the spot, than to outlive the injury. About a third of the farm tractor drivers were under the influence of alcohol.

High mortality of farm tractor drivers can be reduced by introducing a mandatory Rollover protective structures system, by using seat belts in farm tractors with cabs, which would reduce the possibility of the driver dropping out of the farm tractor seat and subsequent injury due to rollover, as well as continuous education of the population.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

The study was conducted in accordance with the Declaration of Helsinki and was approved by the Ethical Committee of the Clinical Centre of Kragujevac (18/10/2016, No 01/13221).

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CONFLICT OF INTEREST

None.

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THYROID AND PREGNANCY

Violeta Mladenovic

University of Kragujevac, Faculty of Medical Sciences, Department of Internal medicine,
Clinical Center Kragujevac, Department of endocrinology, Clinic for Internal Medicine, Kragujevac, Serbia

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Corresponding author:

Violeta Mladenovic, MD, PhD

University of Kragujevac, Faculty of Medical Sciences, Department of Internal medicine, Svetozara Markovica 69, 34000 Kragujevac, Serbia

Phone: +381 34 306800

E-mail: vikicam2004@gmail.com

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ABSTRACT

Hormonal changes and metabolic needs during pregnancy result in profound changes in biochemical parameters of thyroid function, especially if there is preexsisting autoimmune thyroid disease (AITD). Normal thyroid function is important in order to ensure the best outcome. Many changes in the functioning of the thyroid gland occur during pregnancy, and some diseases of thyroid gland can affect both mother and fetus. Hypothyroidism is the most serious disorder that occurs during pregnancy and can go unnoticed as a „non-specific" problem. Hypothyroidism arises from the reduced ability of the gland to adapt to the increased needs during pregnancy. Mild thyroid dysfunction of mothers in the first trimester, which does not threaten during the pregnancy, can damage the psychomotor development of the child. Measurement of TSH is the most practical, simple and cost-effective screening test for thyroid dysfunction. It is necessary to apply the trimester-specific TSH reference values to correctly interpreted thyroid function during pregnancy. The presence of TPOAb is confirmation of existence of AITD, and predicts increased risk of developing subclinical hypothyroidism (SH). Preconceptional education and adequate diagnosis and treatment of thyroid dysfunction in early pregnancy are of great importance, in order to prevent complications during pregnancy and offspring. Current data indicate an increase in pregnancy loss, gestational diabetes, gestational hypertension, pre-eclampsia and preterm delivery in women with SH in pregnancy. The control of thyroid disease reduce complications of pregnancy.

Keywords: *Thyroid, pregnancy, autoimmune thyroid disease, thyroid stimulating hormone, subclinical hypothyroidism*

THE METABOLISM OF THYROID HORMONE DURING PREGNANCY

Iodine is an essential component of thyroid hormones T3 and T4, produced by the thyroid gland. During early gestation, the fetus depends on the mother's thyroid hormones that cross the placenta because of fetal thyroid function starts at 12-14 week of gestation (wg), while synthesisof fetal TSH occurs around 20th wg Even after the onset of the beginning of the production of fetal thyroid hormones,the fetus continues to rely on the mother's thyroid hormone (1). At birth, about 30% of T4 in the umbilical cord comes from the mother. T3 is the active thyroid hormone,and about 80% of T3 is produced from T4 in liver and muscle. Approximately 99.97% T4 and 99.7% T3 is bound to proteins, primarily on thyroxine-binding globulin (TBG), and to a lesser extent to albumin and transthyretin. The reason for the increased need for thyroid hormone during pregnancy is increased the degradation of T4 and T3; and the effect of human chorionic gonadotropin (hCG) inpregnancy. Serum hCG is a glycoprotein produced by the placenta with a peak at the end of the first trimester, resultsin increased secretion of T4 and T3 and partial suppression of serum TSH (2, 3). Thyroxine of mother crosses the placenta in the first half of pregnancy, while TSH and T3 do not cross the placenta, but TRH crosses the placental barrier (4).

The increase in the need for T4 occurs very early (at 4-6 wg), gradually increasing to 16-20 wg, when reaches aplateau, and is held to the delivery (5). Pregnancy has a significant effect on the thyroid gland and thyroid function. Thyroid gland grow about 10% in size during pregnancy, and up to 20-40% in areas with iodine deficiency. Production of T3 and T4 increases to 50%, with an increase of thedaily requirement of iodine up to 50% (6). Fetal fT4 andT4 reaches adult levels at 36 wg, while fetal TSH is higherthan in the adult (7).

The recommended daily intake of iodine for pregnant women is 229 μg, and for women who are breastfeeding 289 μg(8). TSH provides the most sensitive index for detecting abnormalities of thyroid function. Normal thyroid function is important in order to ensure the best outcome (9). A healthy thyroid gland is able to compensate increased needfor thyroid hormones increasing their secretion and maintaining the level of free hormones in the normal range during pregnancy. However, in situations where subtle pathologic abnormalities of the thyroid gland, such as chronic autoimmune thyroiditis (CAT), or hypothyroid woman onL-thyroxine (LT) replacement therapy, is not present to increase production of the thyroid hormones, entailing the risk of a woman to become hypothyroid. Mild thyroid dysfunction of mothers in the first trimester, which does not threaten during the pregnancy, can damage the psychomotor development of the child (10). In early pregnancy, itis necessary to increase the mother thyroid production of thyroxine, about 50% in comparison to the state prior to conception (11). Many changes in the functioning of the thyroid gland occur during pregnancy and some diseases of the thyroid gland can affect both mother and fetus. Hypothyroidism is the most serious disorder that occurs during pregnancy and can go unnoticed as a "non-specific" problem (12).

AUTOIMMUNE THYROID DISEASE (AITD)

Thyroid disease in pregnancy is common, at least 2-3%of women have thyroid dysfunction, and it is estimated that about 5-20% of women of reproductive age suffer from AITD (11). Thyroid antibodies (Ab) may representa marker of generalized autoimmune imbalance which is responsible for the increased rate of spontaneous abortion (SA) (12). AITD is a risk factor for infertility, women with AITD are often older, so older age, per se, may explain theincreased rate of fetal loss (13, 14).

Thyroid autoimmunity is the most common autoimmune disorder in humans. The situation may remain latent, asymptomatic or undiagnosed for years.Approximately 30% reduction in fT4 indicates that nearly half of women who's test is positive, has fT4 below the normal value at the end of pregnancy (15).

Before any clinical decisions based on the basis of TSH 3-4.5 mIU/L, it is necessary to repeat this analysis in a few weeks to turn off transient thyroid dysfunction. Common causes of transient elevated TSH is subacute or postpartum thyroiditis. The presence of TPOAb is confirmation ofexistence of AITD, and predicts increased risk of developingsubclinical hypothyroidism (SH) when TSH >2 mIU/L (16).Studies have shown that the majority of pregnant women with elevated TSH, in the absence of iodine deficiency, withpositive thyroid Ab, indicates that the AITD is the primarycause of decreased thyroid reserve. Studies have shown that about 30-50% women with thyroid Ab develop postpartumthyroid dysfunction (17).

AITD without clear thyroid dysfunction was significantly associated with an increased rate of 3-5 timesof SA. Negro showed that: 1) euthyroid women with thyroid Ab are older when get pregnant; 2) even if in the early pregnant euthyroid, they have a reduced thyroid reserve; 3) have an increased risk for complications of birth (SA and preterm delivery (PD)); and 4) using a LT to normalisethyroid function (14). It was demonstrated the benefit of using LT in patients with AITD, not only correcting the thyroid function of the mother, but also reduce the level ofadverse outcome (AO) (8).

SH is defined biochemically: when the serum TSH is elevated, a thyroid hormone is normal. In about 60-80%of cases, the disorder is associated with positive TPOAb, marker of CAT (18). Women with positive TPOAb who have not developed PPT have a 25% chance to developit after the next pregnancy. During pregnancy, screening to identify women with TPOAb showed 11 times greater risk of PPT (19). CAT that is often only manifest presenceTPOAb and TGAb is associated with 2-4 times higher incidence of PD and SA (20, 21).

EPIDEMIOLOGY OF AUTOIMMUNE THYROID DISEASE

The prevalence of SH among women of reproductive age is 0.5-5%, depending on the criterion reference value (RV) for TSH (18). The prevalence of thyroid autoAb in the population of women of childbearing age varies from 6-45%, in women with SA about 17-33% of women with infertility around 10-31%. It has been shown in studies thatthe presence of thyroid autoAb, especially TPOAb is associated with adverse outcome (AO) (PD, SA, developmentalneurological sequelae in children). The real mechanisms ofthis association are unknown (22). Thyroid disorders are 4-5 times more common in women than men, especially ofchildbearing age. Hormonal changes and metabolic needs during pregnancy result in profound changes in biochemical parameters of thyroid function (23, 24).

The incidence of hypothyroidism is the mother of 0.19-2.5% (25). The need for universal thyroid screeningof pregnant women is controversial. The American College of Obstetricians and Gynecologists (ACOG's) Clinical Guidelines (2002) suggests testing thyroid function only in women with a personal history of thyroid disease, DM1 or the presence of other autoimmune diseases or symptoms of thyroid disease and does not recommend universal screening. Management of Thyroid Dysfunction during Pregnancy and Postpartum: An Endocrine Society Clinical Practice Guideline in 2012 recommended targeted tests on pregnant women (Table 1) (26, 27). American Association of Endocrinologists (AACE) recommends routine screening of thyroid function before pregnancy for all who are planning a pregnancy, or the first trimester (28). AACE and the Endocrine Society did not find sufficient evidence to recommend universal screening for and treatment of SH. Recent results indicate that a limited screening for high-risk women basedon personal or family history may miss the diagnosis of women with hypothyroidism (29, 30).

Table 1. Recommendations for tests of thyroid function in preconceptional period or early in pregnancy (Indications for thyroid function testing during pregnancy)

Women older than 30 years
Women with a family history of AITD and hypothyroidism
The existence of goiter
At positive thyroid (especially TPOAb)
Symptoms or clinical signs of functional disorders of the thyroid gland
Women with DM1 or other autoimmune diseases infertility
Women with a history of previous miscarriage and preterm delivery
Women with previous radiotherapy neck or thyroid surgery
Women who are on estrogen replacement therapy L - thyroxine
Women who live in iodine deficient areas

REFERENCE VALUES (RV) FOR THYROID HORMONE DURING PREGNANCY

Physiological changes during pregnancy often makeit difficult to determine the specific trimester RV for thyroid function. These changes include increased TBG, increased release of T4 and T3 due to the weak agonistic effect of hCG and the consequent decline in TSH. The concentration of TSH in the serum depends on the wg, it is lower in the first trimester as compared to the second and third trimesters of pregnancy. There is substantial data supporting the clinical value of the serum of 2.5 mIU/L and the upper limit during the first trimester, and the lower limit of 0.1 mIU/L (11, 31).

Pregnancy is a stress on the thyroid, resulting in hypothyroidism in women with limited thyroid reserve or iodine deficiency. Most studies show a significant fall in fT4 as the pregnancy progresses. The largest drop in TSH in the first trimester, and weak, obviously linked to hCG, which is the largest in early pregnancy. TSH gradually increases in the second and third trimesters, but remains lower. Recently, the norms for the upper RV 2.5-3.0 mIU/L(6, 31). The concentration of maternal thyroid hormones, T4 and T3 increases from early pregnancy, with a slight increase of free hormones in the first trimester with corresponding lowering TSH (11). Thyrotropic activity of hCG produces a decrease in serum levels of TSH inthe first trimester, so that pregnant women have a lower concentration of TSH in comparison to women who are not pregnant. Expert opinion is committed to the followingrecommendations for a specific value-trimester TSH: 0.1-2.5 mIU/L (first trimester), 0.2-3.0 mIU/L (the second), and 0.3-3.0 mIU/L (third) (31). Lower physiological limitis 0.1 mI/L for the first, 0.2 mIU/L for the second, and 0.3 mIU/L for the third trimester. It is necessary to apply the trimester-specific TSH RV to correctly interpreted thyroid function during pregnancy (14, 27).

Gestational thyroid hormone reference intervals vary according to population ethnicity, iodine nutrition, and assay method and each population should derive trimesterspecific reference intervals for use in pregnancy (32).

In particular, it was found that 9.0% and 8.9% of euthyroid pregnant women had a positive TPOAb test in the first or second trimester, respectively. It is necessary to regularly monitor thyroid function in TPOAb positive women in order to maintain optimal maternal and fetal health (33).

COMPLICATIONS DUE TO AUTOIMMUNE THYROID DISEASE AND HYPOTHYROIDISM

Hypothyroidism arises from the reduced ability of the gland to adapt to the increased needs during pregnancy. Hypothyroidism during pregnancy is associated with a number AO, the most important being: SA, PD and reduced cognitive function in offspring. During the first trimester, when fetal development depends entirely on the mother's thyroxine is a critical period for the functioning of thyroid hormones on the developing brain (34).

Patients with Hashimoto's thyroiditis (HT) are at greater risk for developing hypothyroidism in early pregnancy due to the increased need for thyroid hormones. A recent study has shown a significant reduction of the number of SA and PD in women who are euthyroid, with HT that were treated with LT for the first ten weeks of gestation compared with a control group of pregnant women who did not receive treatment (13). Pregnancy-induced hypertension (PIH) and small birth weight (SBW) was noted in 15% of pregnant women with SH (18).

The diagnosis of primary hypothyroidism during pregnancy is based upon finding of an elevated serum TSH concentration, defined using trimester-specific TSH reference ranges for pregnant women. The implementation of trimester-specific reference ranges is recommended in order to avoid misclassification of thyroid dysfunction during pregnancy (35).

Progeny in TPOAb and TGAb positive mothers have 2-3 times higher perinatal mortality than negative. AITD detected during the first trimester is independently associated with increased perinatal mortality, probably through PD. Careful monitoring of thyroid function is critical for the prevention of potential complications that can occur during pregnancy (36).

Patients with SH have several slightly clinical signs or symptoms of thyroid dysfunction. Possible consequences include cardiac dysfunction, or adverse cardiac events, increasing cholesterol and LDL, and progression to hypothyroidism (37). There is a connection between hypothyroidism and reduced fertility, which is mainly associated with ovulatory disorders, rather than SA. Women with LT therapy have twice the risk of infertility. Repeated SA are associated with serious autoimmune diseases, such as systemic lupus or the antiphospholipid syndrome (38).

Many studies have shown that gestational diabetesmellitus (GDM) as the most common obstetric metabolic disease and functional abnormalities in the thyroid can have a variety of adverse effects on pregnancy outcomes and offspring. Some studies have shown that there is a correlation between thyroid disease and GDM, whereas others have not found this association. However, a recent meta-analysis showed that the incidence of GDM in patients with subclinical hypothyroidism was 1.35-fold higher thanthe incidence in the control group. In summary, this studyprovides new evidence showing that low thyroid hormonelevels increase the risk of developing GDM in early pregnancy (33).

TREATMENT OF HYPOTHYROIDISM DURING PREGNANCY

International guidelines advocate using population based reference ranges; however, if these are unavailable the recommended fixed upper threshold for TSH concentration is 2.5 mIU/L during the first trimester and 3.0 mIU/L during the second and third trimesters (31). According to these diagnostic criteria, is estimated to affect up to 15% of pregnancies in the US and 14% in Europe. This represents a fivefold increase in prevalence comparedwith the 2-3% prevalence of SH before these criteria were established, raising the possibility of overdiagnosis of SH and discussions at the 2016 Endocrine Society meeting about increasing the TSH cut-off limit to 4.0 mIU/L in theupcoming American Thyroid Association guidelines (31). A recent meta-analysis of 18 cohort studies found that pregnant women with untreated SH are at higher risk for pregnancy loss, placental abruption, premature rupture ofmembranes, and neonatal death compared with euthyroidwomen. Current guidelines recommend LT treatment in pregnant women with SH (39).

It is estimated that approximately 1-2% of pregnant women receiving LT for hypothyroidism. Epidemiological studies suggest that 0.4% of pregnant women have TSH>10 mU/mL of 15-18 wg (38).

For women already diagnosed as hypothyreoidism, it is recommended to adjust the dose to a TSH of pregnancy was <2.5 mIU/L, and in the first trimester, while the second and third should not exceed 3 mIU/L (31). To achieve these results, LT dose should be increased at the beginning of pregnancy (after conception) by 30-50%, depending on the cause of hypothyroidism. Increasing the dose is important at thebeginning of pregnancy, although it may be necessary to continue to increase during the second and third trimester. HATwith euthyroidism does not require the application of LT, oron the basis of the risk of hypothyroidism, strict supervisionis necessary during pregnancy (40). In order to minimize thecomplications of hypothyroidism for the mother and fetus,women need to quickly return to the euthyroid state. It is desirable that TSH was maintained at 1.2 mIU/mL (31).

Ideally, women with primary hypothyroidism should be counseled before pregnancy about the appropriate dosageof LT during pregnancy. It is necessary to increase the dailydose of 25-50 mg. There is a consensus that it is necessary tocheck hormone 4-6 weeks, it seems that the need to increasethe dose to half of pregnancy, which is held until delivery (1).The progression of the SH is predictable based on TSH and TPOAb in the first trimester. These parameters areuseful markers for the identification of women at high risk,and careful monitoring of thyroid function during pregnancy. Pregnancy is associated with an increased risk ofthyroid pregnant women with AITD, so that the potentialrelationship between pregnancy and thyroid disorders(41). SH arising before conception or during gestationshould be treated with levothyroxine. The goal of levothyroxine treatment is to normalize maternal serum TSHvalues within the trimester-specific pregnancy referencerange (35). After delivery, the dose of LT should be continued with a dose of a non-pregnant, and is not changedunless there is evidence of a hypo or hyperthyroidism (42).Thyroid hormone treatment was associated with decreased risk of pregnancy loss among women with subclinical hypothyroidism, especially those with pre-treatment TSH concentrations of 4.1-10 mIU/L (39).

TREATMENT OF HYPERTHYROIDISM DURING PREGNANCY

Carbimazole should be avoided in the first trimester of pregnancy due to risk of congenital anomalies, but recent studies would suggest that this risk is present to a lesser magnitude with propylthiouracil. Current international guidelines recommend the use of propylthiouracil in the first trimester and switching to carbimazole for the remainder of pregnancy but the benefits and practicalities ofthis approach is unproven (32).

Graves' disease often shows a characteristic course in pregnancy with amelioration of thyrotoxicosis in the second half of pregnancy and exacerbation after delivery. In addition transplacental passage of maternal TSH receptor antibodies may lead to thyrotoxicosis in the fetus and/or newborn (43).

Both hypothyroidism and thyrotoxicosis may impair the course of pregnancy and may negatively affect the fetus. In particular, maternal hypothyroidism may lead to irreparable and detrimental deficits in the neurocognitive development of the fetus (31, 43).

CONCLUSION

During pregnancy, proper thyroid function of the mother is important for both mother and child. This is particularly important in the first trimester, when fetal development completely dependent on the mother's hormones that are essential for optimal development.

Recommendation for the upper limit for TSH 2.5 mIU/L in the first, and 3.0 mIU/L in the second and third trimester. Lower physiological limit is 0.1 mIU/L for the first and 0.2 mIU/L for the second, and 0.3 mIU/L for the third trimester

Preconceptional education and adequate diagnosis and treatment of thyroid dysfunction in early pregnancy are of great importance, in order to prevent complications during pregnancy and offspring.

In summary, universal screening of TSH, fT4, and TPOAb is essential during the first trimester and second trimester of pregnancy. Taken together, we support implementation of a universal screening strategy for thyroid disorders in pregnant women.

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CONFLICT OF INTEREST

The author declare no financial or commercial conflictof interest.

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ADENOCARCINOMA OF THE JEJUNUM: A CASE REPORT AND LITERATURE REVIEW

Miljan Zindovic[1], Tatjana Culafic[2], Dragan Saric[1] and Dunja Zindovic[3]

[1] *Center of Abdominal Surgery, Clinical Centre of Montenegro, Podgorica, Montenegro*
[2] *Center of Pathology, Clinical Center of Montenegro, Podgorica, Montenegro*
[3] *Clinic for Anesthesia, Resuscitation and Pain Therapy, Clinical Center of Montenegro, Podgorica, Montenegro*

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Corresponding author:

Miljan Zindovic

Center of Abdominal Surgery, Clinical Centre of Montenegro, Podgorica, Montenegro

Phone: +382 683 25 355

E-mail: mzindo@yahoo.com

sciendo

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ABSTRACT

Small intestine cancers account for 1-3% of all gastrointesti- nal tumors, with only 11-25% of these tumors located in the jeju- num. We report the case of a woman who has been experiencing abdominal pain for the last six months, accompanied by nausea, vomiting and appearance of dark-colored stools, who has lost 20 kg in weight during the last few months. Laboratory findings indicated anemia and no significant changes were identified in the abdominal ultrasound. By endoscopic examination of the stomach and duodenum and by colonoscopy, no infiltrations were found. Serum markers were elevated and CT scan of the abdomen showed thickening of a part of the jejunum wall with swollen lymph nodes in the mesentery, along the inferior vena cava and abdominal aorta, in the retroperitoneal space. By opening the abdominal cavity, we observed an infiltration in the initial part of the jejunum with an infiltration of the entire wall. Resection of the jejunum with related mesenthery, vessels and lymph nodes therein was performed. Histopathology revealed an invasive adenocarcinoma of the small intestine, with an invasion of all layers of the wall and mesentery. Adjuvant FOLFOX chemotherapy was introduced, 6 cycles in total, and following each cycle, tumor markers have been constantly decreasing. No relapse has been identified after nine months. Due to often deep position in the small intestine, atypical symptomatology and lack of screening, an early diagnosis is difficult. Surgical resection of the affected small intestine followed by an additional chemotherapy is the optimal treatment strategy.

Keywords: *Adenocarcinoma, jejunum, therapy, prognosis.*

INTRODUCTION

The small intestine occupies 70-80% of the length and about 90% of mucosa of the digestive tract, but small intestine cancers are rare and they are 1-3% of all gastrointestinal tumors, and only 11-25% of these are in jejunum (1, 2).

In the US, malignant tumors of the small intestine account for only 0.6% of all cancers (1-3). Despite its low incidence, small bowel cancer has been on the rise with an estimated incidence growth of over 100% in the last half a century. It has been noted that the incidence of all small intestine cancers in the United States has increased from 11.8 cases / million in 1973 to 22.7 / million in 2004 (4).

Malignant tumors of the small intestine occur more frequently in the male population. In the United Kingdom, the diagnosis of small bowel cancer occurs with a frequency of

3.1 / 100,000 in men and 2.2 / 100,000 in women (5). The incidence of those tumors in Serbia, according to the Batut Institute of Public Health, is 1.2 / 100,000 for men and 1 / 100,000 for women (6). The average age of a patient at the time of diagnosis in the US is 66 years, in the UK it is 80-84 years (5), and in Serbia the highest number of cases is recorded around the age of 60 (6).

The risk factors for small bowel cancer are divided into non-modifiable and modifiable.Non-modifiable risk factors are gender, race and ethnicity, age, inherited mutations and inflammatory bowel disease (5,7-9). Known modifiable risk factors are diet, excessive alcohol use, cigarette smoking, obesity, occupations associated with exposure to radiation, organic solvents and dyes (10-14).

In relation to histogenesis, malignant tumors of the small intestine may be epithelial (carcinomas and neuroendocrine cancers), mesenchymal (sarcomas), lymphomas and secondary tumors (15).

Atypical clinical presentation, lack of screening due to low incidence and inappropriate treatment experience due to lack of prospective randomized trials, make early diagnosis of malignant tumors of the small intestinevery difficult.

CASE REPORT

Patient BR, 62 years old female, with a positive family history (her sister was treated for colon cancer) felt abdominal pain, in the middle upper quadrant, followed by nausea and vomitingover the period of last six months. She was treated with oral proton pump inhibitors and iron preparations drugsdue to gastritis and chronic anemia, but the symptoms became more frequent. The weight loss was over 20kg in the last two months, with the appearance of increasingly frequent dark-colored stools. The patient was admitted to the Center for Abdominal Surgery, Clinical Center of Montenegro.

Physical examination revealed a soft abdomen, with the tenderness in the epigastric region, without muscular defense of the anterior abdominal wall and peritoneal irritation, without palpable neoproliferations in the abdominal cavity. Laboratory findings showed that signs of anemia were present (Red blood cells 3.67 10^{12}/L, hemoglobin 103 g/L, hematocrit 0.325 L/L). Abdominal ultrasound showed no significant changes. Upper gastrointestinal endoscopy was normal, except for antral hyperemia. Colonoscopy was with no pathological findings.

Serum markers increased (CEA = 22.1; Ca 19-9 = 731.5). CT scan showed a thickening of a part of the jejunum wall and manyswollen lymph nodes in the mesentery, adja- cent to the abdominal aorta and inferior vena cava in the ret- roperitoneal space. After adequate preparation for surgical treatment, the patient underwent a surgery. By upper and partly lower midline laparotomy we opened the abdominal cavity and observed an infiltration of the initial part of the jejunum with infiltration of the entire wall, at a distance of about 10cm from the ligament of Treitz. The resection of the jejunumwith the clear marginswas performed with the related mesenthery, vessels and lymph nodes therei. Subsequently, the jejunum was reconstructed with termino-lateral anasto- mosis using an intraluminal stapler of 33mm on the very lig- ament of Treitz (Figure1). We sent the resected jejunum with the mesenthery, vessels and lymph nodes (Figure 2.), the proximal and distal resection margin of the jejunum for his- topathological analysis.

The patient begun with liquid diet“per os” on the second postoperative day and soft diet on the third postoperative day. The patient was discharged from the Center for Abdominal Surgery of Clinical Center of Montenegro on the sixth postoperative day with normal digestive functions in good general condition.

Histopathological examination (Figure 3.) revealed the invasive adenocarcinoma of the small intestine of histologic and nuclear grade 2, with invasion of all layers of the wall andmesentery up to 3 mm. The tumor had infiltrative growth with lymphovascular invasion and absence of perineural invasion. Free surgical margins were achieved. Secondary deposits were histopathologically identified in all the three examined lymph nodes. Adjuvant FOLFOX chemotherapy was initiated for the patient, 6 cycles were administered in total and it was well tolerated by the patient.

The control CTscan showed a significant reduction of the preoperatively swollen lymph nodes along the aorta and inferior abdominal vena cava. The tumor marker CEA and Ca 19-9 have been constantly decreasing after each cycle of chemotherapy.

After nine months of postoperative follow-up, no disease relapse could be identified in the patient.

Figure 1. Stapler T-L anastomosis of the jejunum on the ligament of Treitz

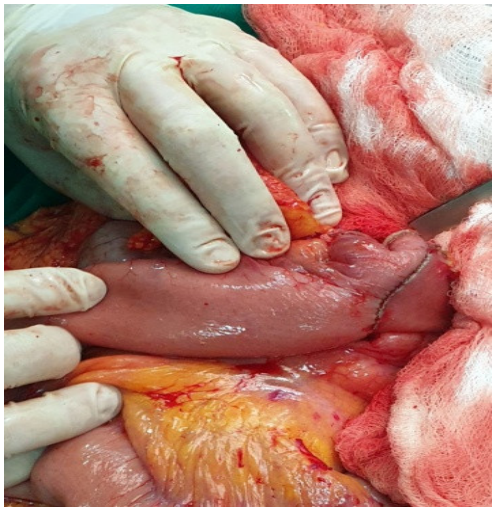


Figure 2. Resected the involved jejunum, mesenthery and vessels.

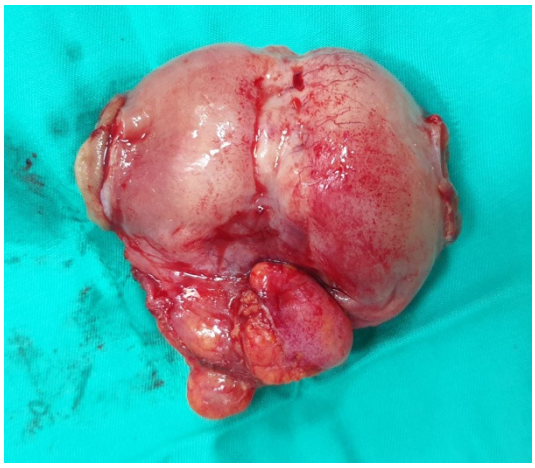
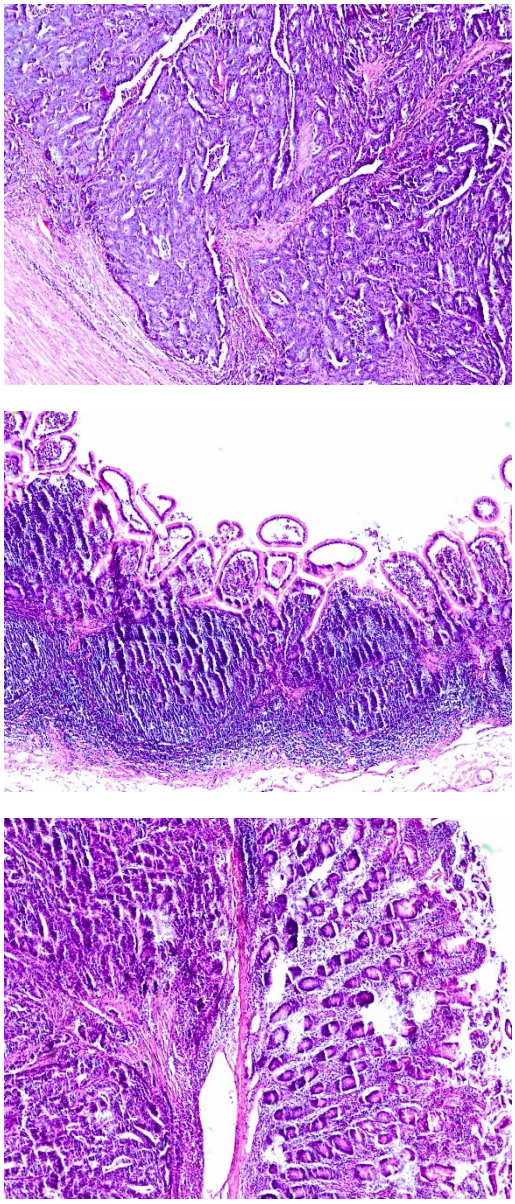


Figure 3. Microscopic images of the tumour from the pathologic specimen; haematoxylin and eosin staining (HE x 40)



DISCUSSION

The small intestine is located between the stomach and the large intestine and is the main site of ultimate absorption of nutrients from ingested foods. It consists of the duodenum, jejunum and ileum. The duodenum is 20-25cm in length and-food, stomach acid, bile and pancreatic juices with enzymes come together there. In the jejunum and ileum, digested food is absorbed and the absorbed substances are transported from the small intestine via the portal vein to the liver.

Malignant tumors of the small intestine are localized in the duodenum in 55-83% of cases, about 11-25% are localized in the jejunum, and 7-17% in the ileum (16-18). In our patient, the tumor was localized in the initial part of the jejunum at a distance of about 10cm from the ligament of Treitz.

Adenocarcinoma is the most common malignant tumor of the small intestine, it accounts for 40% of cases and it is most commonly localized in the duodenum, it metastasizes early to regional lymph nodes and is already in advanced stages at the time of diagnosis. Neuroendocrine tumors are second in frequency, followed by gastrointestinal stromal tumors, sarcomas and lymphomas, which together account for about 20-25% of small intestine tumors (16-18). It has been

observed that neuroendocrine cancers are most commonly localized in the terminal ileum (19).

The incidence of various histological types of small intestine cancer has changed over the last decades. Thus, in the United States, between 1985 and 2005, the proportion of adenocarcinomas decreased from 42 to 33%, and the proportion of neuroendocrine cancers increased from 28 to 44%. In the same period, the proportions of sarcoma and lymphoma did not change. The rise in neuroendocrine cancers is thought to be due to the use of more sensitive diagnostic methods for the recognition of neuroendocrine tumors (20, 21).

Adenocarcinoma occurs by malignant proliferation of small intestinal mucosal epithelial cells, and it is thought that, like those in the colon, small intestine polyps or adenomas can be transformed into adenocarcinomas after a latent period of 10-20 years. Only about 10% of adenomas are known to progress to cancer through the adenoma-carcinoma sequence, which is characterized by numerous chromosomal instabilities associated with mutation accumulation (22). On the other hand, there are suggestions that the pathogenesis of adenocarcinoma in the small intestine differs from that in the colon, since there is a shorter transit time through the small intestine, by which exposure of its mucous membrane to carcinogens from food is limited (23). The low incidence of small intestine adenocarcinoma and lack of screening comparable to endoscopy are also thought to make it difficult to study the pathogenesis of these tumors, but that large differences in the incidence of colon and small intestine adenocarcinoma indicate to different pathogenesis (2).

Small intestinal adenocarcinomas can occur sporadically (without associated intestinal disorders) or associated with various precursor conditions such as familial polyposis, Peutz-Jeghers and Lynch's syndrome or with immunologicalintestinal disorders (Crohn's disease, celiac disease, etc.) (24-27).

Numerous studies reports indicate that primary adenocarcinomas most commonly appear as solitary lesions (28-30), whereas metastatic tumors of the small intestine generally occur as multiple lesions (31,32). Metastasis of breast, lungs, stomach, and melanoma cancers to the small intestine have been described in the literature so far (33-36). Primary adenocarcinoma was presented as a solitary lesion in our patient. With regard to the mode of growth, adenocarcinomas are divided into vegetative, ulcer- ative, and infiltrative ulcerative types (31, 32).

The clinical picture of adenocarcinoma of the small intestine is nonspecific. Often there is painin the upper and middle abdomen, changes in bowel movements, blood in the stool (melena) and weight loss with lack of appetite. Unfortunately, all these symptoms have been treated as functional disorders for a long period of time. As it is often located deep in the small intestine, due to low incidence and atypical symptomatology, early diagnosis is difficult, so these tumors are usually diagnosed at a relatively late stage (37). There is a report in the literature that nearly 60% of patients could not be diagnosed until obstruction or intestinal perforation had occurred (38).

Despite the report that only 50% of patients with small intestine adenocarcinoma have elevated levels of CEA and CA19-9 (37), numerous studies have highlighted their significant role in monitoring the effects of antitumor therapy (38, 40). As regards our patient, tumor markers CEA and Ca 19-9 were constantly decreasing after each cycle of chemotherapy.

Numerous predisposing mutations (such as APC and KRAS), mutations of suppressor genes and oncogenes have been involved in the development of adenocarcinoma of the small intestine, and modern molecular therapies, which include these targets,give hope for future patients. Mutations of the p53 gene were described in about 50% of small intestine adenocarcinomas, the APC gene was mutated in about 10% of cases, and mutations of the βcatenin gene were observed in 10-40% of cases. KRAS, an oncogene that normally functions in cell signaling and proliferation, is present in about 50% of adenocarcinoma cases (39). SMAD4 mutations and activation of the RAS-RAF-MAPK signaling pathway have also been described in this tumor (15).

Contemporary literature points out that surgical resection of the affected part of the small intestine, followed by additional chemotherapy, is the optimal treatment strategy, especially in cases with bleeding or perforation (37,38). Our patient's jejunum was resectedwith the clear margins and with the related mesenthery, vessels and lymph nodes therein, and then adjuvant FOLFOX chemotherapy was introduced, which was well tolerated by the patient.

The length of survival of patients is greatly influenced by the stage of the disease at the time of diagnosis and the choice of anticancer therapy. The noted five-year survival of operated patients is 40-65% as opposed to 15-30% of cases in the non-operated group of patients (38). Median survival time for patients receiving chemotherapy is 32 months (ranging from 20 to 72 months), while for patients not receiving anticancer therapy it is 18 months (ranging from 6 to 60 months). Other factors associated with poor prognosis are tumor size, age, intestinal obstruction or perforation, peritoneal metastases, etc. (18,37). It is noted that median survival time was 28 months (range from 4 to 72 months)in a patient with a tumor smaller than 5cm, while in those with a tumor larger than 5cm median survival time was 14 months (range from 4 to 41 months) (38).

The study of risk factors, pathogenesis and treatment modalities for small intestine cancer is limited as it is a very rare neoplasm.

CONCLUSION

Due to the often deep position in the small intestine, atypical symptomatology and lack of screening, early diagnosis is difficult. Surgical resection of the affected small intestine followed by additional chemotherapy is an optimal treatment strategy.

The study of risk factors, pathogenesis and treatment modalities for small bowel cancer is limited as it is a very rare neoplasm.

ETHICS APPROVAL AND CONSENT TOPARTICIPATE

The study was conducted in accordance with the ethicalstandards of the committee responsible for human experimentation (institutional and national) and the Helsinki Declaration of 1975, as revised in 2013. Voluntary written and informed consent was obtained from the patient prior to enrollment in the study.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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BILATERAL CHANGES IN THE LUNGS OF A PREGNANT WOMAN CAUSED BY BILATERAL SPONTANEOUS PNEUMOTHORAX

Jelena Radojicic[1], Jelena Markovic[2], Zeljko Garabinovic[3], Milan Savic[3,4] and Jelena Stojsic[2]

[1]*City Institute for Urgent Medical Care, Belgrade, Serbia*
[2]*Service of Pathology, Clinical Center of Serbia, Belgrade, Serbia*
[3]*Clinic of Thoracic Surgery, Clinical Center of Serbia, Belgrade, Serbia*
[4]*University of Belgrade, Faculty of Medicine, Belgrade, Serbia*

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Corresponding author:

Jelena Stojsic MD, PhD

Koste Todorovića St. No. 20
11000 Belgrade, Serbia

E-mail: jelena.stojsic@kcs.ac.rs

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ABSTRACT

Lymphangioleiomyomatosis (LAM) is a rare disease that most commonly affects women of reproductive age. The disease is manifested by diffuse destruction of the pulmonary parenchyma with the formation of cysts on a characteristic finding of multidetector computerized tomography (MD-CT) of the chest. It is thought that the presence of cells with estrogen and progesterone receptors among proliferating smooth muscle cells lead to the spread of cystic structures lined by endothelial cells. Towards the end of pregnancy and after childbirth, collapse of the lung parenchyma or rupture of the cyst wall occurs causing a dramatic clinical picture in the form of pneumothorax. Sirolimus is the only drug of choice that should improve and stabilize the patient's pulmonary function and quality of life. Unfortunately, this drug is not always effective enough, so only option for treatment is bilateral lung transplantation. The authors present the patient in the last trimester of pregnancy who was admitted to the hospital with clinical picture of bilateral spontaneous pneumothorax. Dramatic picture of pneumothorax could not be solved solely by drainage in which atypical segmental resection of the lung was performed within a few days so on these samples lymphangioleiomyomatosis were diagnosed.

Keywords: *Lymphangioleiomyomatosis, pneumothorax, pregnancy, dysregulation of estrogen receptor, dysregulation of progesterone receptor.*

INTRODUCTION

The diagnosis of rare lung tumors is a challenge. Some rare lung tumors are diagnosed only once during the years of practicing pulmonary pathology and that only in highly specialized hospitals exclusively dealing with thoracic surgery. According to the latest WHO classification, lymphangioleiomyomatosis (LAM) belongs to the group of mesenchymal tumors, subgroup of PEComatous tumors. In the literature, LAM has been described as a tumor associated with tuberous sclerosis. Pneumothorax and acute respiratory distress syndrome are the leading symptoms of LAM (1, 2). The literature describes patients who have been diagnosed with LAM in pregnancy and who have had to have a premature birth (3, 4). Radiological findings as well as pathohistological, morphological and immunohistochemical findings were characteristic of LAM (5).

We present a patient who developed bilateral spontaneous pneumothorax in the last trimester of pregnancy.

CASE REPORT

We present a case of 32-year-old pregnant woman and non-smoker who was hospitalized at the Clinic for Thoracic Surgery Clinic of the Clinical Center of Serbia, Belgrade, Serbia, in February 2020 for bilateral chest pain and shortness of breath. Because of suspicion to bilateral spontaneous pneumothorax after reviewing radiological findings, it was performed multidetector -computed tomography (MD-CT). MD- CT cross-sectional imaging has determined bilateral diffuse cystic changes of thin and smooth wall both directly below the pleura and deep within the lung parenchyma (Figure 1a, 1b). It was concluded that reexpansion of the pulmonary parenchyma could not be solved only by bilateral drainage but only by surgical procedure.

In order to perform surgery, c-section performed in 37th week of pregnancy at the Clinic for Gynecology and Obstetrics of the Clinical Center of Serbia in Belgrade, was necessary. Thereafter, it was performed atypical segmental resection on the left lung and after a few days, resection of the same type to the right lung.

Pathological findings:

One day before macroscopic examination and sampling, insufflation of 10% buffered formalin was performed in order to make easier examination of distended alveolar spaces by imitating inspirium.

Macroscopic finding: Routine macroscopical examination of sample showed smooth wall cavities with no noticeable content, up to 4mm in diameter, which were present below the pleura and also in the depth of the pulmonary parenchyma. Because of this, both lung samples had a sponge-like appearance (Figure 1c). Tissue samples were routinely processed, embedded into paraffin blocks and classically hematoxylin-eosin stained.

Figure 1. Radiological and macroscopic findings.

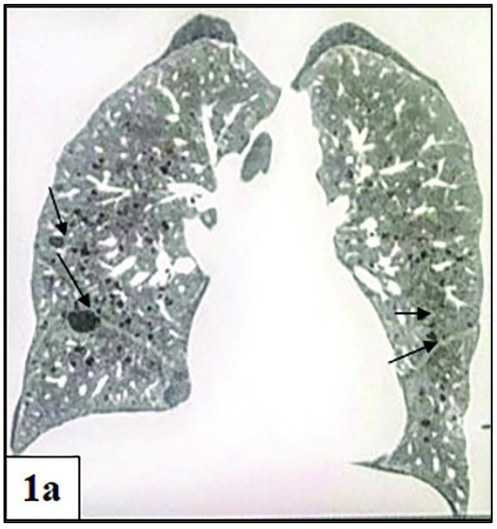

Figure 1a. Apical computed tomography of the chest detected numerous diffuse thin-walled cysts of various dimensions without content (arrows)

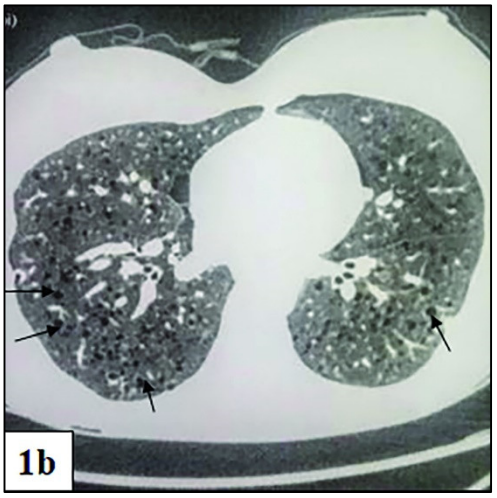

Figure 1b. Apicoaxial computed tomography of the chest: numerous thin-walled cysts without content (arrows) diffusely presented in the lung parenchyma and subpleuraly

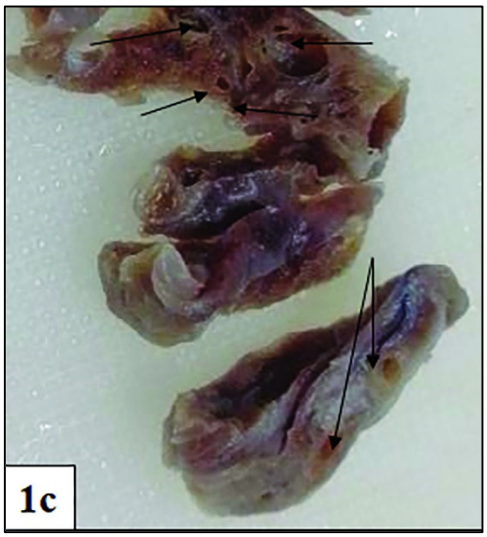

Figure 1c. Macroscopic findings: diffuse cystic spaces of the thin and smooth walls, without content. On this sample, the greatest measured diameter is up to 4mm *(arrows)*

Macroscopic changes were less pronounced on the sample from the right lung, operated one week later.

Suspicion on lymphangioleiomyomatosis arose after H&E staining which has also immunohistochemically proven.

Microscopic finding: In the lung parenchyma were present numerous cystic spaces without content covered by single-row endothelial cells. Under endothelial cells focally have located multiplied spindle cells expressing α-*smooth muscle* (*αSM*) *actin* marker (Figures 2a, b, c). Between these cells are those that express steroid receptors, estrogen and progesterone (Figures 2d, e). The main characteristic is the presence of diffuse cytoplasmic expression of HBM-45 in almost all spindle cells (Figure 2f).

Figure 2. Microscopic findings

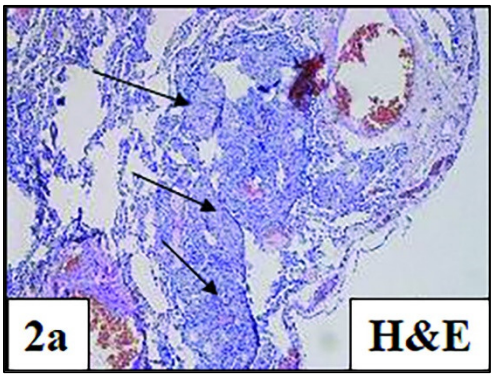

Figure 2a. Diffuse changes in the lung parenchyma in the wall of cystic spaces of proliferative spindle cells (*arrows*) x 10

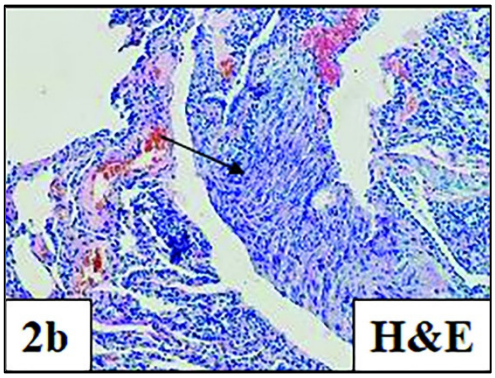

Figure 2b. One of the focuses of proliferating spindle cells in the wall of the cystic lung structure *(arrow)* x 20

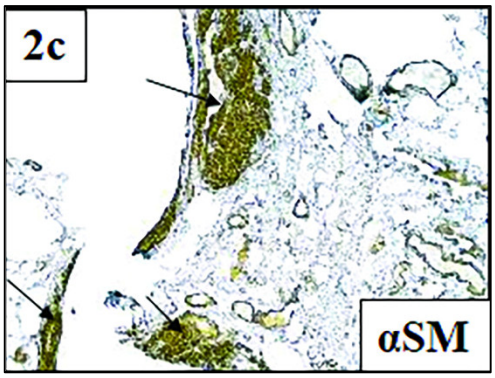

Figure 2c. αSM actin expression in the proliferative spindle cells that confirms their smooth-muscle origin *(arrows)* x 10

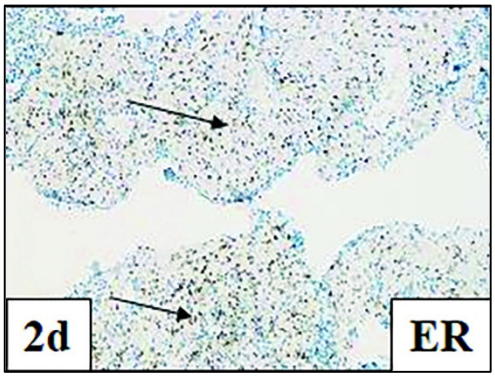

Figure 2d. Expression of steroid estrogen receptor confirms hormonal dependence of LAM and its most common clinical manifestation during hormonal imbalance *(arrows) x 10*

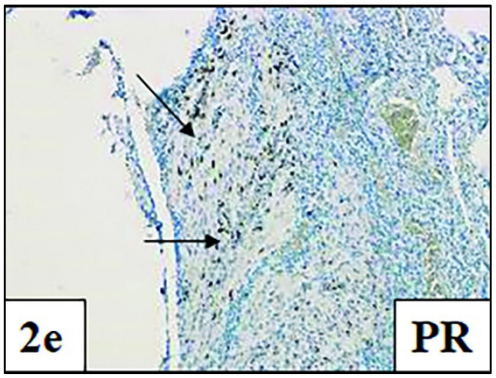

Figure 2e. Expression of steroid progesterone receptor in a few tumor cells *(arrows) x 10*

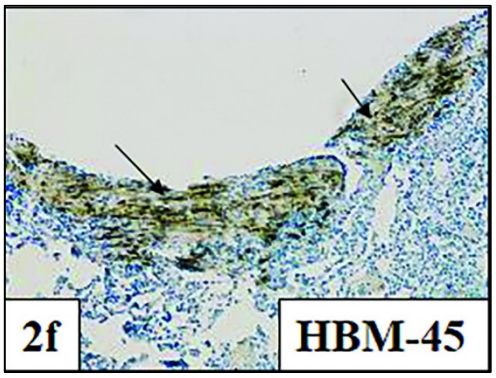

Figure 2f. HBM-45 expression in spindle cell confirms diagnosis of LAM, *(arrows) x 20*

A few days after the first, a second intervention of the same type was done in the right lung which had the same changes present but of some lower intensity.

After bilateral surgery, recovery and concurrent pathohistological diagnosis of LAM, our patient should start with the treatment.

DISCUSSION

According to the 2015 WHO Classification of Lung Tumors, LAM belongs to the group of PEComatous tumors, together with benign and malignant PEComatous tumors and "clear cell" tumor. All tumors in this group are characterized by focal or diffuse expression of HBM-45 (1).

The morphological picture of LAM is characterized by the presence of cystic spaces covered by single-row, flat endothelial cells expressing podoplanin (*clone* D2-40) under which are focally located multiplied proliferations of spindle cells expressing αSM-actin. Between these cells are present those expressing steroid receptors, estrogen and progesterone and those with diffuse and cytoplasmic expression of HBM-45. According to some authors, if there is no HBM-45 expression it is about lymphangiomyomatosis. In the first sample in our patient changes are more pronounced than in the other which has also been described in the available literature. Although changes were of varying intensity, both caused pneumothorax (1, 3, 4, 5).

The literature has described cases of LAM that caused pneumothorax in the last trimester of pregnancy and shortly after birth. This phenomenon is explained by the fact that hormonal stimulation during pregnancy leads to an increase in cystic structures and at the end of pregnancy or shortly after delivery it comes to decline in hormonal stimulation and consequently to the rupture of some of the thin wall cystic structures and onset of pneumothorax. This also explains the fact that LAM is almost typically a female disease (3, 5).

LAM can be associated with other diseases but it can be also an independent disease. The Polish authors analyzed 15 pathohistologically diagnosed LAMs and in 6 of them there was no present an associated disease as well as in our patient (5).

After the diagnosis of LAM, the pulmonologist should start treatment with target therapy. Treatment for LAM is based on its etiology. LAM occurs as congenital (TSC-LAM) or acquired (sporadic or S-LAM) tumor supressor tuberous sclerosis complex (TSC) gene mutation in TSC1 (hamartin) or TSC2 (tuberin). The TSC1–TSC2 complex is incorporated into various signal pathways and it is also involved in the regulation of the target of rapamycin complex. In most LAM tumor tissue, there are either TSC1 or TSC2 mutations, but in 10–15% it is not determined presence of TSC gene mutations. Numerous studies have included the effects of sirolimus which is mechanical target of rapamycin (mTOR) inhibitors. Other molecular - target drugs are also being investigated. If LAM does not respond to these drugs only treatment option is bilateral lung transplantation (6, 7, 8).

CONCLUSION

Lymphangioleiomyomatosis is a rare lung tumor manifested by a dramatic clinical picture. In our patient, end of pregnancy caused a decrease in hormones, estrogens and progesterone, which was manifested by lymphangioleiomyomatosis in the form of bilateral spontaneous pneumothorax.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

The study was conducted in accordance with the ethical standards of the committee responsible for human experimentation (institutional and national) and the Helsinki Declaration of 1975, as revised in 2013. Voluntary written and informed consent was obtained from the patient prior to enrollment in the study.

CONFLICT OF INTEREST

None.

FUNDING

None.

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