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СОДРЖИНА

CONTENTS

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EDITORIAL

KIDNEY DISEASES IN CHILDREN – EARLY DIAGNOSIS AND PREVENTION

Momir Polenakovic[1], Zoran Gucev[2], Velibor Tasic[2]

[1] Macedonian Academy of Sciences and Arts, Skopje, R. Macedonia
[2] University Children's Hospital Medical School, Skopje, R. Macedonia

Corresponding Author: Prof. Momir Polenakovic, MD, PhD, Macedonia Academy of Sciences and Arts, Bul. Krste Misirkov, No. 2, 1000 Skopje, Republic of Macedonia; E-mail maknefpo@t-home.mk

Abstract
Pediatric kidney diseases were in the focus of the World Kidney Day 2016. Macedonian pediatric nephrologists gave their contribution with public appearance in kindergartens, primary and secondary schools, with interactive lectures and discussion with the youngest about the kidney function, healthy life style and simple measures to prevent kidney and urinary tract diseases. Besides promotive appearance in the media, series of lectures were presented in front of the health professionals. The aim was to attract the attention of the professionals for early diagnosis and prevention of kidney disease. The action starts in utero, followed by early postnatal imaging and assessment, conservative treatment and in selected cases surgical treatment. The emphasis is on the multidisciplinary and comprehensive approach to children and adolescents with kidney diseases.

Keywords: World Kidney Day, pediatric kidney diseases, prevention

The 11th World Kidney Day was celebrated on March 10, 2016, around the globe. This event was sponsored in the Republic of Macedonia by the non-governmental renal patient organization (Nephron) and the Macedonian Societyfor Nephrology Dialysis Transplantation and Artificial Organs (MSNDTAO). There are about 1.400 patients on chronic dialysis program in the Republic of Macedonia. The number of transplants is limited and is mainly confined to living kidney transplantation. Due to the limited resources it is mandatory to promote preventive measures for early diagnosis, appropriate treatment and prevention of chronic kidney diseases.

In 2016, the World Kidney Day was dedicated to the kidney disease in childhood and the antecedents of adult kidney disease, which can begin in the earliest childhood [1]. Macedonian pediatric nephrologists gave their contribution with public appearance in kindergartens, primary and secondary schools with interactive lectures and discussion with the youngest about the kidney function, healthy life style and simple measures to prevent kidney and urinary tract diseases. The aim was to attract the attention of the youngest to the kidneys as very important organ for elimination of the waste. The youngest had their exhibition of the paintings with very imaginative presentation of the kidneys (Figures 1 and 2).

Besides the promotive appearance in the media a series of lectures were presented in front of the health professionals. The aim was to attract the attention of the professionals for early diagnosis and prevention of kidney disease. The action starts in utero, followed by early postnatal imaging and assessment, conservative treatment and in selected cases surgical treatment. Macedonian nephrologists presented their data on the spectrum of kidney disease, and pointed that congenital anomalies of the kidney and the urinary tract (CAKUT) are still the leading etiology of the chronic kidney disease (CKD).

The emphasis was on the comprehensive evaluation of the patients, particularly in the case of syndromic cases. Although there is still a high genetic heterogeneity in children with CAKUT the molecular diagnosis is possible. Hyperechogenic and dysplastic kidneys seen on the prenatal ultrasound are often the result of mutation of *HNF1B* gene [2]. Besides nephropathy these patients may develop later in life MODY5 diabetes, hypomagnesemia, hyperuricemia and gout and gynecological problems due to associated urogenital anomalies. Children with mutation in *EYA1, SIX1, SIX5* mutation clinically present as a BOR syndrome; besides CAKUT, a serious problem is the associated hearing impairment [3].

Fig. 1 – Children participants in the World Kidney Day 2016

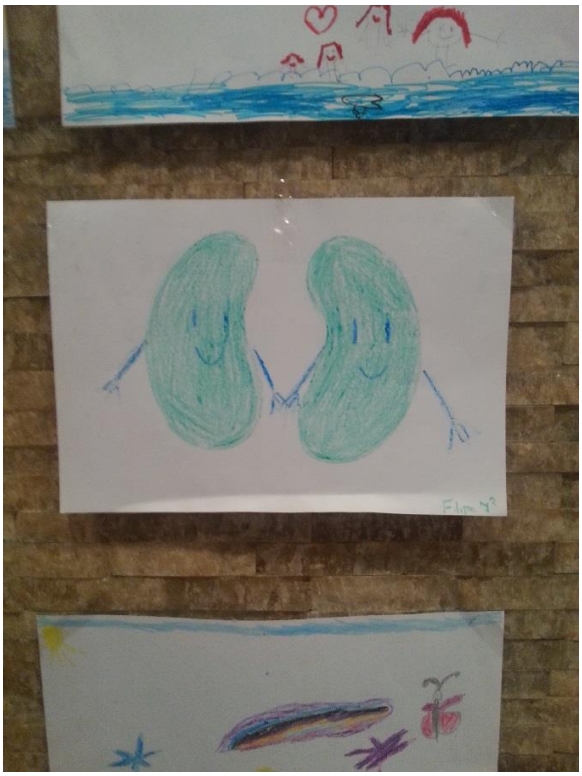

Fig. 2 – Children's paintings at the World Kidney Day 2016

In the presented lectures particular attention was paid to the progression of the chronic kidney diseases in children. The standard factors which may affect progression of CKD in adults are hypertension, proteinuria, glomerular etiology, male gender, anemia, diabetes, dyslipidemia, hyperparathyroidism, malnutrition (hypoalbuminemia) which also operate in children.

The focus of researchers in the last two decades is concentrated on the abnormal birth history [prematurity, low birth weight (LBW), or small for gestational age (SGA)] which are associated with hypertension, chronic kidney disease, cardiovascular morbidity, obesity and diabetes mellitus in adulthood [4]. Low birth parameters are factor for initiation of CKD, but not significant factor for progression to ESRD as it was shown in a large Japanese pediatric study [5].

The other important pediatric risk factor is obesity. There is a global, worldwide epidemic of obesity affecting not only adults but particularly children and adolescents. The sedentary style of life, consumption of junk food, lack of physical activity contributed to the magnitude of this epidemic. There is a clear evidence that obesity is an independent risk factor for progression of CKD as in the case of IgA nephropathy, patients with unilateral renal agenesis or nephrectomy [6]. Even renal allograft dysfunction was noted with higher rate in kidneys from obese donors compared to lean donors' kidneys.

Smoking is another important but underrated risk factor in renal patients. The increasing prevalence of smoking among adolescents has negative impact on the renal functions. The adult studies clearly demonstrated unfavorable effect of tobacco use in patients with IgA neph-

ropathy, diabetic nephropathy and allograft nephropathy. Besides nicotine there are > 4000 toxic chemicals including carbon monoxide, arsenic, vinyl chloride, cadmium, lead, and acrolein, which have negative impact on the kidney function [7].

Instead of conclusion, the global message from the World Kidney Day 2016 in the Republic of Macedonia is early and effective diagnosis of kidney disease, preventive measures which include improving the pre-, peri-, and post-natal health care and struggle against prematurity, obesity and smoking. Health professsionals should be the principal promotors of this action, but the role of society is of utmost importance in education and promotion of the healthy life style.

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Резиме

БУБРЕЖНИ ЗАБОЛУВАЊА КАЈ ДЕЦАТА – РАНА ДИЈАГНОЗА И ПРЕВЕНЦИЈА

Момир Поленаковиќ[1], Зоран Гучев[2], Велибор Тасиќ[2]

[1] Македонска академија на науките и уметностите, Скопје, Р. Македонија,
[2] Универзитетска клиника за детски болести, Медицински факултет, Скопје, Р. Македонија

Педијатриските бубрежни заболувања беа во фокусот на Светскиот ден на бубрегот 2016 година. Македонските педијатри нефролози дадоа свој придонес со посети на градинки, на основни и на средни училишта и со интерактивни предавања дискутираа со најмладите за функцијата на бубрезите, за здравиот начин на живеење и едноставните мерки за спречување заболувања на бубрезите и на уринарниот тракт. Покрај промотивна појава на медиумите, тие презентираа серија предавања за здравствените професионалци. Нивна цел беше да се привлече вниманието на професионалците за рана дијагноза и превенција на бубрежните заболувања. Акцијата започнува уште in utero проследено со ран постнатален imaging и процена, конзервативен третман, а во селектирани случаи и хируршки третман. Акцентот е ставен на мултидисциплинарниот и сеопфатен пристап кон децата и адолесцентите со бубрежни заболувања.

Клучни зборови: Светски ден на бубрегот, педијатриски бубрежни заболувања, превенција

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CHRONIC KIDNEY DISEASE – PEDIATRIC RISK FACTORS

Velibor Tasic[1], Aleksandra Janchevska[1], Nora Emini[1], Emilija Sahpazova[1], Zoran Gucev[1], Momir Polenakovic[2]

[1] University Children's Hospital, Medical Facultu, Skopje, R. Macedonia
[2] Macedonian Academy of Sciences and Arts, Skopje, R. Macedonia

Corresponding Author: Prof. Velibor Tasic, MD, PhD, University Children's Hospital, Medical School Skopje, 17 Vodnjanska, 1000 Skopje, Macedonia, E-mail: vtasic2003@gmail.com

Abstract
The knowledge about the progression of chronic kidney disease is an important issue for every pediatric nephrologist and pediatrician in order to implement appropriate measures to prevent wasting of renal function and the final consequence – end stage renal disease with the need for the dialysis and transplantation. Therefore it is important to know, treat or ameliorate the standard risk factors such as hypertension, proteinuria, anemia, hyperparathyroidism etc. In this review devoted to the World Kidney Day 2016 we will pay attention to the low birth parameters, obesity, hyperuricemia and smoking which emerged as particularly important risk factors for children and adolescent with chronic kidney disease.

Keywords: chronic kidney disease, children, low birth parameters, smoking, obesity, hyperuricemia

Introduction

This review was produced for the World Kidney Day 2016 in order to attract the attention of health professionals to the pediatric aspects of the chronic kidney diseases [1]

The etiology of the chronic kidney disease (CKD) differs between pediatric and adult patients. Diabetic nephropathy, hypertension, and autosomal dominant polycystic kidney disease are prevalent diseases in adults, while in children the congenital anomalies of the kidney and urinary tract (CAKUT) is the most common pathology in 50%, followed by the hereditary nephropathies and the glomerulonephritis [2]. Cardiovascular events are the most common cause of death in pediatric CKD patients, with risk 1,000 times higher in the end stage renal disease (ESRD) population compared to the age-matched non-CKD population. Left ventricular hypertrophy, increased carotid artery intima-media thickness and carotid arterial wall stiffness are early markers of the cardiovascular morbidity in this population. The coronary artery calcification which is a proven early marker of increased cardiovascular mortality in adults with CKD is also demonstrated in children with advanced CKD [3]. The disturbances in the mineral metabolism which are prominent in children also contribute to development of vascular calcification [4].

The standard factors which may affect the progression of CKD in adults are hypertension, proteinuria, glomerular etiology, male gender, anemia, diabetes, dyslipidemia, hyperparathyroidism, malnutrition (hypoalbuminemia) etc. These factors also operate in children. In children, the age is also an important risk factor; those < 2 years and pubertal children more frequently initiate renal replacement therapy. This is the result of the accelerated gro-

wth at that age and the increase in the body mass which cannot be compensated with adequate renal function.

In this review we cannot analyze all these factors, but we will focus to those which attracted the attention of the researchers in the last two decades and which became global health problem, but could be modified or ameliorated by appropriate medical intervention and increased public awareness (low birth parameters, smoking, obesity and hyperuricemia).

Low birth parameters

In the last two decades the low birth parameters attracted the attention of many researchers since there is evidence that the adverse events in utero lead to impaired nephrogenesis and increase the risk of CKD later in life. There is a higher prevalence of children born with low birth parameters in cohorts of CKD patients compared with the subjects without CKD.

The abnormal birth history [prematurity, low birth weight (LBW), or small for gestational age (SGA)] is also associated with hypertension, cardiovascular morbidity, obesity and diabetes mellitus in adulthood. Each kilogram increase in birth weight, results in adult systolic blood pressure lower for 1–2 mmHg. [5, 6] The postnatal rapid weight gain is also associated with the increased risk for future cardiovascular morbidity [6, 7, 8]. It has not yet been defined what is the time required to see these effects because the pediatric studies revealed contradictory results [9]. A possible explanation for these conflicting results is the method of blood pressure measurement. It seems that 24-hour ambulatory blood pressure measurement (ABMP) is more sensitive than office blood pressure measurement to confirm this association.

Lurbe and co-workers performed ABPM in 630 healthy children, all of whom had been born at full term, by ABPM at a mean age of 9.9 years [10]. Although the strongest predictor of the current 24-hr systolic BP was the current weight, the birth weight had a significant inverse relationship on both 24-hour systolic BP and BP variability. Bayrakci et al investigated a group of 41 children born preterm (30 were small for gestational age) and their ABPM compared with a group of children born at term [11]. The preterm group had higher nocturnal systolic BP, elevated nocturnal systolic and diastolic blood pressure loads, and blunted nocturnal dipping.

The researchers from the Chronic Kidney Disease in Children (CKiD) study investigated if a child's abnormal birth history, which included the following parameters (low birth weight, prematurity, SGA, or intensive care unit stay in the neonatal period) had influence on the height and weight in children with CKD [12]. During the four year period 586 children with mild to moderate CKD were enrolled from 48 pediatric nephrology centers across North America. All eligible children were aged 1 to 16 years and had a Schwartz-estimated GFR between 30 and 90 ml/min per 1.73 m^2. This study showed that LBW and SGA were associated with lower weight, especially in children with a glomerular etiology of CKD. Second, children with a history of LBW or SGA, regardless of the type of CKD diagnosis, were disproportionately short in stature. This study clearly proved that LBW and SGA are novel risk factors for abnormal growth in children with CKD.

In a combined study including the Pediatric Nephrology Departments at Hannover Medical School and Charite Hospital in Berlin perinatal data were analyzed in 435 children with CKD stages 3–5 of different etiology [13]. The patients were stratified in three groups [congenital n = 260 (60%), hereditary n = 93 (21%) and acquired n = 82 (19%) CKD etiology]. Low birth parameters (prematurity and SGA) were significantly more prevalent in the three groups compared with the referent population. The prevalence of prematurity/SGA expressed in % in the three groups were as follows: congenital (39.3% / 29.2%), hereditary (24.7% / 22.6%) and acquired CKD (15.5% / 29.3%); these percentages were significantly higher compared to 8% (for both) in the referent population. The authors concluded that both SGA and prematurity predispose for advanced renal disease in childhood and that fetal kidney disease impairs fetal growth. The practical implication from this study is that the acquired renal diseases may have different outco-

mes, those with low birth parameters have higher risk for CKD.

Low birth parameters are factor for initiation of CKD, but are not a significant factor for progression to ESRD, as it was shown in a large Japanese pediatric study [14].

Obesity

In the last two decades there is a global, worldwide epidemic of obesity affecting not only adults but particularly children and adolescents. The new style of life, lack of proper education and aggressive marketing of the junk food industry contributed to the magnitude of this epidemic. In the year 2008, 1.4 billion people worldwide were overweight, and 500 million were obese. The situation is alarming since in 2010, 40 million children under the age of 5 years were overweight or obese [15]. In parallel with these data there is an increasing prevalence of CKD in adults and children.

Obesity is co-morbidity associated with CKD, but vice versa, it can be a strong risk factor for CKD and its progression. It is well known that low birth parameters may be associated with the low nephron numbers and obesity and risk of CKD later in the life. Leptin and adiponectin are elevated in obese subjects and may be involved in pathogenesis and progression of CKD. Additional factors such as hypertension, increased cardiovascular morbidity, insulin resistance, dyslipidemia, and lipotoxicity, may play important roles in the pathogenesis of CKD in obesity [15–16].

It was shown that obesity is independent risk factor for progression of CKD as in the case of IgA nephropathy clinically and pathologically [17]. Worsening of the renal function is clearly demonstrated in patients with unilateral renal agenesis or nephrectomy. Also, the higher rate of renal allograft dysfunction was evidenced in kidneys from obese donors compared to lean donors' kidneys [18]. These results indicate that obesity initiates development and progression of CKD.

It is of note that decrease of the body weight and strong public awareness of obesity as a risk factor for CKD may result in decrease of its prevalence and general population health benefits.

Hyperuricemia

There is evidence that hyperuricemia increases the risk for cardiovascular mortality and morbidity, hypertension and CKD. In experimental models in rats it was shown that hyperuricemia leads to elevated blood pressure, proteinuria, renal dysfunction, and progressive renal and vascular disease. The principal effects of the serum levels of uric acid are endothelial dysfunction, activation of the local renin-angiotensin system, increased oxidative stress, and proinflammatory and proliferative actions [19]. A small number of short-term, single-center clinical studies support the beneficial influence of the pharmaceutical reduction of the serum uric acid on the total cardiovascular risk, as well as on the renal disease development and progression. Hyperuricemia is probably related to the incidence of primary hypertension in children and adolescents, as serum uric acid lowering by allopurinol has an antihypertensive action in this group of patients. Finally, it is clear that the adequately powered randomized controlled trials are urgently required to elucidate the role of uric acid in cardiovascular events and outcomes, as well as in the development and progression of CKD.

Noone and Marks investigated the prevalence of hyperuricemia in a pediatric chronic kidney disease clinic [20]. 116 children (age 0.4–16 years) were enrolled in the study. The prevalence of hyperuricemia in those with an eGFR < 60 mL/min/1.73 m(2) was 70%. This study showed that hyperuricemia was significantly associated with increased body mass index, albuminuria, renal dysfunction with reduced eGFR, and hypertension.

The researchers from the CKiD Cohort study investigated the role of hyperuricemia in progression of CKD in children and adolescents [21]. The following parameters were investigated: age, sex, race, blood pressure status, GFR, CKD cause, urine protein-creatinine ratio (< 0.5, 0.5 – < 2.0, and ≥ 2.0 mg/mg), age- and sex-specific body mass index > 95th percentile, use of diuretics, and serum uric acid level. The study revealed that older age, male sex, lower GFR, and body mass index > 95th percentile were associated with higher uric acid levels. Participants with uric acid levels of 5.5 to 7.5 or > 7.5 mg/dL had 17% or 38% shorter time to

decrease the GFR by 30% or initiation of renal replacement therapy respectively.

Smoking

It is well known that smoking is one of the strongest factors for cardiovascular mortality and morbidity in adults. Smoking is associated with albuminuria, progressive renal insufficiency and graft loss. Second hand smoking (SHS) is underrated pathogenic factor and in children it is associated with blood pressure variability, elevated C reactive protein and poorer neurocognitive functions. The Midwest Pediatric Nephrology Consortium investigated cigarette smoking and second-hand smoking exposure in adolescents with chronic kidney disease [22]. The urinary cotonine/creatinine level was investigated and was found to be higher in those who lived with a smoker or had a friend smoker.

Another American study investigated smoking and second hand smoking [23]. According to the urinary cotinine levels, 22% of the subjects were exposed to SHS. There was a significant correlation between the SHS and the lower maternal education, the African American race, the greater prevalence of the nephrotic range proteinuria and the left ventricular hypertrophy.

Hogan et al. showed that albuminuria was associated with cigarette smoking independent of other comorbidities, such as hypertension and diabetes [24]. Garcia-Esquinas et al. demonstrated a linear reduction in the estimated glomerular filtration rate (eGFR) with rising serum cotinine levels among the healthy adolescents who were active smokers [25]. There are adult studies which proved the effect of tobacco use in patients with IgA nephropathy, diabetic nephropathy and allograft nephropathy [26]. In humans, nicotine, the active ingredient in tobacco, promotes mesangial cell proliferation and extracellular matrix production via recently discovered nicotinic receptors in the mesangial cells. Besides nicotine there are > 4000 toxic chemicals including carbon monoxide, arsenic, vinyl chloride, cadmium, lead, and acrolein [26]. Lead is well known nephrotoxin and is associated with the CKD progression. Acrolein has been shown to induce apoptosis of renal cells and generation of reactive oxygen species. Other mechanisms by which smoking may contribute to proteinuria and CKD progression are induction of hypoxia, intrarenal vasoconstriction and stimulation of proinflammatory cytokines.

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Резиме

ХРОНИЧНА БУБРЕЖНА БОЛЕСТ – ПЕДИЈАТРИСКИ РИЗИК-ФАКТОРИ

Велибор Тасиќ[1], Александра Анчевска[1], Нора Емини[1], Емилија Шахпазова[1], Зоран Гучев, Момир Поленаковиќ[2]

[1] Универзитетска клиника за детски болести, Медицински факултет, Скопје, Р. Македонија
[2] Македонска академија на науките и уметностите, Скопје, Р. Македонија

Знаењето за прогресијата на хроничната бубрежна болест е од големо значење за секој педијатриски нефролог и педијатар заради имплементирање соодветни мерки за превенирање на губење на бубрежната функција и крајна последица терминална уремија со потреба од дијализа и трансплантација. Затоа е потребно да се познаваат, третираат и ублажат стандардните ризик-фактори, како што се хипертензија, протеинурија, анемија, хиперпаратиреоидизам и други. Во овој ревијален труд посветен на Светскиот ден на бубрегот 2016, особено внимание посветуваме на ниските родилни параметри, дебелината, хиперурикемијата и пушењето, кои се наметнаа како особено важни ризик-фактори кај деца и адолесценти со хронична бубрежна болест.

Клучни зборови: хронична бубрежна болест, ниски родилни параметри, пушење, дебелина, хиперурикемија

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MUC1 IMMUNOTHERAPY AGAINST A METASTATIC MAMMARY ADENOCARCINOMA MODEL: IMPORTANCE OF IFN-GAMMA

Catherine J. Lees[1], Nechama Smorodinsky[2], Galit Horn[2], Daniel H. Wreschner[2], Ian F.C. McKenzie[3], Geoffrey Pietersz[4, 5, 6], Lily Stojanovska[7], Vasso Apostolopoulos[7,*]

[1] Current Address, Echuca Regional Health, Echuca VIC Australia
[2] Department of Cell Research and Immunology, Tel Aviv University, Tel Aviv, Israel
[3] Current Address, Emeritus Professor, University of Melbourne, VIC Australia
[4] Bio-organic and Medicinal Chemistry Laboratory, Burnet Institute, VIC Australia
[5] Department of Pathology, University of Melbourne, Parkville, Victoria, Australia
[6] Department of Immunology, Monash University, Melbourne, Victoria, Australia
[7] Centre for Chronic Disease, College of Health and Biomedicine, Victoria University, VIC Australia

***** Corresponding Author: Vasso Apostolopoulos, Centre for Chronic Disease, College of Health and Biomedicine, Victoria University, VIC Australia. Tel: +613 99192025; Fax: +613 99 19 45 65
E-mail: vasso.apostolopoulos@vu.edu.au

Abstract

Immunotherapy using mucin 1 (MUC1) linked to oxidised mannan (MFP) was investigated in an aggressive MUC1$^+$ metastatic tumour, DA3-MUC1 because, unlike many MUC1$^+$ tumour models, DA3-MUC1 is not spontaneously rejected in mice making it an alternative model for immunotherapy studies. Further, DA3-MUC1 cells are resistant to lysis by anti-MUC1 cytotoxic T cells (CTLs). The inability of DA3-MUC1 tumours to be rejected in naïve mice as well as vaccination to MUC1 was attributed to a deficiency of expression of MHC class I molecules on the tumour cell surface. *In vitro* and *in vivo* analysis of subcutaneous tumours and lung metastases demonstrated that DA3-MUC1 tumour cells have a low expression (< 6%) of MHC class I which can be upregulated (> 90%) following culturing with IFN-γ. Results from flow cytometry analysis and immunoperoxidase staining indicated that the *in vitro* up-regulation of MHC class I could be maintained for up to seven days *in vivo*, without affecting the expression levels of MUC1 antigen. Interestingly, MUC1-specific CTL that lyse DA3-MUC1 targets *in vitro* were induced in MFP immunised mice but failed to protect mice from a DA3-MUC1 tumour challenge. These results highlight the importance of MHC class I molecules in the induction of anti-tumour immunity and the MFP immune response.

Keywords: MUC1. MHC class I, interferon-gamma, tumour, immunotherapy

Introduction

Anti-tumour immunity and tumour eradication are induced by cell-mediated immune responses [1, 2]. The activation of tumour-specific CD8$^+$ T lymphocytes and their subsequent differentiation into cytolytic cells is dependent on 2 signals from the antigen-presenting cell. One signal is provided through the interaction of the antigenic peptide (from the tumour) presented on the major histocompatibility complex (MHC) to T cells. The other is the costimulatory signals, efficiently provided by B7 [B7-1 (CD80) and B7-2 (CD86)] binding to CD28 (CD152 or CTLA-4) on T cells [3, 4]. However, malignant cells have evolved mechanisms enabling them to successfully evade the immune system, which in many cases directly affects this 2 signal process. These mechanisms

include inadequate expression of costimulatory molecules, Fas ligand, or adhesion molecules on cancer cells, antigen processing defects, the secretion of inhibitory molecules into the tumour microenvironment or absent or poorly expressed MHC molecules on the tumour cell surface [5–7]. More recently, it has been demonstrated that tumour cells have additional escape mechanisms by expressing PD-L1 (B7-H1, CD275) and/or PD-L2 on their surface, which upon binding to its ligand, PD-1, expressed by activated $CD8^+$ T cells leads to apoptosis of T cells [8].

The failure of tumours to adequately process antigens and present peptide fragments to T cells is greatly attributed to reduced expression of MHC class I molecules on the cell surface of tumour cells [5, 6, 9, 10]. In many tumour models however, this can be rectified transfecting tumour cells with MHC class I gene [11, 12]. Another approach is to transfect cytokine cDNA, in particular IFN-γ, into tumours as it directly causes an up-regulation of cell surface MHC class I expression [11, 13].

This study characterises the DA3-MUC1 metastatic tumour following the failure of mannan-MUC1 (MFP) immunisations to induce anti-tumour immunity in this $MUC1^+$ cancer model. It was demonstrated that DA3-MUC1 was non-immunogenic due to an absence of MHC class I expression on the tumour cell surface, which could be upregulated by IFN-γ but not sustained long enough *in vivo* to cause tumour eradication.

Materials and methods

Mice and immunisations

A MUC1-GST fusion protein containing 5 variable number of tandem repeat (VNTR) regions from the extracellular protein core of MUC1 [14] was produced in a bacterial expression system (pGEX-3X) and conjugated to oxidised mannan to form MFP as described previously [15–23]. BALB/c mice aged 6–10 weeks were given three intraperitoneal immunisations (on days 0, 7 and 14) with either MFP (containing 5μg of MUC1 fusion protein) or a control pH 9.0 phosphate buffer. BALB/c mice immunised with mannan coupled to oxidised GST (M-GST) were included as controls in the lung metastases study. All experiments were approved by the Austin Animal Ethics Committee.

Cell lines

DA3-MUC1 is a metastatic BALB/c DA3 mammary cell line transfected with the cDNA of the transmembrane form of human MUC1 [24, 25]; P815-MUC-1, a DBA/2 P815 mastocytoma cell lines transfected with the cDNA of the transmembrane form of human MUC1 [26, 27] were cultured in RPMI and MUC1 expression selected for every 14–20 days with 1.25 mg/ml G418-sulfate (Gibco BRL, U.S.A).

Flow cytometry

The expression of cell surface molecules on DA3-MUC1 were measured by flow cytometry. The following monoclonal antibodies were used; a) MUC1 (BC2: supernatant) [28], b) MHC class 1 $H2^d$ (34.1.2s, 1/1000 dilution of ascites fluid) [29], c) MHC class II I-A8 (1/500 dilution of ascites fluid), d) B7.1 (4μg) (Pharmingen, San Diego, USA), e) ICAM-2 (1μg) (Pharmingen), f) CD28 (4μg) (Pharmingen), g) LFA-2 (1μg) (Pharmingen) and h) CTLA-4 (1μg) (Pharmingen). DA3-MUC1 tumour cells were prepared for FACS analysis by either a) culturing in growth media, b) culturing with 20 ng/ml vaccinia virus-IFN-γ [22] for 72 h, or c) culturing with 20 ng/ml IFN-γ for 72 h and then removing IFN-γ for subsequent culturing. In preparation for flow cytometry, tumour cells ($2–5 \times 10^5$ cells/ml) were incubated with the specified antibodies for 45 min at 4^0C, washed with phosphate buffer and incubated with either FITC-conjugated sheep $(Fab')_2$ anti-mouse, anti-rat or anti-hamster immunoglobulin (Amersham, UK) (1/50 dilution) for a further 45 min at 4^0C. Cells were washed and analysed by flow cytometry.

Immunoperoxidase staining of DA3-MUC1 tumour cells

Cell surface expression of MUC1 and MHC class I proteins on DA3-MUC1 tumour cells *in vivo* were analysed by immunoperoxidase staining. DA3-MUC1 tumour cells were either, a) injected subcutaneously into BALB/c

mice and grown for > 30 days to establish lung metastasis. Mice were culled and samples taken from both the subcutaneous tumour site and from lung metastasis; or b) cultured with 20 ng/ml IFN-γ for 72 h and injected subcutaneously into BALB/c mice. Mice were culled and samples taken from the subcutaneous tumour site on days 4 and 7 and from lung metastasis > 30 days after injection.

All tissue samples were snap frozen in isopentane and sections 5–6 μm thick were cut using a Microm HM500 cryostat (MICROM Laborgerate, Strässe, Germany), mounted and fixed on silane coated slides [30]. Endogenous peroxidase activity was blocked by incubating with 0.5% H_2O_2 for 40 minutes at room temperature. Tissue sections were incubated for 45 min at 4^0C with biotinylated BC2 to detect MUC1 expression or biotinylated anti-$H2^d$ (1/1000 dilution) to detect MHC class I expression. Excess antibodies were removed by thorough washing and samples incubated with streptavidin-HRP conjugate (Amersham, UK) (1/50 dilution) for a further 45 min at 4^0C. Antibody binding was detected with 1.5 mg/ml 3–3 diaminobenzidine (DAB, Sigma, St. Louis, USA) in phosphate buffered saline containing 0.5% H_2O_2 for 5 min, slides were washed and mounted.

Tumour model

The immunogenicity of DA3-MUC1 tumour was characterised using the following tumour growth experiments and MFP immunisations.

(i) BALB/c mice (x 10) were subcutaneously injected with 5 x 10^6 DA3-MUC1 tumour cells and tumour growth measured with electronic callipers every week for 10 weeks to establish a DA3-MUC1 growth curve. Mice were sacrificed > 30 days after the tumour challenge and lung metastasis determined by microscopically counting the number of metastasis present in random cross sections of similar sizes from formalin fixed lung samples (Anatomical Pathology Unit, Austin and Repatriation Medical Centre, VIC Australia).

(ii) BALB/c mice (x 20 per group) were immunised 3 times on days 0, 7 and 14 with either MFP (5 μg) or M-GST (5 μg) and challenged with 5 x 10^6 subcutaneous DA3-MUC1 tumour cells. A minimum of 4 mice from each group were sacrificed each week for five weeks and the number of metastatic lesions present on each lung determined microscopically.

(iii) BALB/c mice (x 10 per group) were injected subcutaneously with 5 x 10^6 DA3-MUC1 tumour cells until tumours of ~50 mm^2 were established (Day 17). Mice were then immunised intraperitoneally on days 17, 19 and 21 with 5 μg MFP. Tumour sizes were measured every 2–3 days for 30 days using electronic callipers.

(iv) BALB/c mice (x 10 per group) were immunised intraperitoneally on days 0, 7 and 14 with either 5 μg MFP or pH 9.0 buffer, and challenged subcutaneously on day 21 with 3 x 10^6 DA3-MUC1 tumour cells. Prior to challenge, the tumour cells were cultured with 20 ng/ml vaccinia virus-IFNγ supernatant (UV inactivated) [22, 23, 25, 26] for 72 h to increase cell surface MHC class I expression. Tumour growth was measured every 2–3 days for 2 weeks using electronic callipers.

Cytotoxic T cell ^{51}Cr release assay

BALB/c mice immunised (x 3) with MFP (5 μg) were culled and their spleen cells collected and treated with 0.83% NH_4Cl. Two-fold serial dilutions of effector spleen cells from the immunised mice were plated into a 96 well plate beginning at a concentration of 1 x 10^6 cells per well in duplicate. 1 x 10^4 ^{51}Cr labelled DA3-MUC1 cells cultured with IFN-γ (20 ng/ml) for 72 h, DA3-MUC1, P815-MUC1 or P815 target cells were added to the effectors. The spontaneous release of ^{51}Cr from the labelled cells was determined by incubating target cells in RPMIM media and the maximum release was determined by incubation with 10% SDS (BDH Chemicals, Dorset, England). Cultures were incubated for 4 h before transferring 100 μl of supernatant to 96 well flat Optiplates (Disposable Products, Australia) containing 100 μl of Microscint 40 (Packard, USA) for analysis on the microplate scintillation counter (Packard USA). The specific percentage lysis of target cells was determined by; [(experimental-spontaneous) cpm / (maximum-spontaneous) cpm] x 100%.

Results

Immunisation which does not protect against DA3-MUC1 tumour growth

To examine the anti-tumour effects of MFP immunisations on the DA3-MUC1 tumour *in vivo*, 2 immunotherapy models were used.

In the first model, BALB/c mice with an established DA3-MUC1 tumour (~50 mm^2) were immunised 3 times (days 17, 19 and 21) with either MFP or control pH 9.0 buffer, and, tumour growth and lung metastases measured for 30 days. Unlike other tumour models [30] in the DA3-MUC1 model, there was no difference in tumour growth (Figure 1A) or the number of lung metastases (as determine by lung weight) (data not shown). Therefore, therapy with MFP was not effective at treating established DA3-MUC1 tumours.

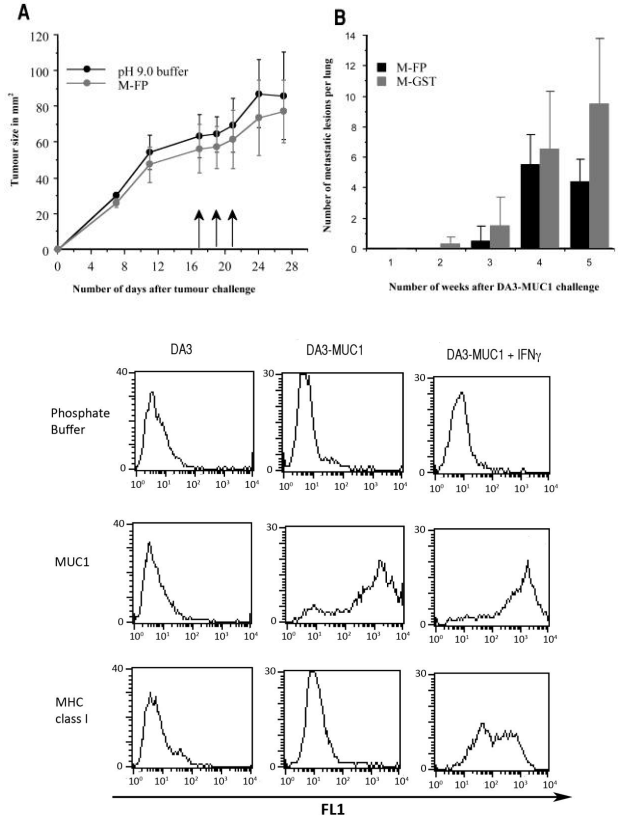

Figure 1. A – Subcutaneous DA3-MUC1 (5 x 10^6 cells/mouse) tumour growth in BALB/c mice immunised with MFP. Mice with an established 17 day tumour were immunised intraperitoneally on days 17, 19 and 21 with 5 μg MFP or pH 9.0 buffer and tumour growth measured. B. Lung metastases in BALB/c mice immunised with either MFP or a control M-GST 3 times (days 0, 7 and 14), then challenged with DA3-MUC1 (5 x 10^6 cells/mouse). 4–5 mice were culled each week for 5 weeks and microscopic lung metastasis counted. Data is presented as mean +/- standard error of the mean

In the second model, BALB/c mice were immunised 3 times with either MFP or a control preparation, M-GST, and challenged with 5 x 10^6 DA3-MUC1 tumour cells subcutaneously. Metastatic lung nodules from 4–6 mice per week were examined microscopically for five weeks (Figure 1B). Immunisation (prophylactic model) with MFP did not protect mice challenged with DA3-MUC1 from developing lung metastases as assessed by the number of lung metastases per lung compared to immunised control mice.

From these studies, it was concluded that immunisation with MFP could not induce tumour protection in mice challenged with DA3-MUC1 tumour cells, nor could it offer protection against an established DA3-MUC1 tumour. These results were in contrast to findings in all other $MUC1^+$ tumour models investigated, where immunisation with MFP was able to successfully induce anti-tumour immunity and tumour protection *in vivo* [14–23, 26, 31]. It was hypothesised that DA3-MUC1 tumours were not immunogenic due to a decrease in either costimulatory or MHC molecules on their surface. To test these hypotheses, the DA3-MUC1 tumour was characterised for cell surface molecule expression *in vitro* and *in vivo*.

DA3-MUC1 tumour cells express high levels of MUC1 but do not express MHC class I

***In vitro* characterisation of DA3-MUC1.** The DA3-MUC1 metastatic cell line was analysed for expression of human MUC1, MHC class I and other cell surface markers by flow cytometry (Table 1 and Figure 2). MUC1 is highly expressed on the surface (> 85%) of DA3-MUC1 cells compared to < 2% on non-transfected parental DA3 cells. In contrast, MHC class I expression was considerably decreased in both DA3-MUC1 cells (6%) and non transfected DA3 cells (< 3%). There was no detectable MHC class II, B7.1, ICAM-2, CD28, LFA-2 or CTLA-4 on DA3 or DA3-MUC1 tumour cells (Table 1). Phosphate buffer was used as a control for non-specific $(Fab')_2$ FITC-conjugate binding .

Table 1

In vitro expression of cell surface markers on DA3 and DA3-MUC1 tumour cells cultured with or without 20 ng/ml IFN-γ for 72 h. Values represent the percentage of cells positive for each antibody determined by flow cytometry

Cell Surface Markers	DA3 (%)	DA3-MUC1 (%)	DA3-MUC1+IFN-γ (%)
negative control	1.94	3.12	2.71
MUC1	3.36	87.73	93.6
MHC class I	2.98	5.67	76.85
MHC class II	3.81	3.72	2.01
B7.1	1.49	3.03	1.67
ICAM 2	3.70	4.42	2.75
CD28	4.13	5.22	4.03
LFA-2	2.27	3.09	2.12
CTLA-4	4.81	6.09	3.70

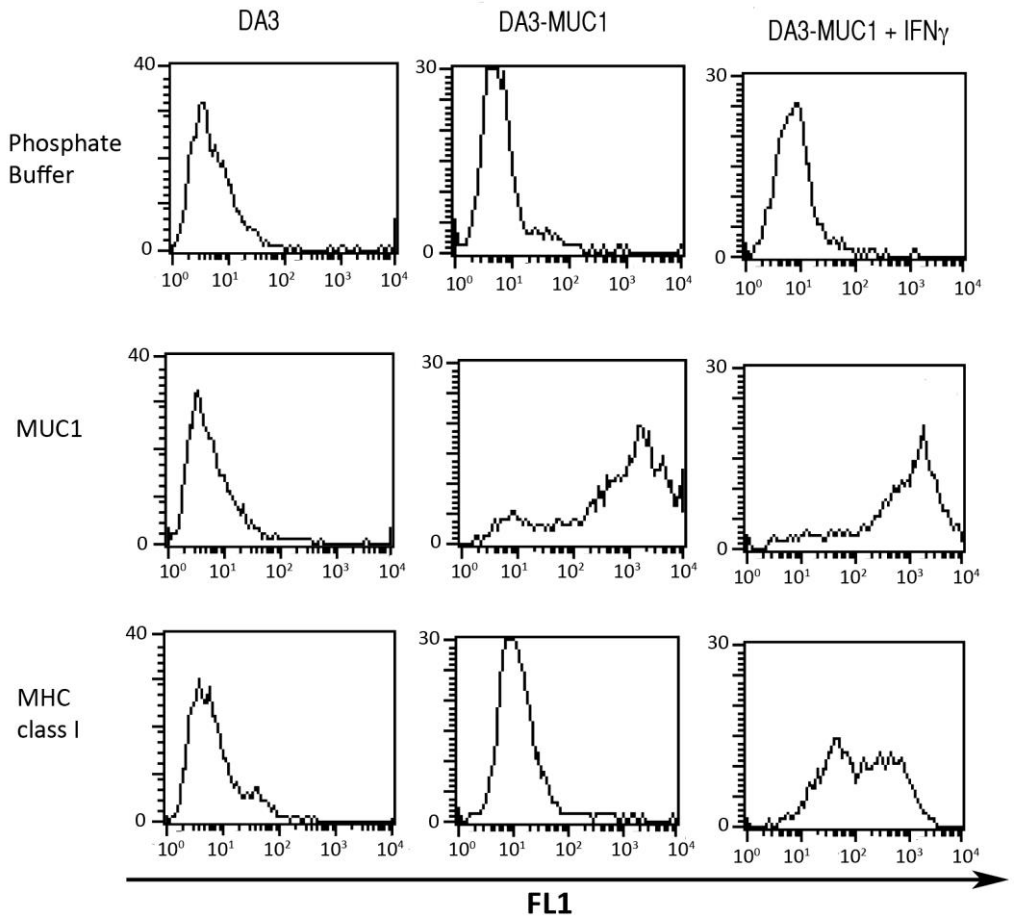

Figure 2 – Flow cytometric analysis of in vitro expression of cell surface MUC1 and MHC class I on DA3-MUC1 cells cultured with or without 20 ng/ml IFNγ for 72 h. The non transfected parental cell line, DA3 was used as a control. Phosphate buffer represents negative control binding of FITC-conjugated sheep $(Fab')_2$ anti-mouse (1/50) to the tumour cell lines

***In vivo* characterisation of DA3-MUC1.** To characterise the DA3-MUC1 tumour *in vivo*, BALB/c mice were challenged with 5 x 10^6 metastatic cells and tumour growth monitored for 10 weeks (Figure 3A). Mice had palpable subcutaneous tumours after 2–3 weeks and were culled after 10 weeks and their subcutaneous tumours and lungs removed for tumour analysis.

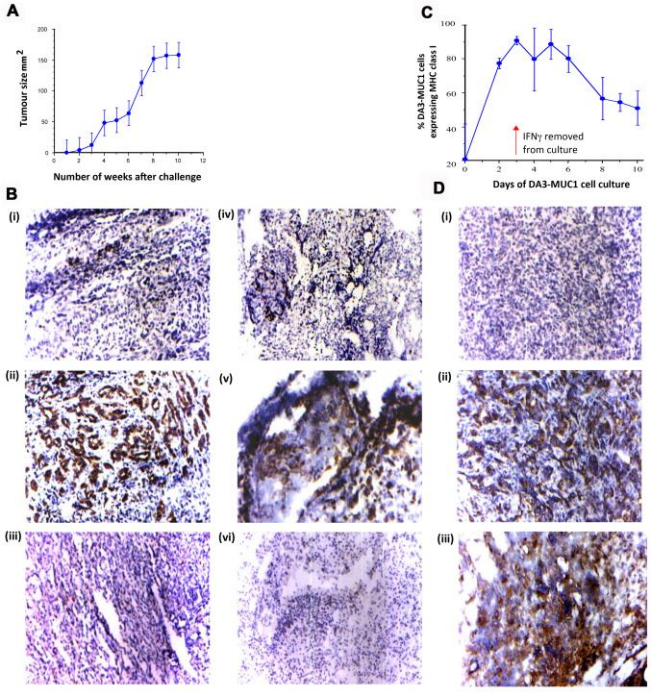

Figure 3ABCD – Tumour growth in BALB/c mice challenged with 5 x 10^6 DA3-MUC1 cells. ***B.*** *MUC1 and class I ($H2^d$) surface expression on DA3-MUC1 tumour cells determined by immunoperoxidase staining; data is presented as mean +/- standard error of the mean. DA3-MUC1 tumour cells from the site of a BALB/c subcutaneous tumour were stained for,* ***B(i)*** *negative control,* ***B(ii)*** *MUC1 expression using biotinylated-BC2 and* ***B(iii)*** *MHC class I expression using biotinylated-34.1.2s. DA3-MUC1 lung metastases were stained for,* ***B(iv)*** *negative control,* ***B(v)*** *MUC1 expression using biotinylated-BC2 and* ***B(vi)*** *MHC class I expression using biotinylated-34.1.2s. All images are shown at 200x magnification. Non-specific binding of $(Fab')_2$ conjugate was blocked with 10% BSA in DME and control samples were incubated with 10% BSA in DME.* ***C.*** *FACS analysis determining the length of time MHC class I expression remained elevated on DA3-MUC1 cells cultured with IFN-γ in vitro. Tumour cells were cultured for 72 h with 20 ng/ml IFN-γ to increase MHC class I expression, IFN-γ removed from culture and MHC class I expression measured daily.* ***D.*** *Immunoperoxidase staining of DA3-MUC1 cells pre-cultured with IFN-γ and grown in vivo. Mice were sacrificed every three days for 10 days, tumours removed, formalin fixed and stained for,* ***D(i)*** *negative control,* ***D(ii)*** *MUC1 expression using biotinylated BC2 and* ***D(iii)*** *MHC class I expression using anti-$H2^d$. Phosphate buffer was used as a negative control – binding of FITC-conjugated sheep $(Fab')_2$ anti-mouse (1/50) to the tumour cells*

As expected, immunoperoxidase staining for MUC1 and MHC class I expression on subcutaneous established DA3-MUC1 tumours were similar to that observed *in vitro* studies. DA3-MUC1 tumour cells express high levels of MUC1 (75–100% of cells) (Figures 3B) and very little, if any, MHC class I (0–15% of cells) (Figure 3B i-iii) on their cell surface. Lung metastases (macroscopic and microscopic) were observed 30–35 days after a subcutaneous injection of 5 x 10^6 DA3-MUC1 cells, however there was no evidence of metastases to the liver (data not shown). Immunoperoxidase staining for MUC1 and MHC class I expression on lung metastases showed MUC1 expression on ~ 50% of tumour cells in the lung but no evidence of MHC class I expression (Figure 3B iv-vi).

Elevation of MHC class I expression on DA3-MUC1 with IFN-γ

It is clear that one of the factors hindering the immunogenicity of the DA3-MUC1 tumour was a decrease in the expression of MHC class I. Numerous studies have shown that MHC class I expression, and therefore tumour immunogencity, can be increased by culturing the tumour with recombinant IFN-γ. To determine whether MHC class I expression could be up-regulated on DA3-MUC1 tumour cells, cells were cultured with 20 ng/ml vaccinia virus-IFN-γ for 72 h and MHC class I expression determined using flow cytometry.

DA3-MUC1 expression of MHC class I molecules on the tumour surface could be greatly increased *in vitro* by culturing the DA3-MUC1 cells with IFN-γ (Table 1 and Figure 2).

Prior to *in vitro* culturing with IFN-γ, only 6% of DA3-MUC1 cells expressed MHC class I on their cell surface (Figure 2), however, after culturing cells with IFN-γ for 72 h, 77% of DA3-MUC1 tumour cells expressed MHC class I (Figure 2) and MUC1 expression still remained high (Figure 2).

To determine the length of time MHC class I expression remained elevated on DA3-MUC1, tumour cells were cultured with IFN-γ for 72 h, removed, and then examined daily for MHC class I expression (Figure 3C). MHC class I expression at 72 h (~ 90%) remained elevated (> 70%) for 3 days after the removal of the cytokine, after which time the expression dropped constantly to plateau at ~ 55% by day 10.

To ensure the elevated class I levels observed *in vitro* could be sustained *in vivo*, DA3-MUC1 cells were cultured with IFN-γ and injected subcutaneously into BALB/c mice. Tumours were examined on days 4 and 7 for MHC class I expression by immunoperoxidase staining (Figure 3D). DA3-MUC1 cells cultured with IFN-γ expressed high levels of MHC class I molecules on 75% of tumour cells removed from the subcutaneous site on day 4 (data not shown), with 50% of tumour cells still remaining positive on day 7 (Figure 3D i, ii). *In vivo* expression of MUC1 on the subcutaneous tumour was not altered after culturing with IFN-γ (Figure 3D iii). Thus, culturing DA3-MUC1 cells with IFN-γ increases the expression of MHC class I molecules on the cell surface for at least 7 days after removal of IFN-γ both *in vitro* and *in vivo*.

T cells from MFP immunised mice lyse IFN-γ treated DA3-MUC1 tumour cell targets

From the data so far, it would appear that the DA3-MUC1 tumour is not immunogenic as it does not express MHC class I on its surface. However, culturing DA3-MUC1 cells with IFN-γ increases MHC class I expression, both *in vitro* and *in vivo* for at least 7 days following removal of IFN-γ. We determined whether the level of increase in MHC class I on DA3-MUC1 cells was adequate to be susceptible to MUC1 specific cytotoxic T cells (CTL) lysis. BALB/c mice were therefore immunised with MFP and 7–10 days following the final immunisation, spleens were isolated and CTL were able to lyse DA3-MUC1 tumour cells (Figure 4A). Lysis of DA3-MUC1 tumour cells (treated with IFN-γ) was similar to that of P815-MUC1 tumour cells (H-$2d^+$ $MUC1^+$ MHC class I^+); non MUC1 transfected P815 cells (H-$2d^+$ $MUC1^-$ MHC class I^+) were used as a negative control. Interestingly, without culturing with IFN-γ, DA3-MUC1 were lysed by MUC1 CTL but the response was considerably weaker. Therefore, *in vitro* MUC1 T cell cytotoxicity to DA3-MUC1 tumours increases substantially with elevated MHC class I expression.

MHC class I is not sustained to induce tumour protection despite induction of CTL

As IFN-γ treated tumours express elevated levels of MHC class I, and *in vitro*, MFP can stimulate $MUC1^+$ CTL capable of lysing DA3-MUC1 target cells (Figure 4A), the anti-tumour effects of MFP immunisation on DA3-MUC1 tumours expressing MHC class I was investigated *in vivo*. Mice were immunised 3 times with either MFP or a control pH 9.0 phosphate buffer and challenged with DA3-MUC1 tumour cells pre-cultured for 72 h with IFN-γ to increase MHC class I expression. BALB/c mice were challenged with 5 x 10^6 DA3-MUC1 cells with elevated MHC class I expression (Figure 4B). Interestingly, a small reduction in tumour growth, which correlated with an increase in MHC class I expression (Figures 2 and 3), was evident between days 2 and 5 in both MFP and control pH 9.0 immunised mice (Figure 4B). A significant ($p < 0.05$) decrease in tumour burden was evident in mice immunised with MFP compared to control mice, on day 3, suggesting that elevated levels (90–95%) of MHC class I expression on DA3-MUC1 tumour cells may increase their susceptibility to CTL lysis. However, no differences in tumour size were noted between MFP and control mice on any other days, and from day 6 onwards, DA3-MUC1 tumours continued to grow steadily which corresponded to a steady drop in surface MHC class I levels (Figure 3C) as the

positive tumour cells lost MHC class I expression. Thus, elevated class I expression could not be sustained *in vivo* to induce anti-tumour CTL responses.

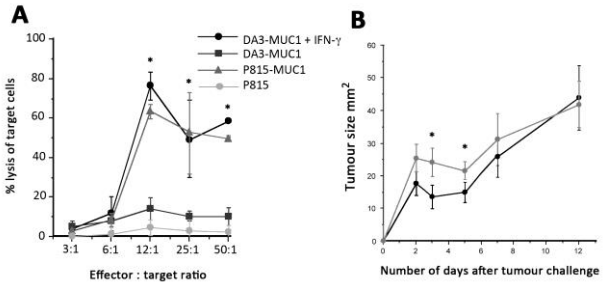

*Figure 4AB – CTL assay of spleen cells from effector BALB/c mice immunised with 5 μg MFP, on ^{51}Cr-labelled DA3-MUC1 cells cultured with IFN-γ for 72 h, DA3-MUC1, P815-MUC1 and P815 tumour cell targets. B. Subcutaneous tumour growth of DA3-MUC1 cultured with IFN-γ in BALB/c mice. Mice (x 10 per group) were immunised intraperitoneally on days 0, 7 and 14 with either 5 μg MFP (-●-) or pH 9.0 buffer (-●-) and challenged with 3 x 10^6 DA3-MUC1 tumour cells previously cultured with 20 ng/ml vaccinia virus-IFN-γ supernatant (UV inactivated) for 72 h. Data is presented as mean +/- standard error of the mean, * p < 0.05*

Discussion

Tumour immunotherapy with mannan MUC1 fusion protein (MFP) induces CD8$^+$ cellular immunity and tumour protection in several immunogenic MUC1$^+$ tumour models (MUC1$^+$ P815, MUC1$^+$ RMA, MUC1$^+$ 3T3) ([14–23, 26, 31–34]. In these models, the transfection of the tumour cell lines with human MUC1 results in the spontaneous rejection of the tumours after approximately 15–20 days [34]. Yet despite this, there is still a window of between 0 and 11 days in which to observe either accelerated rejection or an absence of tumour growth in immunised mice – the basic models with which the MFP anti-tumour immune responses have been described.

In this study, the aggressive MUC1$^+$ metastatic DA3-MUC1 tumour, was investigated as a model to study MFP immunotherapy as it is not spontaneously rejected in mice. However, in contrast to other MUC1$^+$ tumour models where MFP immunisation protected mice from a tumour challenge, DA3-MUC1 tumours grew. This resulted to determined the expression of various cell surface markers on DA3-MUC1, required to induce cell mediated immunity. Both *in vitro* and *in vivo* studies confirmed that in contrast to other MUC1$^+$ tumour models, DA3-MUC1 has a low expression of cell-surface MHC class I which results in reduced immunogenicity *in vivo*. However, treatment with IFN-γ *in vitro* upregulates MHC class I expression which can be sustained for several days in the absence of IFN-γ

Initial immunotherapy studies demonstrated that mice immunised with MFP and then challenged with DA3-MUC1 tumours were not protected from tumour growth. Similarly in a therapy experiment, 3 injections with MFP was also inadequate in decreasing tumour burden in mice with established DA3-MUC1 tumours. These findings were unlike other studies with MFP, whereby mice immunised with MFP were totally protected against a challenge of MUC1$^+$ 3T3 tumours [20, 21] and the induction of a CD8$^+$ cellular immune response caused the regression of established 15 day-old MUC1$^+$ P815 tumours in DBA/2 mice [31].

In vitro and *in vivo* characterisation of DA3-MUC1 indicated that the tumour was weakly immunogenic because even though high surface levels of MUC1 were expressed (> 85%), there were low levels of all other cell surface molecules needed for T cell activation including MHC class I (< 6%), MHC class II, CD80, ICAM-2, CD28, LFA-2 and CTLA-4. Similarly, metastatic lung nodules induced by DA3-MUC1 again demonstrated MUC1 expression to be present on 50% of metastatic cells but there was no MHC class I expression. The absence, or relatively low expression of these molecules on the tumour cell surface causes anergy in any activated T cells and is an effective mechanism many tumours have evolved to evade the immune system [7].

However, tumour immunogenicity can be successfully increased by up-regulating the expression of these molecules (particularly MHC and costimulatory molecules) by either gene transfection or culturing with cytokines – specifically IFN-γ [5, 6, 9, 12]. Therefore, to increase the expression of MHC class I on DA3-MUC1 cells, cells were cultured with IFN-γ. Culturing DA3-MUC1 with IFN-γ increased expression of MHC class I from < 16% to > 90% after 72 h. The MHC class I expres-

sion remained elevated for several days before declining to 50% one week after the cytokine was removed from culture. *In vivo* studies of MHC class I expression on DA3-MUC1 after IFN-γ culturing, revealed a similar pattern whereby levels previously not detected in a subcutaneous tumour, were elevated to 50–75% of cells expressing MHC class I four days later, and still present on day 7. Interestingly, culturing DA3-MUC1 with IFN-γ did not increase cell surface expression of MHC class II, CD80, ICAM-2, CD28, LFA-2 or CTLA-4 as had been previously reported in other tumour models [35].

Following the up-regulation of MHC class I on the surface of DA3-MUC1 tumours, MUC1 specific CTL isolated from the spleen of MFP immunised mice could lyse DA3-MUC1 tumour cells cultured with IFN-γ, but not DA3-MUC1 cells which were not cultured with IFN-γ. This result was considerably higher than untreated tumour cells, demonstrating that DA3-MUC1 immunogenicity is increased in the presence of MHC class I, and can be lysed by MUC1 restricted CTL *in vitro*.

The lack of an effective anti-tumour response in DA3-MUC1 tumours is, in part, a result of the down-regulation in MHC class I expression which can be overcome by culturing the tumour with IFN-γ. As culturing with IFN-γ only temporarily increases MHC class I expression, it is suggested that future studies in this model would focus on the transfection of the IFN-γ gene into DA3-MUC1 cells. Alternatively, the decrease in DA3-MUC1 immunogenicity may also be a result of tumour-reactive T cells receiving inadequate costimulation through the absence of the costimulatory molecules B7-1 and B7-2. This again can be over come through transfection with these molecules [36, 37] and is also suggested for future immunotherapy studies with MFP. Furthermore, we have not investigated whether other relevant receptors may not be expressed by DA3-MUC1 cells. Finally, DA3-MUC1 cells could be used as a model to study other mechanisms and lysis where MHC class I is not required, such as, NK cell lysis.

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Р е з и м е

ИМУНОТЕРАПИЈАТА MUC1 НАСПРЕМА МЕТАСТАТСКИОТ МОДЕЛ НА АДЕНОКАРЦИНОМ НА ГРАДИТЕ: ВАЖНОСТА НА IFN-ГАМА

Кетрин Ј. Лис[1], Некама Смородински[2], Галит Хорн[2], Јан Ф. К. Мекензи[3], Цефри Питерс[4,5,6], Лили Стојановска[7], Васо Апостолопулос[7]

[1] Тековна адреса, Регионално здравство на Ечука, Ечука, Викторија, Австралија
[2] Оддел за клеточно истражување и имунологија, Универзитет во Тел Авив, Тел Авив, Израел
[3] Тековна адреса, почесен професор, Универзитет во Мелбурн, Викторија, Австралија
[4] Биоорганска лабораторија и лабораторија за медицинска хемија, Институт Бурнет, Викторија, Австралија
[5] Оддел за патологија, Универзитет во Мелбурн, Парквил, Викторија, Австралија
[6] Оддел за имунологија, Универзитет Монаш, Мелбурн, Викторија, Австралија
[7] Центар за хронични болести, Школа за здравство и биомедицина, Универзитет Викторија, Викторија, Австралија

Имунотерапија која користи муцин 1 (MUC1) поврзан со оксидиран манан (MFP) беше испитувана кај агресивен метастатски тумор MUC1$^+$, DA3-MUC1, затоа што, за разлика од многу туморски модели на MUC1$^+$, DA3-MUC1 не е спонтано одбиен кај глувците, што го прави алтернативен модел за студии на имунотерапија. Исто така, клетките DA3-MUC1 се отпорни на лизирање од анти-MUC1 цитотоксични Т-клетки (CTLs). Неможноста туморите DA3-MUC1 да бидат отфрлени кај глувците како и вакцинацијата за MUC1 беше припишана на недостатокот на експресија на молекулите на MHC од класа I на површината на туморските клетки. Ин витро и ин виво анализата на поткожните тумори и метастази на белите дробови покажа дека туморските клетки DA3-MUC1 имаат ниска експресија (< 6%) на MHC класа I, кои може да се регулираат (> 90%) по култивирање со IFN-γ. Резултатите од анализата на проточната цитометрија и боењето со имунопероксидаза посочи дека ин витро нагорната регулација на MHC класа I може да се одржува до седум дена ин виво, без засегање на нивоата на експресија на MUC1 антигенот. Интересно, MUC1 специфичните CTL кои ин витро ги лизираат DA3-MUC1 целите беа воведени кај вакцинирани глувци од MFP, но не успеаја да ги заштитат глувците од туморскиот предизвик на DA3-MUC1. Овие резултати ја нагласуваат важноста на молекулите на MHC од класа I во поттикнувањето на антитуморскиот имунитет и имуниот одговор на MFP.

Клучни зборови: MUC1, MHC класа I, интерферон-гама, тумор, имунотерапија

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BIOSIMILAR MEDICAL PRODUCTS – LICENSING, PHARMACOVIGILANCE AND INTERCHANGEABILITY

Aleksandra Grozdanova, Katerina Ancevska Netkovska, Zoran Sterjev, Zorica Naumovska, Rubin Zarevski, Aleksandar Dimovski, Ljubica Suturkova

Pharmaceutical Chemistry Institute, Faculty of Pharmacy, Ss. Cyril and Methodius University, Skopje, R. Macedonia

Corresponding Author: Aleksandra Grozdanova, Faculty of Pharmacy, Ss. Cyril and Methodius University, Skopje, Majka Tereza 47, 1000 Skopje, R. Macedonia; Tel: +38975338786; E-mail: agrozdanova@ff.ukim.edu.mk

Abstract

The use of biological medicine has significantly increased in recent decades and has made substantial contributions to improving the effectiveness of therapies in many diseases. The expiration of patents of biological innovative medicines enables copies of those drugs called similar biological products (biosimilars) to be approved by regulatory authorities and to enter in clinical use. Biosimilars are comparable but not identical and are not a generic version of the innovator biological product. Although biosimilars undergo rigorous characterization as well as clinical studies to prove their safety and effectiveness, specific regulatory requirements for registration apply in the case of biosimilars. They are highly complex molecules and small changes in the production process can have major implications in its safety and effectiveness profile. The availability of biosimilars enhances competition, with the potential to improve patient access to biological medicines and to contribute to the financial sustainability of healthcare systems. In order to be certain that a biosimilar reaches its potential in clinical use, an intensive pharmacovigilance monitoring system must be established in order to prove the true similarity between the original biologic and its biosimilar. There is a need for further guidance and resolution of the ongoing discussions on biosimilar labelling, naming, pharmacovigilance and substitution in order to ensure effective and appropriate use of biosimilars in clinical practice.

Keywords: Regulatory, biosimilars, biologics, licensing, interchangeability, pharmacovigilance, EMA

Background

Biological medicinal products (biologicals) are produced by or derived from living organisms, most often using genetically modified eukaryotic or prokaryotic cell lines. These medicines, often referred to as "biological drug" or "biologicals" are therapeutic or supplementary proteins such as hormones, hematopoietic growth factors, monoclonal antibodies, blood products, immunological medicinal products like sera and vaccines, or advanced technology products such as DNA molecules, genes and cell therapy products. In terms of size and complexity, these molecules are often more than thousand times bigger than chemical medicines, and more complex since they are composed of a string of amino acids, with subunits, and alpha-helix and beta-sheets typically forming the primary, secondary, tertiary or quarterly structure, with loops, pockets and crevices which are critical for their functionality [1].

The production of biologicals is a complex process which involves steps of gene manipulation, fermentation and purification and requires a very high level of technical expertise, sensitivity and precisely controlled condi-

tions for achieving manufacturing consistency in order to guarantee the safety and efficacy of the final product. Still, because of the posttranslational modification such as glycosylation, oxidation and deamination, the final product can be present in different subtitle batch to batch variants which can have high impact on the functionality of the molecule. Process related impurities such as host cell proteins, DNA or endotoxin have to be controlled and to be in the range of safe limit. Therefore, unlike generics where an exact copy can be made, in the case of production of biological medicinal products – "the process defines the product". Beside the manufacturing process and their molecular size and complexity, biologicals differ from the small molecule medicines in their characterrization, stability, variability and immunogenicity.

Since biologicals have high structural variability that can be very subtle, the currently available analytical techniques are not always sufficient enough to fully characterize them. Though every batch in part has product quality differences, the variations from batch to batch need to be monitored to ensure conformance within an allowed range. Still the product even in its purest form is associated with a number of post translational modifications of the product variants. Thorough characterization of these post translational modifications and other structural variations is scientifically challenging [2]. The product quality differences between batches need to be exactly defined in a regulatory application and correlation to clinical trial results needs to be drawn, to avoid any safety issues. Therefore, "one size fits all" approach of the regulatory review of a small molecule does not fit a biological drug and the variability of biologicals is tightly controlled by manufacturers and regulatory authorities and must remain within defined and accepted limits [3].

Due to the nature of the active substance, biological medicines can be recognized by the human body as "foreign" and can have the potential to induce unwanted immune reactions. In a study from 2007, it was shown that recombinant human insulin was less immunogenic than porcine insulin, but still in 44% of the diabetes patients there was an increased titer of antibodies towards recombinant human insulin [4]. Immunogenicity is a key safety concern, and is assessed during the development and production of any biological medicinal product, supported in clinical trials by extensive testing and characterization.

What are biosimilars?

Most of the innovator biological medicine is facing expiration of patents and other data protection and has already lost or will lose its exclusivity in the coming years. This enables copies of those drugs called "similar biological medicinal product" (biosimilars) to be approved by regulatory authorities and thus to enter into clinical use. In general, biosimilars are defined as biological compounds that are highly similar to their already authorized reference biological products, with no clinically meaningful differences in safety, purity and potency. "Biosimilarity" is the regulatory term used first in the European Union (EU) and the European Medical Agency (EMA) to denote the comparability between a biosimilar and its reference medicinal product. Biosimilars are legally approved subsequent versions of innovator biopharmaceutical products following patent and exclusivity expiry. The term "biosimilar" is not a consensus by regulatory agencies and each adopts their own term and definition (Table 1). Despite the existence of slight differences in the guidelines, reference product characteristics and data required for approval among different regions, the basic principles for regulatory requirements are very similar bearing in essence the same meaning world over. However, the definition and nomenclature of biosimilars differs among the various regulatory agencies across the world. For example, they are known as similar biological medicinal products or biosimilars by the EMA [5] and Korean Food and Drug Administration (KFDA), as follow-on protein products or follow-on biologics by the United States of America (USA) Food and Drug Administration (FDA) [6], as similar biotherapeutic product in the World Health Organization (WHO) [7] and as subsequent entry biologics by Health Canada [8].

Table 1

Names and definitions of biosimilars according to different regulatory agencies

Agency	Naming	Definition
EMA/EU	Biosimilar	A biological medicinal product that contains a version of the active substance of an already authorized original biological medicinal product (reference medicinal product) in the EEA. Similarity to the reference medicinal product in terms of quality characteristics, biological activity, safety and efficacy based on a comprehensive comparability exercise needs to be established.
FDA/USA	Follow-on Biologic or Follow-on protein products	A biological product that is highly similar to an U.S.-licensed reference biological product notwithstanding minor differences in clinically inactive components, and for which there are no clinically meaningful differences between the biological product and the reference product in terms of the safety, purity, and potency of the product.
WHO	Similar biotherapeutic product	A biotherapeutic product which is similar in terms of quality, safety, and efficacy to an already licensed reference biotherapeutic product.
KFDA/Korea	Biosimilar	A biological product which demonstrated its equivalence to an already approved reference product with regard to quality, safety, and efficacy.
Health Canada/ Canada	Subsequent entry biologic	A biologic product that is similar to and would enter the market subsequent to an approved innovator biologic product.

Due to these scaled down market authorization requirements for biosimilars, it is expected that pharmaceutical companies can produce biosimilars at a lower cost while ensuring their quality, safety and efficacy. Licensing requirements for biosimilars do not include all elements of a complete dossier for the approval of a new medicine, but are still more demanding than the requirements for the approval of generics [9]. Because of the complexity of their active substance and the specificity of the production process, biosimilars cannot be produce with exactly the same characteristics as the reference medicine. Therefore biosimilars undergo rigorous characterization, preclinical and clinical comparability studies to prove their similarity, safety and effectiveness profile [3]. The extensive product approval process in the EU establishes the therapeutic equivalence and interchangeability of a biosimilar for the innovator biological product and requests additional post-marketing surveillance of biosimilars. Still, substitution of innovator product with biosimilars is not addressed and has been left to be decided by the national authority level by each country. The aim of this review is to provide an overview of the regulatory aspects for licensing of biosimilars in the EU, outlining some key issues for biosimilars like naming, interchangeability, substitution, pharmacovigilance and their impact in the future clinical practice.

Licensing of biosimilars in the EU

The EU is the leader in establishing a regulatory framework for marketing authorization of biosimilars. The EMA and the European Commission were the first to implement a well-documented legal pathway for the approval of biosimilar products that are different from the generic pathway. Specific data requirements in terms of the analytical, preclinical, and clinical data have been specified in the guidelines for biosimilars, which are more detailed than those for generics. If we compare the requirements for biosimilar licensing pathway with regards to the generic (small molecule) and innovator biological products the differences are that for the generic ones the data are for quality, purity and stability, and for the new biologic medicine, beside those three, there are the data for potency, immunogenicity, stability and full preclinical and clinical studies, while for biosimilars there is an extra comparability module and post-marketing monitoring, while the

preclinical and clinical data are abbreviated. After the initial wave of patent expiration of innovator biological drugs, the EMA in 2005 released the first biosimilar guidelines [10], and the approval for the first biosimilar (somatropin) came in 2006. The requirements for marketing authorization of biosimilars were defined in specific guidance for biosimilars, based on the principle of comparative quality and clinical pharmacokinetic and pharmacodynamic studies, nonclinical and clinical studies, and limited toxicology studies, as well as comparative clinical efficacy and tolerability studies. EMA first released general guidelines for quality, nonclinical and clinical issues which addressed the quality, consistency, the manufacturing process, safety, and efficacy considerations. This was followed by more detailed product-specific guidelines, by EMA/Committee for Medicinal Products for Human Use (CHMP), for products like erythropoietin, growth hormone, granulocyte colony stimulating factor (G-CSF), insulin, interferon beta, low-molecular weight heparins, and monoclonal antibodies. Additional guidance and recommendations followed to ensure the safety and efficacy of biosimilar products [11–13]. The list of some of the main guidelines can be found in Table 2 and Table 3 (available at the EMA web site under the section of scientific guidelines, at http://www.ema.europa.eu/docs/en_GB/document library/Scientific_guideline)

Table 2

Overarching EMA guidelines relevant to biosimilar development and approval

EMA/ CHMP/437/04 Rev. 1: Guideline on similar biological medicinal products, October 2014
EMA/CHMP/BWP/247713/2012: Guideline on similar biological medicinal products containing biotechnology-derived proteins as active substance: quality issues. June 2014
EMEA/CHMP/BMWP/42832/2005 Rev1: Guideline on similar biological medicinal products containing biotechnology-derived proteins as active substance: non-clinical and clinical issues. January 2015
EMEA/CHMP/BMWP/101695/2006: Guideline on comparability of biotechnology-derived medicinal products after a change in the manufacturing process - non-clinical and clinical issues. July 2007
EMA/CHMP/BMWP/86289/2010: Guideline on immunogenicity assessment of monoclonal antibodies intended for in vivo clinical use. June 2012
EMEA/CHMP/BMWP/14327/2006: Guideline on immunogenicity assessment of biotechnology-derived therapeutic proteins. January 2008

Table 3

Product-specific EMA guidelines relevant to biosimilar development and approval

CHMP/BMWP/671292/2010 Guideline on similar biological medicinal products containing recombinant follicle-stimulating hormone. March 2013
CHMP/BMWP/652000/20100 Guideline on similar biological medicinal products containing interferon beta. March 2013
EMA/CHMP/BMWP/403543/2010 Guideline on similar biological medicinal products containing monoclonal antibodies: non-clinical and clinical issues. June 2012
EMEA/CHMP/BMWP/301636/08 Guideline on similar biological medicinal products containing recombinant erythropoietins. April 2010
EMEA/CHMP/BMWP/118264/2007 Guideline on similar biological medicinal products containing low-molecular-weight heparins. April 2009
EMEA/CHMP/BMWP/102046/2006 Guideline on non-clinical and clinical development of similar medicinal products containing recombinant interferon alpha. June 2009

The EU guidelines for biosimilars to date stand as one of the most stringent regulatory norms for biosimilar product development. Until several years ago, the production of biosimilars was mainly directed towards the development of simple protein therapeutics like granulocyte colony stimulating factor, erythropoietin, somatropins, and follitropins. However, recently, in 2013 two biosimilar monoclonal antibodies of infliximab have been granted marketing

authorization, indicating that complex molecules are starting to gain biosimilar status in the EU. The EMA has the longest list of approved biosimilars after the implementation of biosimilar guidelines. At the moment in the EU/EMA there are current market authorization for 19 biosimilars, in 6 classes as follow: one insulin, five epoetins, two follitropin alfa, one somatropin, seven filgrastim and two monoclonal antibodies (Table 4).

Table 4

EMA-approved biosimilars (October, 2015)

	Product Name	**Active Substance**	**Marketing Authorization Holder**	**Authorization date**
1	Abasaglar (Abasria)	insulin glargine	Eli Lilly Regional Operations GmbH	09/09/2014
2	Epoetin Alfa Hexal	epoetin alfa	Hexal AG	28/08/2007
3	Binocrit	epoetin alfa	Sandoz GmbH	28/08/2007
4	Abseamed	epoetin alfa	Medice Arzneimittel Pütter GmbH & Co.KG	28/08/2007
5	Silapo	epoetin zeta	Stada Arzneimittel AG	18/12/2007
6	Retacrit	epoetin zeta	Hospira UK Limited	18/12/2007
7	Ovaleap	follitropin alfa	Teva Pharma B.V.	27/09/2013
8	Bemfola	follitropin alfa	Finox Biotech AG	27/03/2014
9	Omnitrope	somatropin	Sandoz GmbH	12/04/2006
10	Tevagrastim	filgrastim	Teva GmbH	15/09/2008
11	Ratiograstim	filgrastim	Ratiopharm GmbH	15/09/2008
12	Biograstim	filgrastim	AbZ-Pharma GmbH	15/09/2008
13	Zarzio	filgrastim	Sandoz GmbH	06/02/2009
14	Filgrastim Hexal	filgrastim	Hexal AG	06/02/2009
15	Nivestim	filgrastim	Hospira UK Ltd.	08/06/2010
16	Grastofil	filgrastim	Apotex Europe BV	18/10/2013
17	Accofil	filgrastim	Accord Healthcare Ltd	18/09/2014
18	Remsima	infliximab	Celltrion Healthcare Hungary Kft.	10/09/2013
19	Inflectra	infliximab	Hospira UK Limited	10/09/2013

The EU is the leading biosimilar market, which is evident from the number of approved drugs, market size, launched guidelines, and other regulatory aspects. The EMA requirements to approve biosimilars vary according to the class of molecule, and decisions occur case by case. The EMA requirements for comprehensive comparability studies between the biosimilar and reference products are elaborately defined in the EMA guidelines. The EMA guidelines are some of the most stringent, considering the diversity and complexity of the biological products. The regulatory legislation for biosimilars in the EU have clarified many aspects of their development, and many countries have adopted their own guidelines based on the EMA publications. The EMA has a robust regulatory process and the guidelines are continually revised and updated based on the experience obtained with the approval of biosimilars over time [14]. However, there are some challenges in areas such as bio analytics and comparability assay development, the definitions of biological activity under clinical view, the global harmonization of acceptable data, and product commercialization strategies of biosimilars [15]. Immunogenicity, one of the most serious adverse effects, has been observed during the therapeutic use of innovator biological products, which causes additional safety concerns among biosimilar regulators. Up to now there have been no clear specifications for analytical tests or preclinical and clinical studies to demonstrate biosimilarity in relation to a reference product. The reference product for demonstration of biosimilarity continues to be the one approved by the European Economic Area (EEA). To promote global development of biosimilars and to avoid the repetition of clinical trials, the revised guideline states that alternati-

vely certain clinical studies and in vivo non-clinical studies could be conducted with non-EEA authorized reference product providing justification and bridging studies. This reference is acceptable for a product authorized by a regulatory authority with similar scientific and regulatory standards as the EMA [16]. The development of biosimilar processes is very flexible due to the numerous possibilities and benefits available, such as disposable technology for production, supply chain logistics, and modern in-process analytical methods for process development and validation [17]. Changes that occurred over the time which were added by the experience gained by application reviews, led to an updated draft guideline released in 2013 and adopted by the Committee for Medicinal Products for Human Use (CHMP) on October, 2014. [16]. Case-by-case analysis is an approach used by the EU to deal with the diversity and complexity of biologics taking into consideration the class of the biologic which is under review [9].

Nomenclature of biosimilars

Currently, according to the International Nonproprietary Names (INN) mode of nomenclature established by the WHO, biological medicines are classified in a therapeutic group based on the active substance, and this scheme has been recognized by all the regulatory agencies. On the basis on this policy, the INN for a new biosimilar can be the same as that of the original biologic medicine [18]. In such case, when only the INN without a distinguishable identifier is used when prescribing and using a biologic and biosimilar medicine, an adverse event may be difficult to attribute to a specific product. That would lead to questions on what medicine caused the adverse event, leading to an unclear ability to document long-term product safety [19]. Each biosimilar product should be readily distinguishable from the reference product and other biosimilars in order to ensure appropriate use, traceability, and accurate reporting of adverse drug reaction (ADR) [20]. Different countries have addressed this issue on specific way and have adopted their own policies for biosimilar naming. For example in Japan, according to the guidance released by the Pharmaceuticals and Medical Devices Agency, the Japanese regulatory agency non-proprietary names of biosimilars should contain, at the end of the name, the respective follow-on number (e.g., biosimilar 1, 2, or 3), and the proprietary name should contain the BS letters, in addition to the dosage form, dosage, and name of the manufacturer [21]. Another issue is that some national regulatory authorities have licensed biologicals that are intended to be a copy of the innovator so called “intended copies”, even though the development there was not conducted in a rigorous, stepwise comparison with the reference product, according to the EU guidance and the WHO recommendations. In those countries, particularly in new emerging markets, the lack of specific regulatory guidance for naming of biologicals and biosimilars, or the existence of different approval pathways currently represents a substantial challenge for all involved parties and stakeholders, including clinicians, pharmacists and patients, who may be confronted, as a consequence, with the approval and use of ‘intended copies’ or non-comparable versions of biologic products. The fact that these copies of biologics often share the same INNs as the reference products underscores why having an INN qualifier unique to the manufacturer would be advisable from a traceability and patient-safety perspective.

Pharmacovigilance of biosimilars

Naming of biosimilar products has important implications for physicians’ prescriptton, potential patient bias, and interchangeability, as well as pharmacovigilance. As indicated by the WHO, national regulatory agencies need to ensure accurate ADR reporting for marketed biological medicines, by requiring inclusion of proprietary (brand) name, manufacturer’s name, lot number, and country of origin, in addition to the INN [22]. Often the pharmacovigilance system in a given country is not implemented based on high regulatory and efficiency standards, and it may not be possible to detect safety signals related to specific intended copy products or for biosimilars. To address this issue, the WHO is currently developing guidance on a risk-based approach. At the last meeting in 2014, the WHO acknowledged the need to distinguish biosimilar products. In August 2014, the WHO released a draft proposal for adding a 4-letter code, or “biological qualifier” to all biologics, in addition to the INN, and to establish a more

robust system for the identification of biosimilars and other biologics [23]. While the adoption of the biological-qualifier system would be a voluntary decision by individual regulatory authorities, it would represent an important tool for global harmonization.

Interchangeability and substitution

Other regulatory challenge is related to the interchangeability and/or substitution. These terms are often used as synonyms in the US, but not in the EU. According to the European Generic Medicines Association (EGMA), interchangeability refers to the prescription of a biosimilar in place of the reference product by prescribers, while substitution means that pharmacists are allowed to dispense a biosimilar [24]. Substitution of generic drugs for reference drugs is used because the two medicines are considered identical if they have demonstrated bioequivalence. However, since biosimilar drugs are not exact copies and the generic approach cannot be applied in the case of biosimilars, the question whether they can be substitutes of original biologics remains unclear. Interchangeability and substitution of biosimilars are not within the scope of the EU regulatory approval and, hence, there is no an agreement on the definition of what interchangeability actually means and no inclusion of such information in the European public assessment reports (EPAR) [14]. The EU regulators are considering biosimilars as "therapeutic alternatives" to the reference product, which would allow a biosimilar to be switched for the reference product either at the initiation or during the therapy. However, the European Consensus document released by the European Commission notes that interchangeability implies an initiative or agreement by the prescriber, and that patients should speak to their physician and pharmacist about switching decisions and changing therapy from one biologic product to another [14]. EMA does not have the authority to designate a biosimilar as automatically substitutable and currently, regulatory decisions concerning substitution are left to individual countries [25]. The EMA does not guarantee interchangeability and established that these aspects are beyond its competence. Therefore, authorities of each Member State should decide after scientific evaluation performed by the CHMP and other data submitted to the regulatory agency on support of the request [26].

Automatic drug substitution is the decision to switch a product for another product at the pharmacy level without the consent of the prescribing physician. Automatic substitution is generally confined to true generic drugs, which are chemically derived products that can be identical to their reference product in terms of chemical composition. Specific implementtation of automatic drug substitution is independently regulated by each European country. In practice in the case of biologics, substitution is construed as the legal authority for a hospital or a pharmacy filling a prescription to switch from dispensing the innovator product to a biosimilar or the reverse. A lack of clear guidelines on this issue and the consequence of substitution of one biological medicine for another can severely impact patient safety and make post-marketing pharmacovigilance more difficult. The issue of substitution is also closely tied to naming because if doctors prescribe biologics by a unique identifier, rather than by the currently used INN, the substitution of a biosimilar product when dispensed by a pharmacist would likely occur much less often. In practice, substitution by a pharmacist of a biosimilar for a reference biopharmaceutical medicine is not allowed in any European country [27] and is not recommended by the World Health Organization or by medical societies. The EMA also advises that the physician should be in charge of the decision to switch between the reference and biosimilar, or vice versa [28]. The major concern about interchangeability is that repeated switches between the biosimilars and the reference biological may increase immunogenicity, leading to adverse reactions. Some inherent differences arising due to a post approval process or a formulation change could lead to differential immunogenicity that may not necessarily be accessed through characterization or clinical trials during the time of application/approval, but may become evident during the post approval surveillance and pharmacovigilance. Even during pharmacovigilance the studies are typically designed on patient population and never follow a single patient, making it very difficult to track the status of interchan-

geability issues. Therefore, it would be difficult for regulatory bodies to certify that the drug is truly interchangeable without adequate data. There has been considerable debate over this issue in all regulatory agencies. According to the EMA, the approved biosimilar status signifies that the biosimilar can be used interchangeably with reference drugs. However, automatic substitution is not possible according to the EU pharmaceutical law governing similar biological medicinal products. The EMA in a public consultation for the revision of the 2005 Guideline has included a new element where in a statement it mentions that a biosimilar application when assessed for marketing authorization does not certify the interchangeability status. Since these provisions come under the national laws of the EU Member States, the EMA does not have the power to make such determination. According to the EMA, automatic substitution does not apply for any approved biosimilar. The EU Generics Association also claims that more than 12 countries have rules against automatic substitution. However, France has now permitted the switching of biosimilars and generics with the originals as part of a new Social Security Budget Legislation (article 47), which came into effect on January 1, 2014 [29]. Overall, due to the intense efforts from the originator manufacturers concerning health risks and differences of biosimilars in relation to the reference product, giving uncertainty for prescribers and patients, the application of interchangeability and/or substitution is limited. Still the use of biosimilars in the clinic practice may have a positive impact in the near future, leading the way towards adequate decisions [30].

Conclusion

The EMA has established a tied regulatory framework for the licensing of biosimilars, but these requirements are inadequate for fully establishing the efficacy, safety and clinical use of biosimilars. The questions of naming and nomenclature of biosimilars, interchangeability and substitution, pharmacovigilance and the degree of comparability between a biosimilar and the reference need to be considered. The differences in the national health care systems, regulatory and reimbursement authorities in the EU member states and in other counties, can lead towards different policy decisions on use of biosimilars. There is a need for more comparative studies in order to collect the data necessary to follow up on and evaluate uncertainties surrounding the longer-term safety, effectiveness, and cost-effectiveness of a biosimilar, as compared to the current standard treatment. Biosimilars are here to stay, and beside approval from quality and safety regulatory aspects, even more important is the confidence of health authorities, doctors, and patients in the efficacy and safety of biosimilars.

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Резиме

БИОСЛИЧНИ МЕДИЦИНСКИ ПРОИЗВОДИ – РЕГИСТРАЦИЈА, ФАРМАКОВИГИЛАНЦА И ЗАМЕНЛИВОСТ

Александра Грозданова, Катерина Анчевска-Нетковска, Зоран Стерјев, Зорица Наумовска, Александра Капедановска-Несторовска, Рубин Заревски, Александар Димовски, Љубица Шутуркова

Институт за фармацевтска хемија,
Фармацевтски факултет, Универитет
„Св. Кирил и Методиј", Скопје, Р. Македонија

Употребата на биолошките лекови е зголемена во последните декади и тоа резултираше со значителен придонес кон подобрување на ефективноста од терапијата кај многу заболувања. Истекувањето на патентната заштита на биолошките иноваторни лекови овозможи да се јават на пазарот таканаречените слични биолошки производи (биослични) кои беа одобрени од регулаторните агенции за клиничка употреба. Биосличните лекови се слични, но не се идентични и не се генеричка верзија на иноваторните биолошки производи. Иако биосличните лекови минуваат ригорозна карактеризација како и клиничките студии со цел да се докаже нивната безбедност и ефективност, сепак, постојат специфични регулаторни барања за регистрација на биосличните лекови. Ова се многу комплексни молекули и мали промени во процесот на производство може да имаат големо влијание

на нивната безбедност и ефикасност. Појавата на биосличните лекови ја зголеми компетитивноста, со можност да се подобри достапноста на биолошките лекови до пациентите и да продонесе кон одржување на финансиска стабилност на здравствените системи. За да се искористи потенцијалот на биосличните лекови во клиничката пракса, мора да се обезбеди интензивен фармаковигиланца мониторинг-систем со цел да се потврди вистинската сличност меѓу оригиналниот и биосличниот лек. Оттука е и потребата од насоки и водичи за јасно дефинирање на постоечката дебата за обележување, именување, фармаковигиланца и супституција со цел да се обезбеди ефикасна и соодветна употреба на биосличните лекови во клиничката пракса.

Клучни зборови: регулатива, биосимилари, биолошки лекови, лиценцирање, заменливост, фармаковигиланца, EMA

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ANALYSIS OF INDEPENDENT COMPONENTS OF COGNITIVE EVENT RELATED POTENTIALS IN A GROUP OF ADHD ADULTS

Silvana Markovska-Simoska, Nada Pop-Jordanova, Jordan Pop-Jordanov

Macedonian Academy of Sciences and Arts, Skopje, R. Macedonia

Corresponding Author: Silvana Markovska-Simoska, Macedonian Academy of Sciences and Arts, Addres: Krste Misirkov, No 2, P.O. Box. 428, 1000 Skopje, R. Macedonia, Tel. +389 70 83 73 75, E-mail: silvana@manu.edu.mk

Abstract

In the last decade, many studies have tried to define the neural correlates of attention deficit hyperactivity disorder (ADHD). The main aim of this study is the comparison of the ERPs independent components in the four QEEG subtypes in a group of ADHD adults as a basis for defining the corresponding endophenotypes among ADHD population.

Sixty-seven adults diagnosed as ADHD according to the DSM-IV criteria and 50 age-matched control subjects participated in the study. The brain activity of the subjects was recorded by 19 channel quantitative electroencephalography (QEEG) system in two neuropsychological tasks (visual and emotional continuous performance tests). The ICA method was applied for separation of the independent ERPs components. The components were associated with distinct psychological operations, such as engagement operations (P3bP component), comparison (vcomTL and vcom TR), motor inhibition (P3supF) and monitoring (P4monCC) operations.

The ERPs results point out that there is disturbance in executive functioning in investigated ADHD group obtained by the significantly lower amplitude and longer latency for the engagement (P3bP), motor inhibition (P3supF) and monitoring (P4monCC) components. Particularly, the QEEG subtype IV was with the most significant ERPs differences comparing to the other subtypes.

In particular, the most prominent difference in the ERPs independent components for the QEEG subtype IV in comparison to other three subtypes, rise many questions and becomes the subject for future research.

This study aims to advance and facilitate the use of neurophysiological procedures (QEEG and ERPs) in clinical practice as objective measures of ADHD for better assessment, subtyping and treatment of ADHD.

Keywords: ERP independent components, ADHD, adults, executive functions

Introduction

In the last decade, the aim of many studies was to define the neural correlates of attention deficit hyperactivity disorder (ADHD). In this context, Event Related Potentials (ERPs) have been investigated in a large number by many researchers and a substantial number of ERP correlates of ADHD have been identified (Barry et al., 2003).

Cognitive ERPs are among the most important characteristics of the function of the brain. Using the independent component analysis (ICA) method, ERPs can be decomposed into functionally different components which have been shown to provide features that could be used for characterizing clinical populations (Kropotov, 2009; Müller et al., 2010; Müller et al., 2011). Thus, the use of ICA substantially improves the traditional method of the separation of signals of ERPs (Kropotov, 2009). These independent components (ICs) have different latencies, different topographies and dif-

ferent functional meanings (Kropotov et al., 2011).

The components were associated with distinct psychological operations, such as engagement operations (P3bP component), motor inhibition (P3supF), monitoring (P4monCC) and comparison components (vcomTL and vcom TR). Amplitude and latency of these components at Pz, Fz, Cz, T_5 and T_6 leads were measured and analyzed in each test subject.

Activation (engagement) component – P3bP (P3bParietal component) is generated in the parietal cortex and is associated with an operation of action engagement i.e. activation of the cortex. This feature from neurophysiological perspective is associated with activation of cortical and subcortical structures in the frontal-parietal cortex involved in performing of the selected action. From a psychological and functional point of view it is associated with combining all brain resources to implement the action. Previous experiments with the use of this modification of the test have shown that the ERPs in the interval of 200–300 ms have a positive component distributed in the parietal central areas and are related to the mental process of the initiation of action (the component of action engagement) (Pronina et al., 2011). It was confirmed that ADHD children exhibit lower amplitudes of GO and NOGO P300 components in comparison to normal groups (Kropotov et al., 1999; Overtoom et al., 1998; van Leeuwen et al., 1998; Ponomarev et al., 2000).

Inhibition component (inhibition of preparatory activity) – P3supF (P3 suppression Frontal): From a neurophysiological aspect, inhibition of the response includes a special circle in the right ventral prefrontal cortex, basal ganglia and thalamus axis. Ventral prefrontal cortex receives information from the sensory systems that detect the mismatch between the expected and actual sensory stimuli. For example, when the information comes from the visual parts, the cortex receives additionally information from the anterior cingulate, where executive action is compared to the planned (prepared) action. The ventral prefrontal cortex is active when we need to stop or inhibit commenced behavioral pattern. The damage to the inhibition of reaction is conceptualized as the core symptom of ADHD by many authors including Barkley (1997). However, attempts to test his hypothesis experimentally proved quite controversial. The team of Banaschewski et al., (2004) failed to find any deviation of inhibition component in Go/NoGo paradigm. Unlike them Satterfield (1988, 1990, 1994) and his associates have shown a significant reduction of inhibition component in the stop signal task with ADHD group compared with the control group.

Self-monitoring component – P4monCC (P400 monitoring Cingulate Cortex): Error correction or monitoring of what we have done is very important executive function. From neuropsychological point of view, monitoring is based on neural mechanism of comparison of the expected action compared to the behavioral response. If an action does not meet the expectations, a change in behavior to correct the difference is appearing. Scientists from Ghent University in Belgium (Wiersema et al., 2005) found that children with ADHD have normal monitoring in relation to the detection of the error, but show abnormal and inadequate strategy of adjusting the response.

In the study of Kropotov et al. (2005) which analyzed 150 ADHD children, significantly reduced P400 monitoring component was noted compared to a normative database for appropriate age.

Comparison component – vcomTL (visual comparison temporal left) and vcomTR (visual comparison temporal right): Other indirect indicator of working memory is comparison component, which appears in Go/NoGo task. This component is appearing in response to the second stimulus in NoGo attempts when presented stimulus does not match expectations. In the research of Kropotov et al., 2005 depletion of this component in 36 (25%) of 150 examined ADHD children was found.

According to sLORETA the sensory mismatch component was generated in the left and right temporal areas, the action suppression component was generated in the supplementary motor cortex, and the conflict monitoring component was generated in the anterior cingulate cortex [Kropotov et al, 2011].

The main aim of this study is the comparison of the ERPs independent components in the four QEEG subtypes of ADHD adults as a basis for defining the corresponding endophenotypes among this population. The paper ap-

plies a methodological approach developed in the Institute of Human Brain (St. Petersburg) for assessment of electrophysiological indexes of executive functions of ADHD adults.

Methods

Subjects

Two groups, the ADHD adults and the control group that participated in the study were recruited in the framework of the EU COST Action B27 "Electric Neuronal Oscillations and Cognition – ENOC". The 67 ADHD adults and 50 normal controls (between the ages 18 and 50 years) were enrolled in the study. The control group was recruited from the local community and matched by sex and age. All subjects gave their informed consent for participation in the study.

A female to males' ratio was equal for the ADHD (33 females and 34 males) and the control group (25 females and 25 males). The mean age of the ADHD group was 33.4 ± 8.39 years, and for the control group the mean age was 32.8 ± 8.22 years.

In the ADHD group 45 subjects were referred by their psychiatrist (with the previous diagnosis of ADHD) and 22 adults were new patients. Subjects were included in the ADHD group only if they had been diagnosed as ADHD by an independent psychiatrist. The ADHD diagnosis was confirmed according to the DSM-IV criteria with at least 4 symptoms of inattention or at least 4 symptoms of hyperactivity/impulsivity, frequently present during the past 6 months, affected in at least 2 areas of life, with no history of epilepsy and no history of head injury.

It can be noted that the four symptoms of inattention and/or hyperactivity/impulsivity is less than DSM-IV requires for ADHD diagnosis, but according to Barkley this is acceptable for adults. All subjects met the criteria of the Barkley's Semi-structured Interview for adults with ADHD. In order to ensure diagnostic validity, additional information was collected from parents, partners, relatives and friends. The determination of the presence of adulthood ADHD symptoms during the assessment resulted in 26 ADHD subjects being diagnosed with the inattentive subtype, 4 with hyperactive/impulsive and 37 with combined behavioral subtype. All subjects had normal or corrected to normal vision and were right-handed.

According to Kropotov's QEEG spectrum classification for ADHD population (Kropotov, 2009), we have made grouping on our subjects according to the following four subtypes: I.) Abnormal increase of delta-theta frequency range centrally or centrally-frontally; II.) Abnormal increase of frontal midline theta rhythm; III.) Abnormal increase of beta activity frontally; IV.) Excess of alpha activities at posterior, central, or frontal leads (Markovska-Simoska and Pop-Jordanova, 2010).

All participants were briefly interviewed before testing to exclude those with a history of head injury with subsequent loss of consciousness, substance abuse, neurological, systemic medical diseases and/or severe psychiatric disturbances. Except for symptoms of psychosis, comorbidities were no reason for subject exclusion.

Subjects were unmedicated, or they had refrained from taking methylphenidate during 48 hours before testing. Control subjects also did not receive any medication at the time of testing. All participants were asked to restrain from coffee and cigarette intake on the day of the testing. Subjects taking other psychotropic substances were not included in the study.

The study was approved by the local ethics committee. Subjects voluntarily participated in the study and written informed consent was obtained from all participants after having provided an explanation of the procedure.

Procedure

All participants were individually assessed in two sessions with neuropsychological and neurophysiological testing in an environment free from distractions. The testing was carried out in a quiet, air-conditioned room with the experimenter and the recording equipment present. In the first assessment the interview and questionnaires as Current and Childhood Symptoms Scale (Barkley); Brief Symptom Inventory (Derogatis); Health History (Barkley); Trauma questionnaire (Müller & Thomann) and Semi-structured Interview for Adults with ADHD (Barkley) for excluding the ADHD symptoms were applied. The results of

the neuropsychological testing performed with Amsterdam Neuropsychological Testing (ANT) and CogMed test, at the first session, are not relevant to this paper.

EEG data were acquired by the Mitsar 19-channel QEEG 201 system (Mitsar Ltd.), while the subjects were in an eyes-closed and in an eyes-open resting condition, lasting five minutes each (sufficient for 2 minutes artefact-free data EC and EO). Then data was recorded while subjects were performing a visual continuous performance task – VCPT (two-stimulus Go/NoGo paradigm) and emotional continuous performance test – ECPT from Psytask program. The duration of the tasks was approximately 22 minutes for each one.

Separate channels for recording a signal from the button were used for monitoring the accuracy of the test performance and measuring the response trial. The input signals referenced to the linked ears were filtered between 0.5 and 50 Hz and digitized at a sampling rate of 250 Hz. The Impedance was kept below 5 kOhm for all electrodes. Electrodes were placed according to the International 10–20 system using an electrode cap with tin electrodes (Electro-cap International Inc.). The quantitative data were obtained using WinEEG software. The linked ears reference montage was changed to average reference montage prior to data processing. In addition, epochs of the filtered electroencephalogram with excessive amplitude (> 100 μV) and/or excessive fast (> 35 μV in 20 to 35 Hz band) and slow (> 50 μV in 0 to 1 Hz band) frequency activities were automatically marked and excluded from further analysis. Finally, the EEG was manually inspected to verify artefact removal.

ERPs were computed off line. The epoch of analysis included 300 ms before the first stimulus and 900 ms after the second stimulus. Trials containing electrooculogram artefacts (exceeding 100 μV threshold) were discarded from further analysis. Trials with omission and commission errors were automatically excluded from averaging. To get reliable ERPs, more than 70 trials for each condition were needed.

Behavioral tasks

The Visual Continuous Performance Task (VCPT) and the Emotional Continuous Performance Task (ECPT), were administered using the standard protocol. During the test, a subject sat in a comfortable armchair with armrests. Pictures were presented in a pseudo-randomized order in the center of a computer monitor placed 1.5 m from the subjects' eyes. The stimuli were presented on a 17 inch monitor using the Psytask (Mitsar Ltd.) software. Before each session, the test was explained to the subject in detail and 10–20 trials were performed. Accuracy and speed were encouraged. There was a 5-minute rest between the tests. If it was necessary, subjects rested for a few minutes after each 200 trials.

Three categories of visual stimuli were selected for VCPT: 1) 20 different images of animals; 2) 20 different images of plants; 3) 20 different images of humans presented together with an artificial "novel" sound. All visual stimuli were selected to have similar size and luminosity.

For ECPT the three categories of visual stimuli were: 1) 20 different images of angry faces; 2) 20 different images of happy faces; 3) 20 different images of neutral faces presented together with an artificial "novel" sound.

The trials consisted of presentations of paired stimuli with inter-stimulus intervals of 1000 ms and inter-trials intervals of 3000 ms. Duration of stimuli was 100 ms. Four categories of trials were used: Animal-Animal, Animal-Plant, Plant-Plant, and Plant-(Human+Sound) for VCPT and Angry Face-Angry Face, Angry Face–Happy Face, Happy Face–Happy Face, Happy Face – (Neutral Face+Sound). The trials were grouped into four blocks with one hundred trials each. In each block a unique set of five animals (angry faces) stimuli, five plants (happy faces) stimuli, and five humans (neutral faces) stimuli were selected accordingly. Each block consisted of a pseudo-random presentation of 100 pairs of stimuli with equal probability for each stimulus category and for each trial category. The task was to press a button as possible in response to all Go trials. A–P (AF-HF) pairs represented the NoGo condition, in which the person should withhold from responding. Thus, after the presentation of the first Go stimulus, the subject was ready to press the button, and presentation of the second stimulus inhibited the prepared motion. For the P–P (HF-HF) and P–H

(HF-NF) it was assumed that the first stimuli would signal that no preparation for action was needed and that the trial could be ignored. It must be stressed here that in pairs A-A (AF-AF) and P- P (HF-HF) the first and the second stimuli were physically the same.

Mean reaction time (RT) with a standard deviation (SD) of RT was calculated across trials for each participant. Omission- (not pressing the button to Go trials) and commission errors (pressing the button to NoGo trials) were also computed for each participant separately. A response was considered correct if it occurred in relation to the appropriate second stimulus and took place during the time interval from 200 to 1000 ms after the second stimuli presentation.

Decomposition of collection of ERPs into independent components.

The goal of the Independent Component Analysis (ICA) is to utilize the differences in scalp distribution between different generators of ERP activity in order to separate the corresponding activation time courses (Makeig et al., 1996). The components are constructed by optimizing the mutual independence of all activation time curves, leading to a natural and intuitive definition of an ERP component as a stable potential distribution which cannot be further decomposed into independently activated sources. The ICA method used in the present study was implemented in the analysis software described by Kropotov (2009). The 700 ms interval after the second stimulus in the two conditions (Go and NoGo) with sampling rate 250 samples/second was selected.

Statistical analysis

Amplitudes and latencies of the components were computed for each condition and each subject separately. One-way ANOVA was used for assessing statistical significance of the difference between groups (all ADHD , ADHD I, II, III, IV subtype and control) and conditions (EC, EO, VCPT, ECPT). To explain the significant interactions post hoc Bonferroni test was performed. Due to space reasons only the significant effects and interactions between groups and conditions are presented.

Results

Table 1 shows the behavioral performance of participants in VCPT and ECPT. The ADHD group showed a significantly higher number of omission and commission errors and a significantly higher RT and its variance, compared to the control group.

Table 1

Behavioral performance for the ADHD and Control groups in VCPT and ECPT

	Controls	ADHD group
VCPT		
Omission errors (SD)	1.40 (1.62)	5.89*** (2.98)
Commission errors (SD)	0.69 (1.28)	1.24* (1.46)
RT[a] (ms) (SD)	364.63 (54.67)	417.52*** (72.26)
Var[b] RT (ms) (SD)	7.22 (2.27)	10.82*** (3.20)
ECPT		
Omission errors (SD)	3.50 (1.77)	14.70*** (3.80)
Commission errors (SD)	1.96 (1.78)	2.33 (1.32)
RT[a] (ms) (SD)	411.23 (25.6)	456.18** (74.04)
Var[b] RT (ms) (SD)	9.80 (3.50)	13.94*** (3.41)

[a] Reaction time; [b] Variability of reaction time – The stars mean the level of significant difference between both groups (***p < 0.001, **p < 0.01, *p < 0.05)

P3bP (activation component)

a) Amplitude

When considering the amplitude of the P3bP component in Normal and ADHD groups we have obtained statistically significant difference [F (1,228) = 3.18, p < 0.05, (p = 0.043)], with lower value of the P3bP amplitude in the ADHD group (Figure 1, left).

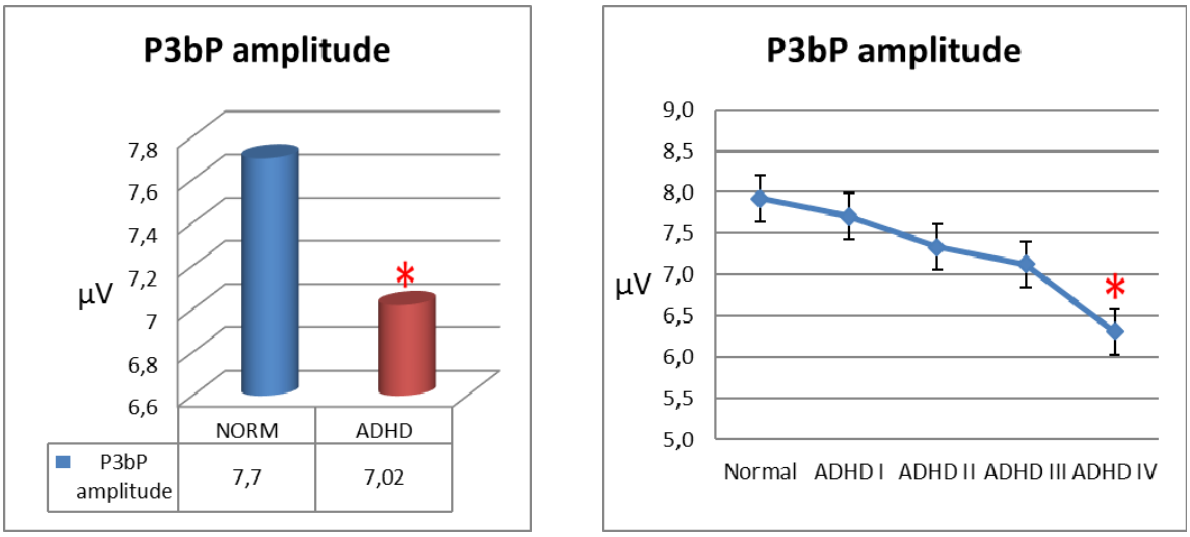

Figure 1 – Amplitude of P3bP component

In terms of the dependence of the P3bP amplitude from the pertaining to the QEEG subtypes of ADHD group, ANOVA showed a statistically significant difference between groups F (4,225) = 2,7660, $p < 0.05$, ($p = 0.03$). Specifically, Bonfferoni post hoc test localize the lowest value of the P3bP amplitude in ADHD IV subtype with $p < 0.05$ (Figure 2, right). As it can be seen, a linear decreasing of the amplitude depends on the belonging to the subgroup.

As for the dependence of the P3bP amplitude to the test condition (VCPT or ECPT) significant difference was not received between these two tests (although in VCPT amplitude was higher). So, in this case the different stimuli applied in the tests do not affect the amplitude of the P3bP component.

b) *Latency*

In terms of the latency of P3bP component, a significant difference between the Normal and the ADHD group [$F(1,228) = 3.03$, $p < 0.05$, ($p = 0.03$)], expressed through longer P3bP latency in ADHD group (Figure 2, left) was found.

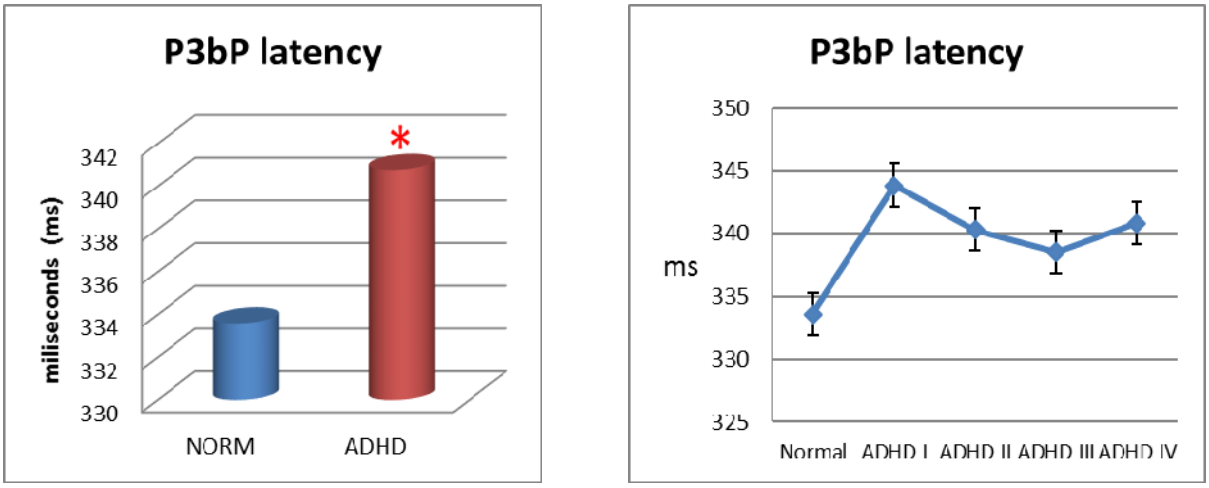

Figure 2 – Latency of P3bP

When the ADHD subtypes and the VCPT and ECPT conditions were analyzed as between subject variables, significant differences were not obtained.

P3 supF (inhibition component)

a) Amplitude

When considering the amplitude of P3supF in Normal and ADHD groups we found statisti-

cally significant difference [F(1,228) = 11,145, p < 0.01, (p = 0.001)], with lower amplitude of the component for ADHD group (Figure 3, left).

Additionally, a significant difference was obtained and shown in distribution (Normal, ADHD I, ADHD II, ADHD III, ADHD IV) with [F(4,225) = 5,26 and p < 0.001 (p = 0.000)] (Figure 3, right). Specifically, Bonferoni post hoc test showed that for p < 0.05, the amplitude of the P3supF component is with the lowest value in the ADHD IV subtype. Here, as previously found with the P3bP component, the linearly decreasing amplitude depends on the group affiliation (Figure 3, right).

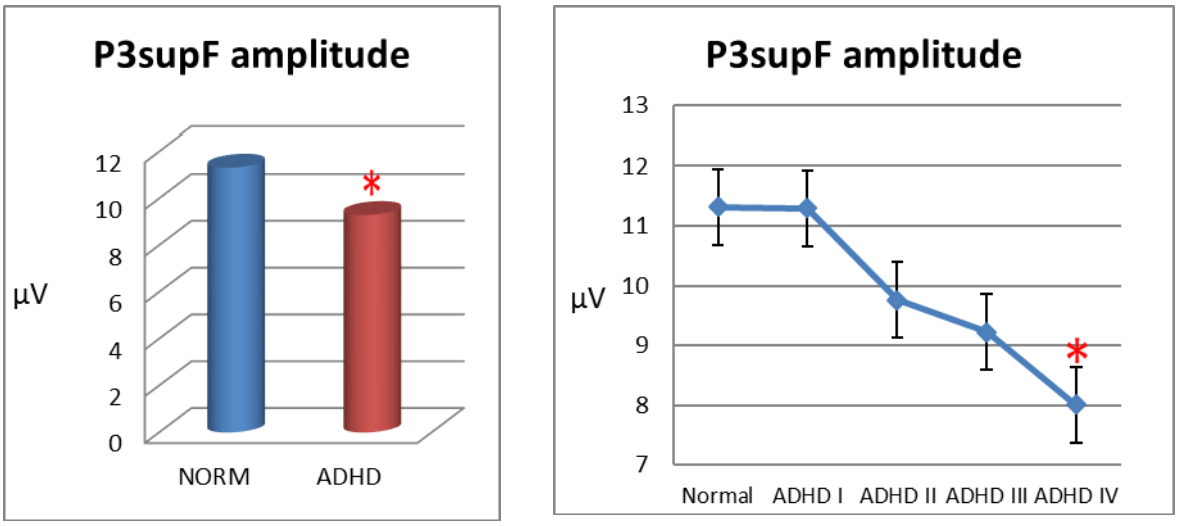

Figure 3 – Amplitude of P3supF component

The effect of task is with no significant difference on the amplitude of the P3supF component [F (1,228) = 0.004, p > 0.05, (p = 0.95)].

b) Latency

In terms of latency we have not obtained a significant difference between Normal and ADHD group and the Normal and ADHD subtypes (although ADHD IV subtype as seen in Figure 4. left, is with the longest latency).

However, in terms of the task, the ECPT showed longer latency in comparison to VCPT [F (1,228) = 28.54, p < 0.01, (p = 0.000)] (Figure 4. right).

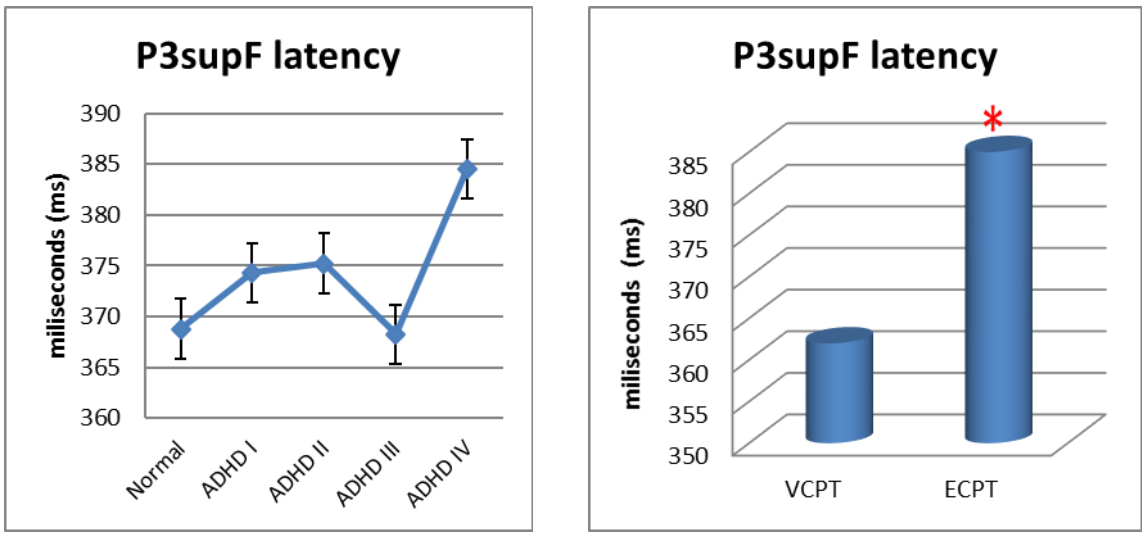

Figure 4 – Latencies of P3 supF component in groups (left) and the recording conditions (right)

vcomTL (visual comparison temporal left component)

a) Amplitude

When analyzing the amplitude of vcomTL in groups Normal and ADHD any statistically significant difference [F(1,228) = 1.98, p > 0.05, (p = 0.16)], between the two groups has not been found.

Additionally, no significant difference was obtained in the shown distribution (Normal, ADHD I, ADHD II, ADHD III, ADHD IV) with [F (4,225) = 0.69 and p > 0.05 (p = 0.60)].

While, vcomTL amplitude does not depend on the effect of belonging to a group, the statistical analysis showed that the effect depends on Task condition, with lower amplitude in ECPT compared to VCPT [F(1,228) = 64.72, p < 0.001, (p = 0.000)] (Figure 5).

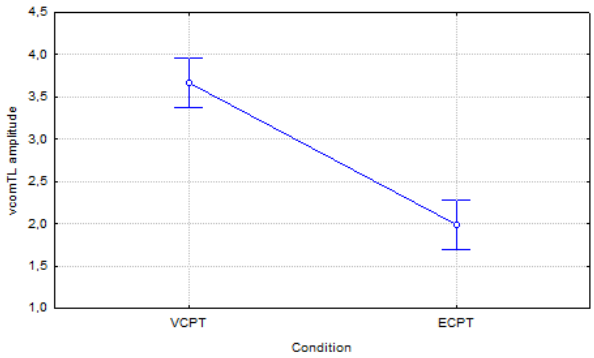

Figure 5 – Amplitude of vcomTL in VCPT and ECPT

b) Latency

Concerning the latency, no significant difference between the normal group and the ADHD, as well as between the ADHD subtypes was found [F (4,225) = 1.54, p > 0.05, p = 0.19] (Figure 6).

For the Condition effect there is a significant difference, with a longer latency of vcomTL in ECPT regarding VCPT with F (1,228) = 55.05, p < 0.001, (p = 0.000) (Figure 6).

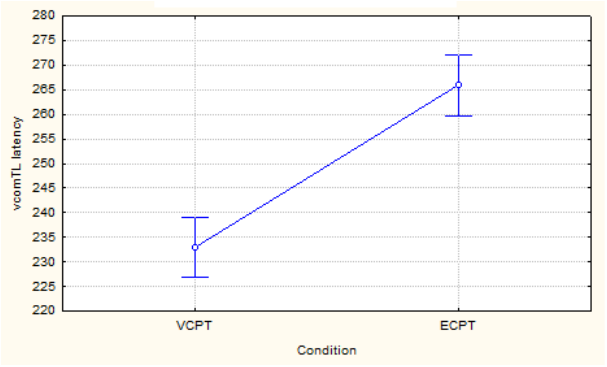

Figure 6 – Latency of vcomTL in VCPT and ECPT

vcomTR (visual comparison temporal right component)

a) Amplitude

When analyzing the amplitude of vcomTR in groups Normal and ADHD there was no statistically significant difference [F (1,228) = 0.38, p > 0.05, (p = 0.54)], between the two groups. Also, no significant difference was obtained in the shown distribution (Normal, ADHD I, ADHD II, ADHD III, ADHD IV) of [F (4,225) = 1.35 and p > 0.05 (p = 0.25)]. For the effect Condition no significant difference between conditions was found F (1,228) = 0.07, p > 0.05, (p = 0.78).

b) Latency

Like vcomTL, vcomTR regarding latency showed no significant difference between the normal group and ADHD. Concerning the tasks, there is a longer latency in ECPT [F (1,228) = 33.29, p < 0.001, (p = 0.000)] (Figure 7).

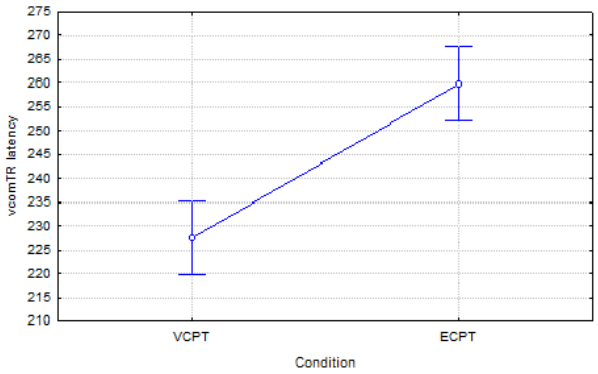

Figure 7 – Latency of vcomTR in VCPT and ECPT

P4monCC (monitoring component)

a) Amplitude

We obtained statistically significant difference when considering amplitudes of P4monCC component in groups of the Normal and the ADHD adults [F (1,228) = 10.25, p < 0.01, (p = 0.002)], with lower amplitude for the ADHD group (Figure 8, left).

Also, a significant difference was obtained and shown in the subtype distribution (Normal, ADHD I, ADHD II, ADHD III, ADHD IV) with [F (4,225) = 4.16 and p < 0.01 (p = 0.003)] (Figure 8, right). More specifically, Bonfferoni post hoc test showed that for p < 0.01 (p = 0.001) the normal group has greater P4monCC amplitude than other subtypes, especially ADHD IV subtype.

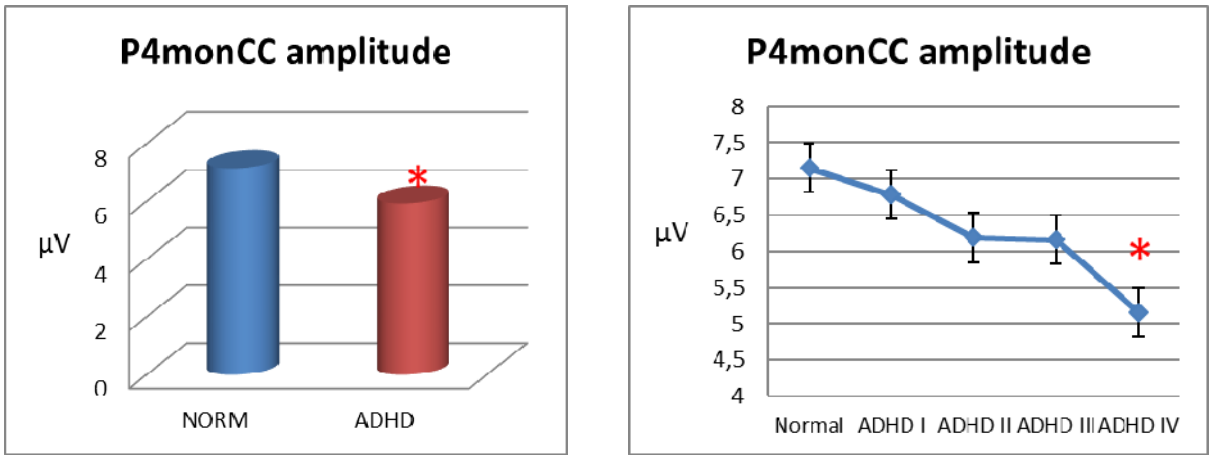

Figure 8 – Amplitude of P4monCC component

Regarding the tasks we did not found a significant relationship between VCPT and ECPT, F (1,228) = 0.01, p > 0.05, (p = 0.90).

b) Latency

In terms of the latency there was a significant difference between the normal and the ADHD group, F (1,228) = 7.85, p < 0.01, (p = 0.005) with a longer latency in ADHD (Figure 9. left). Significant subtype group effect was obtained, F (4,225) = 2,71, p < 0.05, p = 0.031 (Figure 9, right), specifically for ADHD IV subtype (p = 0,04), which has significantly longer latency than the normal group.

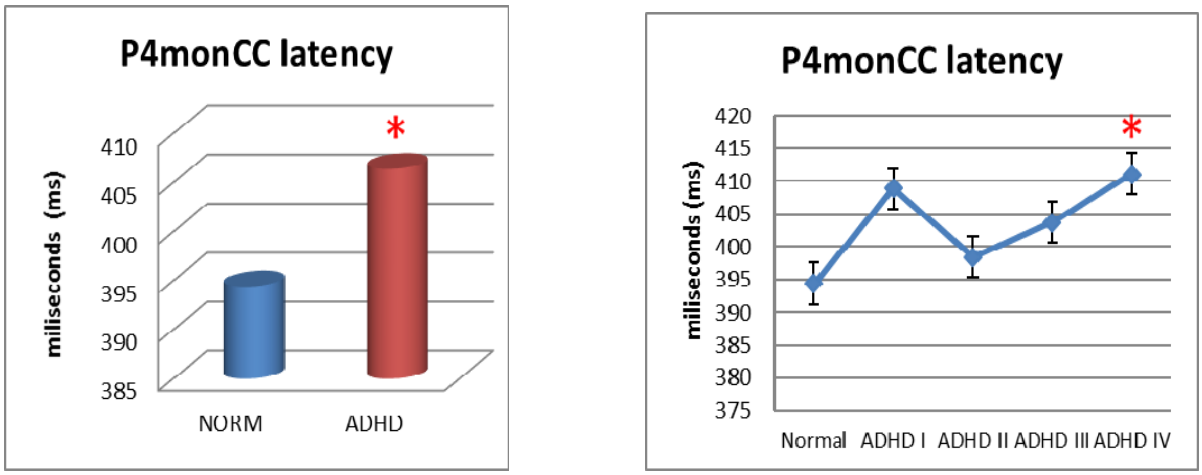

Figure 9 – Latencies of P4monCC component

Finally, compared to VCPT the latency of P4monCC in ECPT was longer with F (1,228) = 39.25, p < 0.001 (p = 0.000).

The topographies of all components are presented on Figure 10.

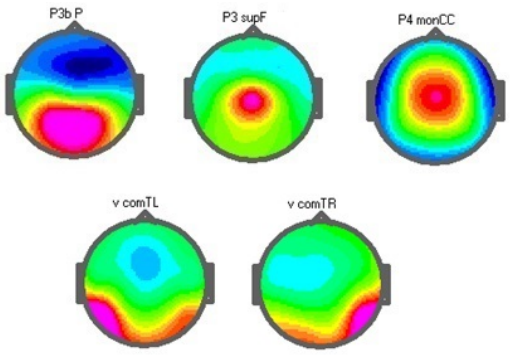

Figure 10 – Topographies of ICA ERP components

Discussion

The ERP results of this study point out that there is a confirmed disturbance in the executive functioning in the investigated ADHD group. Significantly lower amplitude and longer latency for the engagement (P3bP), motor inhibition (P3supF) and monitoring (P4monCC) components were obtained. Particularly, the QEEG subtype IV showed the most significant difference compared to the other subtypes.

The term executive functions refer to the coordination and control of motor and cognitive actions in order to achieve specific objectives. The executive functions are implemented by complex brain system consisting of several cortical and subcortical structures related to each other. Along with the basal ganglia, prefrontal areas are the seat of executive functions associated with activation, deactivation (inhibition), monitoring and working memory. Although the components associated with the executive functions overlap in time and space, recently developed independent components analysis provides a powerful tool for their separation and the detailed study. Using normative HBI database, we were able to separate and analyze the components of these executive event related potentials: P3bP, P3supF, vcomTL, vcom TR and P4monCC.

The P3bP component of evoked potentials is theoretically and clinically the most studied component in the scientific literature. There are several reasons for this: 1) the P3bP component is created in odd ball task, a task that is easily performed in nearly all categories of neurological and psychiatric patients; 2) P3bP is relatively large component and can be easily distinguished as the wavelength difference between the responses of target and non-target deviant standards; 3) P3bP has a diagnostic power because impairments of this component were found as several executive dysfunctions in several disorders, i.e. schizophrenia and ADHD (Kropotov, 2009). Otherwise, there are several functional meanings of P3bP component. The most significant of these is the concept of recovery working memory proposed by Donchin (1981). P3A and P3bP components are normally considered as an index of attention and for this reason are widely applied in the diagnosis of brain disorders in which there is disturbance of attention systems. Many studies suggest reduced P3bP component in the ADHD population compared to the normal. The latest diagnostic application was confirmed in this study through reduced amplitude and longer latency to P3bP parietal component (as an index of activation processes) in adults with ADHD, compared to the normal control group. This interpretation also matches the received neuropsychological results and indicates reduced working memory in the target group especially in tasks with increased cognitive effort.

The P3supF inhibiting motor component occurs after NoGo attempts and is expressed through the frontally distributed negativity. Weakening of the inhibition of the response is conceptualized by many authors as the core symptoms of ADHD (including Russel Barkley, a leading name in the field of ADHD). However, some controversial experiments which are testing this hypothesis exist. An international team from the University of Gottingen, Germany, and the University of Zurich (Banaschewski et al., 2004), failed to find any deviations of the ADHD group compared to the normal. Unlike them, another study at the University of Texas, demonstrated significant reduction of this component in the ADHD group. Accordingly (somewhere in between), the results of this paper showed that there is a reduced amplitude of the P3supF component in the ADHD group, but there was no significant difference in latency.

The left and right comparison components (vcomTL and vcom TR) are no significantly different in both examined groups even in terms of amplitude or in terms of latency. But there is a significant difference in terms of the recorded condition that is VCPT vs. ECPT, with lower amplitude and higher latency in ECPT, which shows the dependence of this component of working memory overload with emotional stimulus. This component is an indirect index of working memory, sign for detecting a change in the current stimulus compared with the memory trace stored in working memory. It occurs in response to the second stimulus efforts in NoGo when presented stimulus does not coincide with the expected stimulus. The results of this study did not match the

results of Kropotov et al., 2005, where they have obtained reduced amplitude of the comparison component of 150 ADHD children.

The monitoring P4monCC component is with its highest amplitude and by s-LORETA (Pascual-Marqui et al., 1994) is generated by the medial prefrontal cortex and anterior cingulate cortex with maximum in Cz-Fz. Its functionality includes dynamic adaptation of human behavior through continuous assessment of ongoing activities and their consequences. The ability to monitor and compare current activities with internal standards and objectives is critical for optimal decision making. In this paper, we have obtained a significant reduction in the amplitude of monitoring component in the group with ADHD and significantly longer latencies. Similar results are found in children with ADHD in the above mentioned study of Kropotov et al., 2005. With regard to the recording condition, only significantly longer latencies in ECPT, suggest more difficult nature of the task, which also requires and longer self-processing of the information thus the entity acted properly or not.

In terms of QEEG subtypes the results showed that amplitudes of P3bP, P3supF and P4monCC were significantly generally reduced, with the longest latencies in the fourth QEEG subtype, suggesting impaired activation, inhibition and monitoring components. One possible explanation for this association of ADHD symptoms with QEEG subtype with increased alpha brain activity, may be the fact that the deep state of inactivity (idling) of the brain corresponds to a lack of inhibition, resulting in impulsiveness, hyperactivity and inattention (which was also obtained with the obtained results in the means of reduced amplitudes and longer latencies of evoked potentials in almost all tested components).

These valid subtypes may have different reasons for their occurrence, and therefore can react differently to medication and neurotherapy. These opportunities merit further investigation for future research.

Conclusion

From the obtained results, it can be concluded that the applied neurophysiological measures relatively clearly differentiate the ADHD into four subtypes, illustrating the heterogeneous and multifactorial character of this disorder with different clinical expression, related to different underlying neuropsychological and electrophysiological abnormalities, and consequently the different responses to treatment regimes. This study aims to advance and facilitate the pace of using neurophysiological procedures in clinical practice as objective measures of ADHD for better assessment, subtyping and treatment of ADHD. Especially, the most prominent difference for the IV subtype raises many questions and becomes the subject for the future research.

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Competing interests

SMS, NPJ report no potential conflicts of interest.

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Резиме

АНАЛИЗА НА НЕЗАВИСНИТЕ КОМПОНЕНТИ НА КОГНИТИВНИТЕ ЕВОЦИРАНИ ПОТЕНЦИЈАЛИ КАЈ ГРУПА ВОЗРАСНИ СО АДХД

Силвана Марковска-Симоска, Нада Поп-Јорданова, Јордан Поп-Јорданов

Македонска академија на науките и уметностите, Скопје, Р. Македонија

Во последната деценија многу студии се обидоа да ги дефинираат нервните корелации на

дефицитот на внимание кај хиперактивното растројство (АДХД). Главна цел на оваа студија е споредбата на независните ERPs-компоненти кај четирите QEEG поттипови кај група возрасни со АДХД како основа за дефинирање на соодветните ендофенотипови.

Во студијата учествуваа шеесет и седум возрасни дијагностицирани како АДХД според DSM-IV критериумите и 50 испитаници од контролната група на иста возраст. Активноста на мозокот беше снимена со 19-канален систем на квантитативна електроенцефалографија (QEEG) при изведба на две невропсихолошки задачи (визуелен и емоционален изведбен континуиран тест). Беше применета ICA-методата за поделба на ERPs на независни компоненти. Овие компонентите се поврзани со различни психолошки операции, како што се ангажираност (P3bP компонентата), споредбена компонента (vcomTL и vcom TR), моторна инхибиција (P3supF) и мониторинг (P4monCC) компонентата.

ERPs резултатите укажуваат дека постои нарушување на егзекутивното функционирање кај групата АДХД и се добиени значително помала амплитудата и подолга латентност за активационата компонента (P3bP), моторната инхибиција (P3supF) и мониторинг (P4monCC) компонентата. Особено, QEEG поттип IV се покажа со најзначајни ERPs разлики во споредба со другите поттипови, што поттикнува многу прашања зошто е тоа така и останува како тема за идните истражувања.

Оваа студија има цел да го унапреди и да го олесни користењето на неврофизиолошките постапки (QEEG и ERPs) во клиничката пракса, како објективни мерки на АДХД користејќи ги за подобра процена, типизирање и третман на АДХД.

Клучни зборови: ЕРП независни компоненти, АДХД, возрасни, егзекутивни функции

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DOPPLER VELOCIMETRY OF THE UTERINE ARTERIES: AN EARLY SCREENING TEST FOR MISCARRIAGE

Ilian Trayanov, Elena Dimitrakova

UMBAL St. George – Plovdiv, Department of Obstetrics and Gynecology
Chair of Obstetrics and Gynecology, Plovdiv, R. Bugaria

Corresponding Author: UMBAL St. George – Plovdiv, Department of Obstetrics and Gynecology Chair of Obstetrics and Gynecology GSM 0898233776; E-mail: lilianski2000@yahoo.co.uk

Abstract

Introduction: According to the WHO, miscarriage is defined as "the loss of a pregnancy before the age of 20 weeks of gestation or fetal weight below 500 g". Only 50–60% of all conceptions fail to survive 20 weeks of gestation. It is estimated that about 75% of all unsuccessful conceptions are due to failed implantation and are not reported as spontaneous abortions.

Material and Methods: We performed a prospective observational study at the Department of Obstetrics and Gynecology of the Medical University Plovdiv between October 2014 and September 2015. We studied two groups of pregnant women: women with threatened abortion and those with normal pregnancy. All women underwent a transvaginal ultrasound evaluation of the uterine arterial blood flow. We measured peak systolic and end-diastolic velocity and calculated the average IR (resistance index) and S / D (systolic to diastolic ratio).

Results: There was no association between pregnancy outcome and the mean difference between uterine arterial IR and S / ($P > 0.05$).

Conclusion: In our study, Doppler ultrasound could not differentiate between women at risk for miscarriage and normal women. Further studies are necessary to answer this question.

Keywords: Doppler ultrasound, index of resistance, pregnancy, threatened abortion

Introduction

According to the World Health Organization (WHO), "miscarriage is the loss of a pregnancy before 20 weeks of gestation or fetal weight below 500 g" [1]. Only 50–60% of all conceived embryos fail to exceed 20 weeks gestation. It is estimated that about 75% of all unsuccessful conceived pregnancies are due to failed implantation and are not reported as "spontaneous abortion" [2]. Despite the constant progressive growth of the embryo and the trophoblastic tissue, the anatomy of the pelvis does not change dramatically in the first trimester after implantation. During this period, the main changes are reflected in the uterine blood flow. Uterine perfusion increases during pregnancy starting from the first quarter and this change can be documented using Doppler ultrasound [3].

Many congenital and acquired changes in the vascular wall of the endometrial spiral arteries can lead to inadequate fetal implantation of the fertilized oocyte. Spiral artery blood flow is affected by endothelial factors which change the velocity of the blood flow and can predispose to thrombosis [4].

Normal implantation and placentation are very important for the successful outcome of pregnancy. Suboptimal implantation and placentation can lead to miscarriage or complications in late pregnancy, such as preterm delivery or preeclampsia [5]. Placentation provides uteroplacental circulation and allows the tro-

phoblast to come in direct contact with maternal blood. A multitude of cellular and molecular signals affect implantation. Blastocyst invasion involves sex steroids, peptide hormones, growth factors, cytokines and immunological factors [6–8]. Excessive invasion resulting from improper countering the invasion of blastocyst by maternal tissues leads to placenta accreta or percreta. Inadequate invasion can result in miscarriage, preeclampsia or premature birth. Although the regulatory mechanisms are not fully understood, there is an urgent need to develop and validate tests that can identify pregnancies that are at high risk for miscarriage or complications [5].

Objective: To determine the prognostic value of Doppler ultrasound of the uterine arteries in patients diagnosed with threatened abortion.

Materials and methods

We performed a prospective observational study in the Department of Obstetrics and Gynecology at the UM Plovdiv for the period October 2014 – September 2015. We excluded pregnant women with subchorionic hematoma, slow heart rate of the fetal, abnormal yolk sac (missing, with a solid ultra-sonographic density, abnormal size, duplicate), less-than-gestational-age fetus, lack of heartbeat, multiple pregnancy, ectopic pregnancy, structural changes in cervical comorbidities that increase the risk of miscarriage (thrombophilia, anti-phospholipid syndrome), or parents with genetic diseases.

98 pregnant women (mean age, 26.54) were included in the study. The women were presented to the "DKC-6 Central Region Plovdiv" outpatient department or were inpatients at the "St. George's Multiprofile Hospital for Active Treatment". 27 did not meet inclusion criteria and were excluded. The remaining 71 pregnant women were divided into two groups. 35 patients were diagnosed with threatened abortion (group 1) and 36 women had normal pregnancies (group 2).

After explaining the method and completing the informed consent form, the women underwent ultrasound transvaginal examination. In order to measure blood flow of the uterine arteries we used GE Voluson E6, Voluson 730 and Philips ClearVue 650 equipment.

For the research we used color and pulsed Doppler velocity. After visualization of the left and right uterine artery at the level of corpocervical junction, in real time under the minimum angle (if necessary, the angle can be manually adjusted) the Doppler beam is directed to the blood vessel. There was a recording from 3 to 6 wave curves of uniform shape and a quality (filters between 50 and 100 hertz were used to help) performance of the measurement. After registering the peak systolic and end diastolic frequency with computer program we recognized the average value of the IR (index of resistance) and S / D (the ratio systole and diastole).

Based on the purpose, the objectives of the survey, the volume and type of data we used the following statistical methods:

We applied descriptive statistics to describe the results. The results are presented by arithmetic mean, standard deviation and standard error (mean, Std. Deviation and Std. Error).

We made an evaluation of the percentage and the frequency allocations in qualitative data and we grouped the data performed by an alternative analysis.

For comparison of the results in more than two independent samples analysis of variance (ANOVA) and the Kruskal Wallis Test were used.

For comparison of two independent samples we used criteria and the Mann–Whitney U test. For comparisons of the results in two-dimensional distributions we applied the criterion χ^2.

In order to search for a relationship between variables – Pearson and Spearman's rho;

In order to illustrate the addictions we used the capabilities of the graphical analysis.

The level of significance of the null hypothesis was accepted at $P < 0.05$. The data were processed by statistical software package SPSS ver.16.0.

Results

We studied 71 pregnant women divided into two groups. Thirty-six women were diagnosed with threatened abortion and thirty-five women had a normal pregnancy. The threa-

tened abortion group had an average age of 27.03, the healthy group age was 26.04 years. We did not find a significant difference between the two groups by age P > 0,05 (t = 0.44) (Table 1 and Table 2). We did not find an association between the pregnancy outcome and the values of the difference between IR and S / D of the left and right uterine artery P > 0.05 (Table 3).

Table 1

Mean age of patients in the two examined groups

Groups	**N**	**Mean**	**Std. Deviation**	**Std. Error Mean**
Healthy	35	26,40	5,637	0,953
Threatened abortion	36	27,03	6,245	1,041

Table 2

Subdivision of age in two groups

Groups		Age		
	below 20 yrs	21–30 yrs	> 31 yrs	Total
Healthy women p ± Sp	7	21	7	
	20,0 ± 6,8	60,0 ± 8,3	20,0 ± 6,8	35 100,0%
Threatened abortion number p ± Sp	9	16	11	36
	25,0 ± 7,2	44,4 ± 8,3	30,6 ± 7,7	100,0%
Total number p ± Sp	16	37	18	71
	22,5 ± 5,0	52,1 ± 5,9	25,4 ± 5,2	100,0%

Table 3

Obtained results for the evaluated indicators

Indicators	**groups**	**N**	**Mean**	**Std. Deviation**	**Std. Error**	**u**	**P**
IR.DEX	Healthy	35	0,79317	0,110161	0,018621	1.61	> 0.05
	Threatened abortion	36	0,75128	0,109488	0,018248		
S.D_DEX	Healthy	35	5,61257	3,114046	0,526370	0.65	> 0.05
	Threatened abortion	36	5,08083	2,410583	0,401764		
IR.SIN	Healthy	35	0,78503	0,089534	0,015134	1.00	> 0.05
	Threatened abortion	36	0,76189	0,104281	0,017380		
S.D_SIN	Healthy	35	5,29657	2,641164	0,446438	0.93	> 0.05
	Threatened abortion	36	4,80000	1,575601	0,262600		
IR=IRsin-IRdex	Healthy	35	0,05489	0,042280	0,007147	**4.37**	**<0,001**

Discussion

When we compared the two groups, there was a difference concerning the IR = SIN-DEX $P < 0.001$. The controls have smaller averages compared to the experimental group. Probably the difference in blood flow between the left and right uterine artery leading to dissonance in the blood supply to respective sections of the large decidual cells of the superficial layer of the endometrium and consequently, appears to be a difference in the delivery of the progesterone from the corpus luteum. After the first trimester the placenta produces enough estrogen and progesterone and the role of the corpus luteum is ignored. In the first 12 weeks of gestation, the stability of the uterine lining is extremely dependent on progesterone, which is secreted by the corpus luteum. If one part of the endometrial cells does not receive adequate amounts of estrogen, progesterone and growth factors, which require good blood supply, those layers of the decidual lining begin to drop, and it bleeds ex utero. Similar processes lead to the menstrual bleeding of the endometrial cycle, but there is a total exfoliation of the endometrium because of the complete atrophy of the corpus luteum, and a total cessation of its hormonal secretion.

All vessels that provide blood flow to the uterus have extensive anastomoses with each other, causing even violations in the bloodstream of the uterine artery and may not drastically respect the outcome of the pregnancy. Establishing a difference in the blood supply would rather orient us concerning which women would expect complications such as bleeding and pain, but it enables us to forecast the outcome of pregnancy. These women may be advised to reduce physical stress, and if their work requires it to be reassigned from the beginning of the pregnancy. The increased physical activity is responsible for the redistribution of a large part of the circulation to the lower extremities, which will lead to an even greater deterioration in the uterine blood flow.

There is no correlation between the difference in the blood flow to the left and right uterine artery and the interruption of pregnancy. From the very beginning of the pregnancy, one of the most notable changes in the uterus that occurs is constant and progressive increase in blood flow. Depending on the location of the trophoblastic reaction a possible increase in the blood flow in one uterine artery occurs before the improving of the perfusion of the contralateral. The results are similar from the conducted four studies of uterine artery blood flow in women with threatened miscarriage. The authors concluded that Doppler arteria uterina is not beneficial positive predictor of differrentiation concerning which case of the threatened abortion will end with the termination of pregnancy before 20 GS [9–11]. The results obtainned do not match those obtained by Manal and colleagues at the University Hospital in Zagazig [12]. One should not ignore the fact that they use the combined study of human chorionic gonadotropin and Doppler velocimetry of the left and right uterine artery. The Doppler study of the uterine artery improves the specificity and sensitivity of the prognostic value of the dynamics in the levels of human chorionic gonadotrophin on the outcome of early pregnancy. There was not shown a significant correlation with statistical processing of the results of the increased difference in the index of resistance and the ratio S / D in pregnant women diagnosed with threatened abortion compared to the control group.

Conclusion

Apparently the Doppler study is not convenient to independently distinguish risk groups for pregnant miscarriage of normal ongoing pregnancies, but it would be useful as an additional study.

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Р е з и м е

ДОПЛЕР ВЕЛОСИМЕТРИЈА НА АРТЕРИИТЕ НА МАТКАТА: ТЕСТ НА РАН СКРИНИНГ ЗА СПОНТАН АБОРТУС

Илијан Трајанов, Елена Димитракова

УМБАЛ Св. Ѓорѓи – Пловдив,
Оддел за акушерство и гинекологија,
Катедра за акушерство и гинекологија, Р. Бугарија

Вовед: Според СЗО, спонтаниот абортус е дефиниран како „губење на бременоста пред возраста од 20 недели од бременоста или тежина на фетусот под 500 g“. Само 50-60% од сите зачнувања не успеваат да преживеат 20 недели од бременоста. Се проценува дека околу 75% од сите неуспешни зачнувања се должат на неуспешната имплантација и не се пријавени како спонтани абортуси.

Материјал и методи: Извршивме проспективна опсервациска студија на Одделот за акушерство и гинекологија на Медицинскиот универзитет во Пловдив меѓу октомври 2014 и септември 2015 година. Проучувавме две групи бремени жени: жени со ризик за абортус и оние со нормална бременост. Сите жени беа подложени на трансвагинална евалуација со ултразвук на протокот на артериската крв на матката. Ја меревме најголемата систолна и крајната дијастолна брзина и го пресметувавме просечниот IR (индекс на отпор) и S/D (однос систолен и дијастолен).

Резултати: Не постоеше поврзаност помеѓу исходот на бременоста и средната разлика помеѓу артериските IR на матката и S / (P > 0,05).

Заклучок: Во нашата студија, со доплер ултразвукот не може да се направи разлика помеѓу жените со ризик за спонтан абортус и нормалните жени. Понатамошни студии се потребни за да се одговори на ова прашање.

Клучни зборови: Доплер ултразвук, индекс на отпор, бременост, ризик од абортус

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GOAL DIRECTED BEHAVIOR AND DYSLEXIA

Giuseppe Augusto Chiarenza

Centro Internazionale Disturbi di Apprendimento, Attenzione e Iperattività, (CIDAAI), Milano, Italy

Corresponding Author: Giuseppe Augusto Chiarenza, Centro Internazionale Disturbi di Apprendimento, Attenzione e Iperattività, (CIDAAI), Milano, Italy

Abstract

Goal directed behavior is explained by two approaches: the first, which can be named as cybertetic (behavior is wieved as homeostatic and reflexive), and second, as cognitive approach, a learned response, (skills developed by whaching the behavior of another individual).

The aim of the paper is to present a noninvasive method described as an interaction of human beings with environment, recording the electrical activity of the brain from the human scalp.

Obtained results are in agreement of psychological theories that place at determined levels of age the acquisition of the capacities of abstract thinking and with the functional neuroanatomic studies according to which biological maturation is necessary for learning processes to develop. An acquired level of learning is in close relationship with the maturation level of the cerebral structures.

Keywords: directed behavior, dyslexia, oscilloscope

Goal directed behavior is teleologically purposive. It often seems to be a search for a goal previously defined by a model or idea in the brain (Granit 1977). Exactly how this goal is achieved, however, can vary. Formulation of strategies, their realization through actions, evaluation of results and comparisons with past experiences can be explained in different ways. Fundamentally, there are two approaches to account for purposive behavior. One is the cybernetic approach, which views behavior as homeostatic and largely reflexive (Wiener 1971). According to this model, an organism is endowed with innate patterns of behavior explained as reflexes triggered by the stimulus or as the reduction of drives.

Numerous observations have established the great power of this approach to account for many complex as well as simple behaviors in humans and other mammals, as well as in insects, fishes and birds. As we ascend the philogenetic scale, the cybernetic approach becomes unsatisfactory. Behaviors emerge that cannot be explained plausibly as innate or conditioned reflexes. For example, response learned to a specified stimulus can be elicited by generalization to a novel stimulus that activates very different afferent pathways; learned responses can be executed by using muscles that achieve the desired purpose but which were never before used for that behavior; animals and humans can learn new skills by watching the behavior of another individual.

In 1980 the Italian neurophysiologist Giacomo Rizzolatti and his colleagues have found that neurons in the rostral-ventral part of the premotor cortex of the monkey (area f5 corresponding to Brodmann 44 Broca's area in man) not only respond when they perform certain gestures like taking some peanuts, but even when they see other monkeys to perform the same gesture. These cells are called mirror neurons (Rizzolatti and Fogassi 2014). This system of neurons allows a person to recognize the

gestures of others and their meaning thus contributing to social contact and interaction. Researchers who have studied mirror neurons have identified, since 1990, two new features of the motor system. First, it has been shown that the motor system is not active only during the actual execution of the movement, but some parts of this system are active even when the action is imagined. Secondly there are neurons that are active not only when performing a well circumscribed but when we look at another perform the same action or when we hear sounds that belong to that action. Mirror neurons therefore represent the multimodal nature of the actions, play a role in the concept of what a monkey or a man is doing and can distinguish various types of action to help their planning. The observation of the movements and actions of others also encourages imitation. Mirror neurons foster understanding of motor behavior of others, the imitation of gestures and learning actions.

The authors show that the mind is basically relational and deal with some of the practical consequences of this, for example that a child will not learn to speak by watching television.

Therefore, as one tries to explain such behaviors, another approach must be considered that assumes that higher animals possess consciousness, have ideas and can think about the significance of the information for the environment. The newborn individual can survive only by the action of species specific reflexes and homeostatic processes. As the individual develops, other mechanisms may serve to distribute information to additional regions of the brain.

In this manner multisensory and multivariate transactions begin to modulate genetically specific processes that were initially more simply determined and a cognitive model of the environment is built gradually, incorporating features of individual experience as well as species characteristic features.

One can view behavior as resulting from a cognitive process which involves an interaction between neural events representing the previous experience, the present state of the individual and the occurrence of particular features in the environment. Such behaviors consist of the attempt to match new experience against an idea reflecting past experiences. It is cognitive rather than reflexive, involving thinking rather than activation of specific neural pathways constituting stimulus response circuits (John 1980).

The aim of this paper is to present a non invasive method with which we can describe the interaction of human beings with environment, recording the electrical activity of the brain from the human scalp.

When it is the subject that begins a voluntary action to act upon or to interact with the environment and when the consequent modifications can be evaluated and compared with the preprogrammed strategies and past experience, we have all the necessary and sufficient conditions to observe the interaction between the environment and the human being and how the relative information are represented in the brain. When a subject is engaged in a self-paced, voluntary and skilled task and receives a visual feedback in real time about his motor performance, exactly as it happens in our days with videogames, a characteristic sequence of brain macropotentials can be recorded from the scalp both in children and adults.

The task is self-paced, voluntary, goal-directed and interactive. To perform it adequately, it requires the following skills: bimanual coordination, bimanual ballistic movements, adaptive programming, learning a proper timing and performance improvement. The task provides online knowledge of results and feedback (Chiarenza et al., 1982a, 1982b). In particular, the subject sat in an armchair 70 cm in front of an oscilloscope and held a joystick-type push button in each hand. The excursion of the button was 5 mm. The task consisted in starting the sweep of the oscilloscope trace with the left thumb and stopping it in a predetermined area of the oscilloscope by pushing the other button with the right thumb. The sweep velocity was 1 mm per ms and the target area corresponded to a time interval between 40 and 60 ms. The brain electrical activity associated with this task is called movement-related brain potentials.

On the basis of the spatial-temporal characteristic of these potentials and their relationship to electromyographic activity and perfor-

mance it has been proposed that there are four successive time-periods during a skilled task (Figure 1). A premotor period that precedes the EMG activities is characterized by the Bereitschaftspotential (BP) (Kornhuber and Deecke 1965). Its amplitude is higher during skilled and goal oriented tasks than during unskilled and not purposive ones (Papakostopoulos 1978). Its scalp distribution is prevalent in the central and precentral areas. During unimanual action the BP is predominantly on the contra lateral hemisphere, while during bimanual skilled action is symmetric in the right hand subjects but not in the left hand subjects (Papakostopoulos 1980a). This potential seems to reflect the strategic organization of the ideokinetic elements necessary to achieve the goal. The sensory motor period starts from the onset of EMG activities and lasts for 80 ms after the peak of the EMG. It is during this period that behaviour is manifested. During this period the electrical cerebral activity is dominated by a further negative potential, Motor Cortex Potential (Papakostopoulos and Crow 1984). This potential seems to be an index of reafferent peripheral activity. This potential is followed by the N100, the expression of the early stages of visual perceptual processing of the brain response to the visual stimulus, i.e. the oscilloscope's sweep.

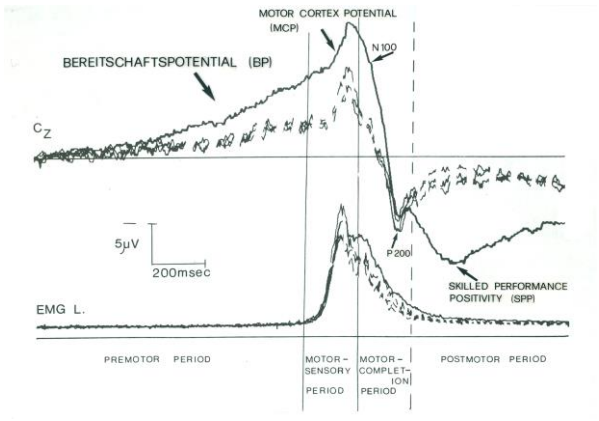

Figure 1 – Movement related potentials and left forearm electromyograms during unskilled (- - -) and skilled (– actions, recorded from the vertex (Cz). Modified from Papakostopoulos 1978 J. of Physiology

The motor completion period is characterized by the decline of the EMG activities and by a positive peak with a latency of 200 ms: P200 (Vaughan et al. 1968). During this period the visual evoked potential, evoked by the sweep of the oscilloscope, during a motor perceptual task is suppressed in the precentral areas (Papakostopoulos et al. 1975).

The postmotor period is characterized by the return of the electromyographic activities to the preceding rest conditions and by a presence of a large positive potential with a latency of 460 ms denominated skilled performance positivity (SPP) (Papakostopoulos 1980b). This potential has a central and parietal scalp distribution. It is observed in healthy subjects, when they are asked to perform a motor perceptual task that requires precision, timing, and improvement of performance level by providing adequate real time feedback information on the outcome. The SPP is absent during unskilled tasks and when adequate feedback is not provided to the subject (Papakostopoulos 1986). From a chronological standpoint it may be said that the SPP coincides with the subject's awareness of the success or failure of his performance. This positivity appears when the subject looks for information about his outcome, that it is to say, information relevant to the efficiency of his psychomotor pre-programmed organization. The knowledge of the outcome is likely to be used to influence future actions.

The various stages of organization of goal directed behaviour can be appreciated following the developmental features of the Movement Related Potentials from childhood to adult life. (Figure 2)

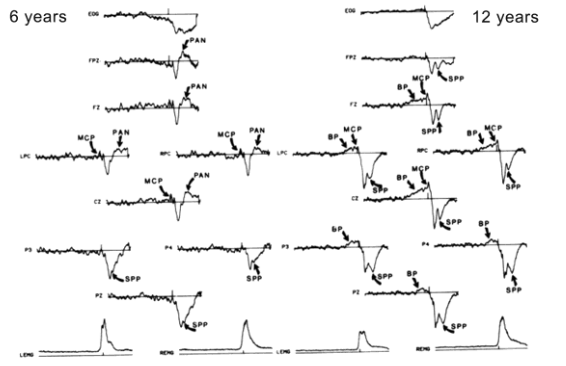

Figure 2 – Figure 2 shows the averaged movement-related brain potentials during the execution of a complex motor perceptual task, in normal subjects of 6 and 12 years old, along with (bottom) left arm (LEMG) and right arm (REMG) electromyographic activity. In this and following figures, BP = Bereitschaftspotential; MCP = Motor Cortex Potential, SPP = Skilled Performance Positivity; PAN = Post Action Negativity

In children of six-seven years, only MCP N100 and P200 are present; they represent the sensory information coming from the subject and environment. Therefore, in spite of the awareness of the movement carried out by the children, the BP characteristic of the preparatory period, is absent in these young subjects. At these ages, during the postmotor period, another potential is recorded with a negative polarity, denominated Post-Action Negativity (PAN) most prominent in the frontal and central regions, while the SPP is present only in the posterior areas. This negativity might indicate the preconceptual stage of the representational intelligence in which the reality depends and extinguishes in the moment of the immediate perception (Inhelder and Piaget 1958). In these children the surprise or the novelty about the outcome of the performance predominates. PAN decreases with age and disappears by the age of 8–9; in parallel, the BP and SPP start to emerge in the frontal and central areas. During this period of age, the children acquire the adult capacity of abstract thinking. They conceive many possible ways in which they could operate and many alternative ways in which they could be better. We suppose that these different possibilities, these strategic qualities, i.e. the intentionality of action to achieve a goal are reflected in the BP amplitude. Furthermore after eleven years of age thinking is propositional (Inhelder and Piaget 1958). In the period of formal operation the children take the outcome of their performance, put them in sentence form, and begin to find relationships between sentences. According to this view, the emergence of SPP in frontal areas corresponds to a new way of brain functioning: the outcome and the knowledge of results are used to match them with the projects and past experience in order to improve the goal directed behaviour.

Ageing affects these potentials. In particular, the MCP slowly decreases in amplitude and disappears, the SPP also decreases in amplitude and slightly increases in latency. The BP seems not to be affected by ageing (Chiarenza 1993).

One clinical aspect of dyslexia that has been little explored is the lack of fluency and prosody during reading, namely aspects related to the organization of movement. Various difficulties in the execution of neuromotor acts, such as simple repetitive movements or alternating complex movements such as bimanual coordination have long been observed in dyslexic children. Furthermore, clinical signs such as dysrhythmia, the presence of synkinetic movements, have often been described in dyslexic individuals (Adams et al.1974, Kennard 1960, Rutter et al., 1966, Stine et al., 1975, Wolff and Hurvitz, 1973, Denckla 1973). These difficulties were interpreted as a disorder of the temporal organization of motor skills (Klicpera et al., 1981, Denckla 1973). These observations were also recently confirmed by Punt et al. (2010), who reported that 87% of dyslexics exhibit minor neurological dysfunction, especially in fine manipulative skills, the regulation of muscle tone, and the excessive presence of associated movements. All of these observations support the hypothesis of an important involvement of cerebellar function in reading and writing. It is therefore possible to maintain that we are facing a considerable heterogeneity in the dysfunction of skills in dyslexic children, not only visual and auditory, but also motor: Nicolson and Fawcett (2005) stated that children with dyslexia show difficulties when they have to acquire new skills quickly and fluently, and when they have to assemble two or more actions.

In our opinion, the reason for the neglect of the motor component of dyslexia lies in the fact that all experimental designs, both neurophysiological and behavioural, were built on the stimulus-response model. This is able to describe *only* phenomena that occur in the interval between the stimulus and the response of the subject, without being able to observe the phenomena before the onset of the stimulus and after the onset of the response. In this way, only phenomena related to the processing of auditory and visual stimuli have been described.

To study in detail the organization of a motor act, both simple and complex, such as reading and writing, it is necessary to devise other experimental models that take into account not only what happens during the processing of a stimulus, but also phenomena that take place before and after it. This is the fundamental and unique contribution of movement related potentials. Therefore, the performance

of a complex perceptual-motor task appears to be particularly well suited to provide information on those systems and subsystems that regulate and organize the functions of reading and writing (Chiarenza et al., 1982a). In addition, since the assumptions in dyslexia predict poor reading skills, a test of perceptual-motor skills, which lies outside the domain of reading, would be particularly suitable to test this hypothesis.

Using this task, we have shown that dyslexic children, besides being slow and not very accurate from a behavioural point of view, present a deficit of programming movements, a deficit of visual and kinaesthetic sensory processes, a deficit and a reduced capacity to evaluate their performance and correct their errors (Figure 3, 4, 5) (Chiarenza 1990, Chiarenza et al. 1982a, 1982b, 1986, 2006, Casarotto et al. 2007).

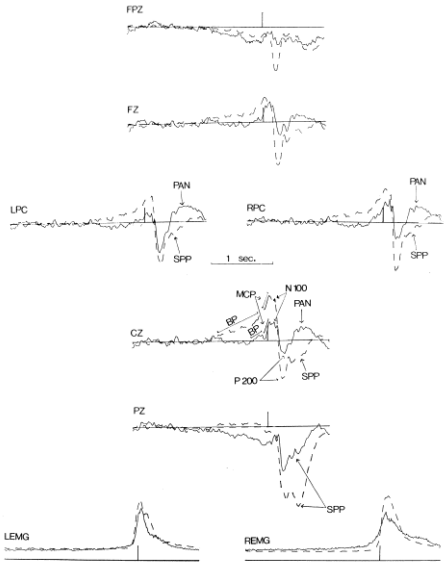

Figure 3 – Figure 3 shows the average movement-related brain potentials during the execution of a complex motor perceptual task, in normal subjects (thin line) and dyslexic subjects (thick line), recorded in Fpz (Fpz = middle prefrontal), Fz (Fz = middle frontal), and Pz (Pz = middle parietal), along with (bottom) arm electromyographic activity (EMG). Dyslexic subjects showed a reduced BP amplitude of very short duration, indicating a non-adequate preparation. MCP reduced amplitude, indicating a lack of kinesthetic processing; N100 and reduced P200 amplitude indicating a deficit of visual perception and reafferent activity respectively; SPP reduced amplitude on the parietal regions and the presence of PAN on the central and frontal regions suggesting a reduced ability to evaluate target performance and non-target performance respectively (for more details see Chiarenza Journal of learning disabilities, 1990)

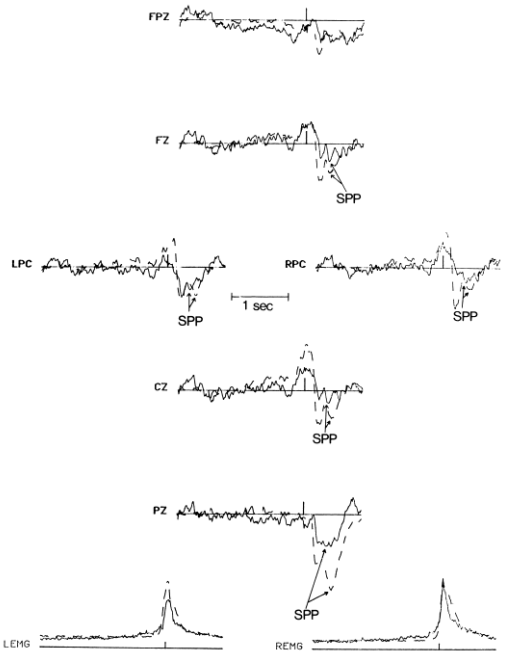

Figure 4 – Figure 4 shows the average of the movement-related brain potentials associated with target performance in normal subjects (dashed line) and dyslexic subjects (continuous line). The potential associated with knowledge of results (SPP) is present in all areas of the brain but of reduced amplitude

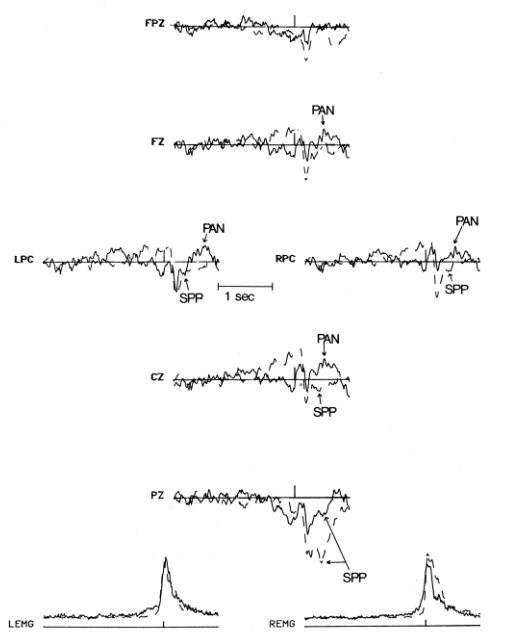

Figure 5 – Figure 5 shows the average of the movement-related brain potentials associated with non-target performance in normal subjects (dashed line) and dyslexic subjects (continuous line). The potential associated with the assessment (SPP) is only present on the parietal areas (perceptual activity), while on the central and frontal areas a negative potential (PAN) is recorded, expression of the failure to process the error (Chiarenza 1990)

These studies clearly demonstrate that dyslexia is not only a phonological or a gestalt deficit, but also a praxic disorder in which praxic abilities, such as motor programming, sequential and sensory-motor integration and evaluation processes, are required and somehow defective in dyslexia (Chiarenza et al. 2014). Dyslexia can be defined from a psychophysiological point of view, as a disorder of programming and integrating ideokinetic elements, associated with a deficiency in the fast processing and integration of sensory information, with a reduced efficiency of error systems analysis. All these phenomena occur at different levels of the central nervous system and at different times during reading (Chiarenza 1990; Chiarenza et al. 1982a, 1982b, 1986).

All these results are in agreement with the psychological theories that place at determined levels of age the acquisition of the capacities of abstract thinking and with the functional neuroanatomic studies according to which a biological maturation is necessary for learning processes to develop. An acquired level of learning is in close relationship with the maturation level of the cerebral structures. When these structures due to different conditions are not able to accomplish their functions, the behaviour is always affected during its preparatory period, or its realization or its evaluation period.

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Р е з и м е

ЦЕЛНО НАСОЧЕНО ОДНЕСУВАЊЕ И ДИСЛЕКСИЈА

Цузепе Аугусто Кијаренца

Меѓународен центар за проучување на нарушувањата, вниманието и хиперактивноста, Милано, Италија

Целното насочено однесување се објаснува со два пристапа: првиот кој може да се нарече кибертетски (на однесувањето се гледа како на хомеостатско и рефлексивно) и, вториот, како когнитивен пристап, научен одговор, (вештини развиени со гледање на однесувањето на друго лице).

Целта на трудот е да се претстави неинвазивна метода опишана како интеракција на луѓето со средината, снимање на електричната активност на мозокот од човечкиот скалп.

Добиените резултати се во согласност со психолошките теории кои на утврдени нивоа на возраст го ставаат стекнувањето на капацитетите на апстрактно мислење и со функционалните невроанатомски студии според кои биолошкото созревање е неопходно за да се развие процесот на учење. Стекнатото ниво на учење е во тесна врска со нивото на созревање на церебралните структури.

Клучни зборови: насочено однесување, дислексија, осцилоскоп

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EMOTIONAL HEALTH IN CHILDREN AND ADOLESCENTS WITH CYSTIC FIBROSIS

Nada Pop-Jordanova[1], Aneta Demerdzieva[2]

[1] Macedonian Academy of Sciences and Arts, Skopje, R. Macedonia
[2] Acibadem Sistina Hospital, Skopje, R. Macedonia

Corresponding Author: Macedonian Academy of Sciences and Arts, Bul. Krste Misirkov, br. 2, 1000 Skopje, R. Macedonia, E-mail: popjordanova.nadica@gmail.com

Abstract

Although modern therapeutic procedures have considerably improved the survival and the quality of life of children with cystic fibrosis, the relevant psychological aspects have been still insufficiently considered similarly to the other chronic diseases.

The aim of this research was to evaluate the emotional health: psychological characteristics and adjustment of CF children and their family coping.

The study comprises 25 CF children, mean age 13.13 ± 2.29 years (23 boys and only 2 girls), selected from total 60 actually treated children for CF. Children were examined in the period of improved health conditions (without superinfection, wheezing or gastrointestinal problems). Obtained results are compared with a control group of 25 healthy children of the same age, selected by random from primary schools.

The psychometric instruments used were: Kohs Design Test, Child Behavior Checklist, Eysenck Personality Questionnaire, General Anxiety Scale, Emotional Profile Index, MMPI-201 and Human Values Test, together with two projective tests of drawing (Machover and Corman).

The unexpected good psychological results obtained from psychometric instruments could be explained by the fact that CF children accept the real situation and express vivacity. However, their deep feelings of fear impose on them high level of self-control and resistance. The results obtained for CBCL presented CF children as immature, with accentuated aggressiveness in interpersonal relations. The most important problem is related to the delay of puberty changes, leading to low self-esteem.

Generally, family members cope relatively well with the disease in children, in spite to discrepancies in mother/child reports for child psychopathology. Divorces also occurred in some families.

Psychological support for both, children and family members are necessary. The need for a holistic approach in the assessment and treatment, including biofeedback techniques was pointed out.

Keywords: cystic fibrosis, chronic disease, psychology, holistic approach

Introduction

Cystic Fibrosis [CF] is a genetic disease that can be life threatening. It is considered to be the most common genetically based disease in white race population with an incidence of 1 in 1,500–2,500 live births, while in other races this ratio is 10 to 50 time lower. In Macedonia the incidence of CF is estimated to be 1 in 3,000 live births.

CF primarily affects the digestive system and the lungs, but can be systemic in nature as well. It develops because of a defective gene that forces mucus to build up in the lungs and other organs in a very thick layer. This mucus is difficult to process and it can begin to clog up airways so that it becomes difficult to breathe. In addition, many other complications such as chronic liver disease, diabetes, distal

intestinal obstruction, nasal polyps, rectal prolapse, pancreatitis, infertility, cardiac failure etc. may also occur.

In early childhood the meconium ileus, malnutrition, diarrhea and repeated respiratory difficulties and infections are the most common signs. Although the early diagnosis is crucial for the prognosis and life expectancy, the number of cystic fibrosis cases that are diagnosed by the age of 2 is only 5.75%.

The major diagnostic test is the sweet chloride determination (over 60 mmol/l), but after 1989 DNA testing is also used. The most frequent CF gene mutation is ΔF508, present in about 70% of CF chromosomes worldwide, but altogether over 800 associated mutations have been described [34].

In our country are available genetic diagnosis as well as the antenatal carrier determination.

Medical therapy in CF is very extensive in order to maintain optimal health and to improve survival. Additionally, strong physical therapy together with balanced nutrition are very important.

All patients, as well as all family members must be educated and encouraged to participate in therapeutic procedures. Thus, patient's coping strategies and adaptation are of particular importance [12, 13, 15, 17, 19, 27].

Patients diagnosed with cystic fibrosis in the 1980s typically had a life expectancy of fewer than 20 years. Patients today can expect an average life expectancy of about 40 years. Babies born today with cystic fibrosis have an increased life expectancy from just four years to more than 30 years. Figure 1 shows changes in life expectancy starting from 1930 until nowadays.

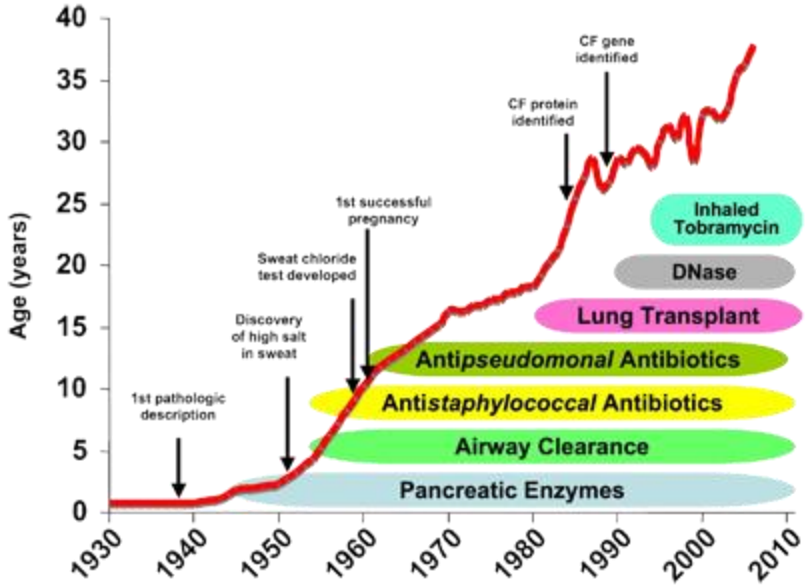

Figure 1 – Life expectancy in ages for CF patients
(Adapted From 2005 Annual Data Report to the Center Directors. Cystic Fibrosis Patient Registry, Bethesda)

Studies measuring psychological distress in individuals with cystic fibrosis have found high rates of depression and anxiety. Psychological symptoms in both individuals with CF and parent caregivers, have been associated with decreased lung function, lower body mass index, worse adherence, worse health-related quality of life, more frequent hospitalisations and increased healthcare costs [37]. However, Thomson et al [35] note that CF children manifest fewer depressive and dysthymic diagnoses than psychiatrically referred children.

Important psychosocial problems in CF children's adjustment comprise of: a) acceptance of the disease, including understanding and compliance, b) freedom from severe psychopathology, c) normal or age-appropriate personality functioning and d) age-appropriate functioning in school, with family and peers. Maladjusted children manifest excessive anxi-

ety, depression, school-related behavior problems or disciplinary problems at home [26, 36].

In the school period physical, intellectual and emotional changes are happening simultaneously. Peer group seems to be probably more important than ever, and it is normal to want to fit in, and not stand out as 'different'. Having CF, with all its challenges and time-consuming treatments can be a huge burden. Although patients could go through periods of depression, it must be remembered that they are not alone. Teens with CF report being depressed for many reasons such as: feeling physically unwell, being frustrated by the challenges of endless daily treatments, experiencing negative side effects from medications, missing major life events (sports, parties, concerts, proms) due to hospitalizations, and feeling different from their peers.

Children with CF may be teased or picked on at school because they can be underweight and small for their age, and might have a persistent cough. Taking tablets and capsules with meals and eating a different diet from classmates can also be embarrassing. Physiotherapy is time consuming, sometimes at the expense of a child's social life, although children with cystic fibrosis often find supportive friends who help with care and physiotherapy.

During teenage years there is a chance that children may neglect their physiotherapy and diet. Some people with CF experience delayed onset of puberty, which may cause anxiety or insecurity. Teenagers may need sympathetic treatment and counselling to help them deal with some of these issues.

Cystic fibrosis requires a level of special involvement from teachers, which could include consultations with parents or even practical help.

The important issue is related to parental coping and adaptation to CF disease in children. Many parents cope relatively well and do not manifest major psychopathological problems. But there are critical times during which parental functioning is trained, especially the role of mother [24]. Most of the family problems appear in the first year after the diagnosis of CF. Potential crises times for parents are cited in the following [32]:

- Diagnosis and explanation of the meaning of CF
- Future pregnancies
- Learning to cope with new demands, which may occur each time a new treatment is instigated
- First course of intravenous antibiotics and isolation of new bacteria from the sputum
- Problems with school and employment
- Increased need for antibiotics
- Change from pediatric to adult care
- Loss of responsibility such as allowing the children to perform their own treatment
- Decision about possible transplantation
- Deteriorating health of a sibling with CF, friend, or one's own child
- Death of sibling with CF, or own child
- Bereavement

Parents of CF children may express signs of depression, anxiety but also anger and hostility. Within a few years the family has more or less adjusted to the disease and equilibrium is restored, but still the process of stress and coping is dynamic and continues through the life span of the child and even after his death.

The aim of this study is a comparative evaluation of the personality profiles of school age children with cystic fibrosis with special attention to internalizing symptoms, and relating them to maladjustment and coping. Some controversial aspects of family functioning and child/mother discrepancies are also considered.

Subjects and methodology

The study comprises 25 CF children, mean age 13.13 ± 2.29 years (23 boys and only 2 girls), selected from total 60 actually treated children for CF. Children were examined in the period of improved health conditions (without superinfection, wheezing or gastrointestinal problems). The obtained results are compared with a control group of 25 healthy children of the same age, selected by random from primary schools.

The used psychological battery comprised Child Behavior Check List (CBCL), Kohs Design test, Eysenck Personality Questionnaire (EPQ), General Anxiety Scale (GASC), Emotional Profile Index (EPI), Human Values Test

(HVT) and two projective drawing tests – the drawing man test (Machover) and the drawing family test (Corman). Only for adolescents we applied Minnesota Multiphase Personality Inventory (MMPI-201).

CBCL [1] is designed to obtain the parent's descriptions of their own child behavior in a standardized format. There are 118 behavior problem items plus spaces for parents to write and score additional physical problems with no known medical cause. Two broadband grouping are in the focus: internalized and externalized. They reflect a distinction between fearful, inhibited, over controlled behavior and aggressive, antisocial, under controlled behavior. The profile can contribute to a formal diagnosis by showing the degree of child's deviance in behaviors that parents are more likely to observe than clinicians, as well as help to structure effective training. CBCL the most used test for selection of behavior problems in chronically ill children but the agreement between the scales and the mental diagnosis was shown to be moderate [5, 15].

Kohs Design Test [20, 38] presents a simple test for global intellectual functioning. The results are correlated with the logical thinking (analytical-synthetic performance) of the person. The standardization in Macedonia was made in the early 1978.

EPQ [14, 22] evaluates the four classical characteristics of the personality: N (level of emotional stability/neurosis); E (dimension of extraversion/introversion); P (psychotic behavior/psychopathy) and L (degree of dissimulation or social adaptability). Our previous experience with this psychometric test confirmed the validity, reliability and discriminativity of the obtained results, especially in preadolescents (10–12 years).

EPI [3] shows the emotional structure of the patients in correlation with their personal characteristics. The basic theoretical concept of the test is the hypothesis that the personality traits are the result of primary emotions and emotional states. The obtained emotional profile that indicates the main conflicting area of the person is defined through eight dimensions (related to eight respective emotional states): incorporation (acceptance), non-control (impulsiveness), self-protection (fear), deprivation (sadness), opposition (refusal), aggressiveness (destruction) and reproduction (vivacity), while bias represents the scale for assessment of socially favorable answers.

GASC [33] is a simple questionnaire chosen to show the actual anxiety level, correlated with fears from different situations, persons and objects.

The drawings are used as projective tests in addition to other psychometric instruments [4, 11, 23]. Two projective techniques are used: Machover's analysis of the man drawing and Corman's analysis of the family drawing. Machover's man drawing test is selective for actual problems and conflict within the child himself; Corman's family drawing shows the social and intimate relation of the family members through the development of the patient as well as in the actual situation. Our previous experience in the psychological assessment of children with organic diseases through projective techniques is very positive [28, 29].

HVT [31] gives quick overview of the motivational structure of the personality related to the super-ego component. The hierarchical values are correlated to the real personal needs. It is also standard for assessment of the interplay of social situations and the self.

MMPI-201 [2, 16] contains ten clinical scales: Scale 1- Hypochondriasis scale which measures a person's perception and preoccupation with their health and health issues; Scale 2- the Depression scale measures a person's depressive symptoms level; Scale 3- the Hysteria scale measures the emotionality of a person; Scale 4- the Psychopathic Deviate scale measures a person's need for control or their rebellion against control; Scale 5- Paranoia scale measures a person's inability to trust; Scale 6- the Psych asthenia scale measures a person's anxiety levels and tendencies for somatization and obsession; Scale 7- the Schizophrenia scale measures a person's unusual/odd cognitive, perceptual, and emotional experiences, and Scale 10- the Mania scale measures a person's energy, euphoria or hyperactivity.

The three scales L, F and K are validity scales and measure the readiness of the responders to this kind of examination. The L

scale refers to rigidity or naiveté of responder's approach to the test material; the F scale refers to confused thinking/ lack of understanding the questions or malingering; the K scale refers to responses chosen to be socially acceptable.

Raw scores on the scales are transformed into a standardized metric known as T-scores (Mean or Average equals 50, Standard Deviation equals 10), making interpretation easier for clinicians. Before the analysis of clinical scales, some criteria should be satisfied: L and K scales must be with the score ≤ 70 and F scale ≤ 80. A significant advantage of the MMPI over other self-report and observer rating scales is that it provides valid and reliable estimates of response bias.

This type of psychometric battery was chosen to obtain the global intellectual, emotional, behavioral and social functioning of CF children.

Results

The global intellectual functioning of CF children is evaluated with Kohs Block-Design test. The obtained mean score is IQ = 104 ± 32.78 which means that children had normal intellectual level.

CBCL obtained from mothers showed 'normal' profile for the children's age (Figure 2). However, three aspects of behavioral problems are more expressed: aggression, depression and compulsivity, still below the 65th percentile (normal T-scores).

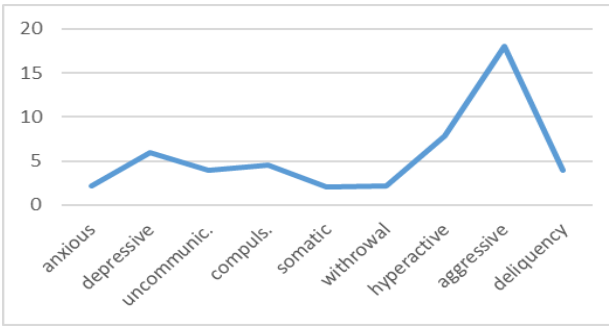

Figure 2 – CBCL profile for CF children

The results obtained for EPQ in CF patients compared with the control group are presented on Table 1.

Table 1

EPQ in patients with CF and control group

Group	**P**	**E**	**N**	**L**
CF	6.67 ± 2.90	15.91 ± 2.54	11.67 ± 4.23	15.0 ± 3.81
Control	11.87 ± 6.23	13.16 ± 5.75	13.84 ± 5.31	12.64 ± 4.62
Student t-test	t = 2.87 p < 0.05*	t = 1.66 p > 0.05	t = 1.18 P > 0.05	t =1.45 p > 0.05

Generally, patients with CF are similar in scores obtained for extroversion, neuroticism and lie dimension of EPQ with the control group. Only P scores are statistically lower than in healthy children, which corresponds to lower psychopathological traits.

The score obtained for GASC is M = 17.17 ± 11.69 (out of total 35), which corresponds to the moderate degree of actual anxiety. The large standard deviation is related to large differences in the level of anxiety between patients. For example, two of the examined children manifested very high actual anxiety (obtained scores were 32 and 33 respectively); the reason was presumed to be the socioeconomic deficiencies in the families.

As it is well known, the treatment and the diet of CF children are quite expensive, so that in a situation of unemployment the procedure may be compromised, influencing malnutrition and emotional instability. However, generally, chronically ill children are overprotected, in attempt to satisfy most of their needs and wishes.

The results obtained for the Emotional Profile Index (Figure 3) showed high bias scores related to dissimulation. Incorporation and impulsivity are pretty high, which is in accordance with the results obtained with other tests. The scores obtained for aggression and depression are also accentuated, which corresponds to the insecurity related to the illness.

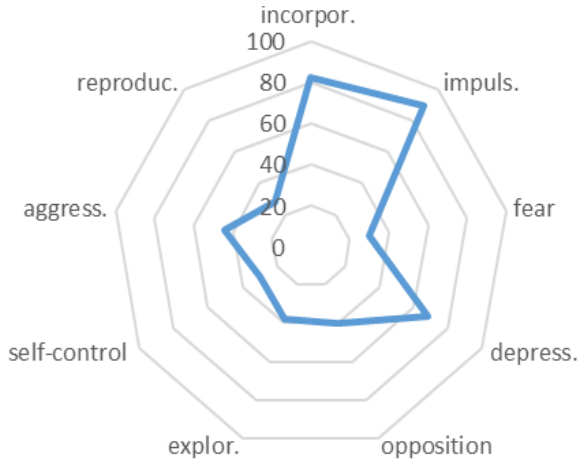

Figure 3 – EPI for CF children

MMPI-201 is applied only in adolescents (mean age 17.5 ± 2.6 years). Figure 4 shows MMPI profiles for girls and boys. The so called 'neurotic' profile (Hs-D-Hy), as it can be seen, is more accentuated in boys. Girls showed peak on Hy which can be interpreted as accentuated tendencies for conversion reactions.

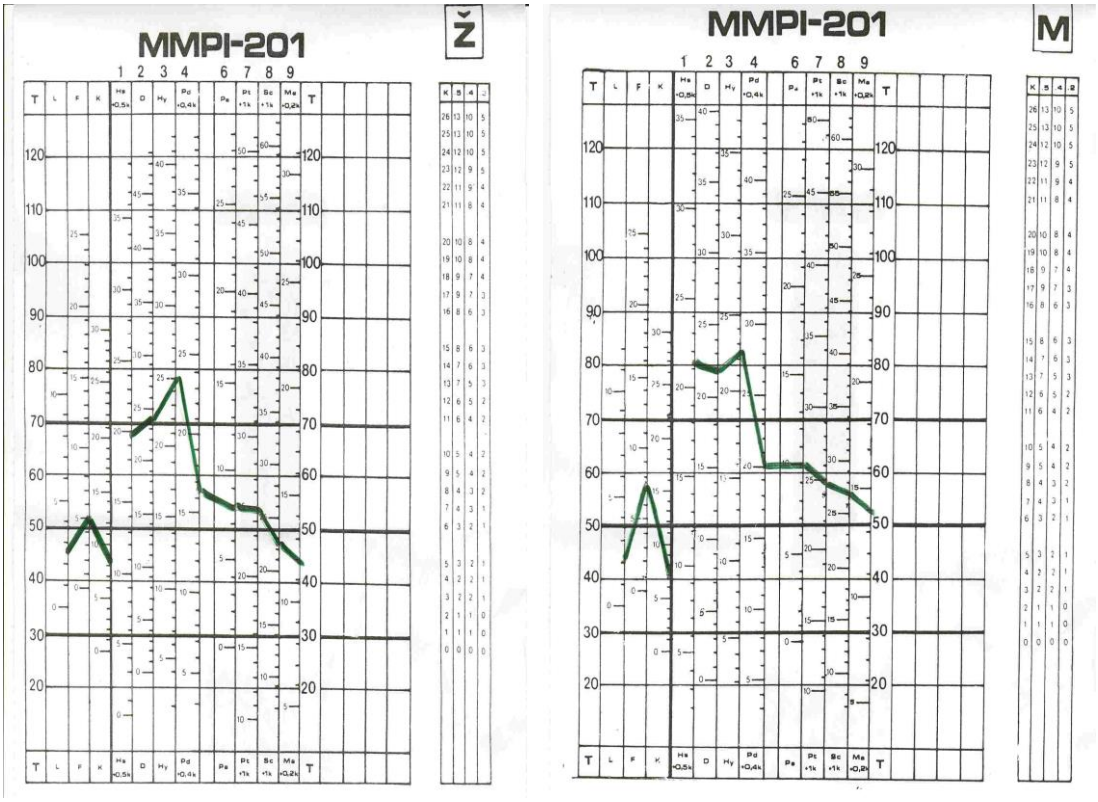

Figure 4 – MMPI profiles for girls and boys with CF

The drawings (Machover and Corman) obtained from CF children do not differ in general from those obtained from the control group. Good family functioning, the interrelationship between family members as well as the identity of the patients are interpreted as 'normal'. In three drawings obtained, the figures with open arms could be related to an accentuated need for protection and love.

Finally, all children fulfil Human Values Rank [31]. HVR obtained from CF children was very interesting. As it can be seen on Figure 5, the main value for children is good health, which is clearly understandable. The high values given to the friendship and love express the need of support, and the following – freedom is stressed as necessary for living: these children need a feeling of freedom, the possibility to act and make decisions. Personal

happiness is also ranked high in the human values, related to self-development. The values quoted are generally highly ranked in adolescents and students, so that the obtained results could be interpreted as the earlier mental maturation of CF children. The low value given to beauty, professional success, power and comfort in life could be related to low self-esteem and disappointment.

Figure 5 – Human Values Rank obtained for CF children

Discussion and Conclusions

The results obtained in our evaluation showed the normal intellectual functioning of CF children, which corresponds to the findings of other authors. In spite to chronic hypoxia, significant deficits in cognitive performance were not manifested. The obtained scores correlate to school achievement, and in spite of the frequent absenteeism, CF children are good pupils. Moreover, some psychopathological problems happen to be even less frequent than in healthy children (results obtained for EPQ).

Thomson et al. [35] reported that CF children have higher internalized scores on a child behavior checklist and experience distress, anxiety and depression, albeit not more than non-chronically ill children. However, from our test results, interviews with parents and the observation of children, we could generally conclude that the behavior of CF children in our study appeared to be within the normal range for the age. Only two children manifested psychological problems. One of them manifested a depressive reaction in his 17th year of

age, after his mother's and grandmother's deaths. This boy also manifested staturoponderal deficit and sexual retardation. He has not adjusted to all therapeutic procedures for CF. Another boy, 14 years old, also with staturoponderal deficit, manifested emotional immaturity and sado-masochistic attitude toward his mother. This boy's scores for aggressiveness were very high. Generally, the results obtained showed that CF children have relatively high cognitive skills, they are moderate extraverts, do not manifest major psychopathological behavior and they are not significantly anxious. This corresponds to Blair's [5] finding that most patients with CF are in robust psychological health.

The unexpected good psychological results could be explained by the fact that CF children accept the real situation and express vivacity, but the deep feelings of fear demand from them high self-control and resistance.

Most problems arise in the period of puberty and adolescence. The dysthymic feelings and fear causing accentuated self-control and self-defense are frequently present. Disturbed self-image, delated sexual characteristics, staturoponderal deficit, insecurity about the long-term prognosis are factors inducing behavioral and emotional problems in adolescents CF patients. None of our patients showed any sentimental relationship with the opposite sex, confirming the previous findings about the avoidance of close relationships with the opposite sex in adolescence [17–18].

All the families were well informed about the specificities of the disease. In general, they had no significant problems concerning normal family functioning. But, in three families divorce was noticed in the period of 1–2 years after the diagnosis, while in two the mothers died (from suicide and cancer, respectively). The poor economic situation is the biggest problem in some families.

As demonstrated in many studies like our own, the main psychological problems in the families are related to the mothers. The feelings of guilt, the everyday pressure related to nurture, medication and drainage procedures, the uncertainty of the future, were factors which influenced the mother's mental stability. In addition, the differences between the aggressiveness expressed in patient self-evaluation and the opinion of mothers have been noted. Canning [6–8, 10] also concluded that a discrepancy between parent and child reports concerning child psychiatric problems existed. This was also noted by Canning [9] where maternal distress was correlated with the number of disorders identified by mother, but not with those identified by child. In our everyday experience fathers are rarely present during control/hospitalizations. In some families we did not have any contact with the father.

Parental coping with the disease appeared to be problematic, which corresponds with the findings of other authors [21, 24, 32]. Our results also agreed with Eddy's [13] concerning the agreement between parents associated with problems in compliance with treatment, which have in adverse impact on the disease and health status of the child with CF.

We introduced the psychological assessment and support of CF children and their families with the general therapeutic procedures. Psychological interventions varied depending on the type of problem presented and the environmental context in which the child resides. Cognitive and behavioral treatment, combined with biofeedback modalities for relaxation were used, and have shown to be quite efficient [30]. Of course, parents and other family members were motivated to encourage the treatment. In our experience, the more frequent psychological problems during therapy were related to the child's adaptation and coping, especially in the period of puberty and adolescence, together with adherence to the medical regime and feeding, as well as parental coping linked to family dysfunction.

However, the main problems arise during the transfer of patients from pediatric to adult medical institution. Neither medical staff nor patients are satisfied to this transfer which is indispensable.

We can conclude that the multidimensional approach in the diagnosis and treatment of CF is 'condition sine qua non' [25]. Mental health professionals must be involved in the team.

The essential responsibilities of a psychologist working in a CF team are: (a) evaluating the psychological effects of living with CF

within the team, (b) undertaking comprehensive assessment and intervention when emotional, behavioural and psychological difficulties arise, (c) integrated post-diagnosis and annual reviews (comprising of assessment, screening and support), either face-to-face (which is preferable), and/or utilising psychometrically sound measures, (d) assessing the patient's and family's psychological resources, (e) providing support and where necessary, appropriate psychological intervention before and after the possible lung transplantation, and (f) actively participating in transition programmes (to high school and adult services) if these programmes exist or try to establish them if they do not.

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Резиме

ЕМОЦИОНАЛНОТО ЗДРАВЈЕ КАЈ ДЕЦА И АДОЛЕСЦЕНТИ СО ЦИСТИЧНА ФИБРОЗА

Нада Поп-Јорданова[1], Анета Демерџиева[2]

[1] Македонска академија на науките и уметностите, Скопје, Р. Македонија
[2] Аџибадем Систина, Скопје, Р. Македонија

Иако модерните терапевтски процедури значително го подобрија преживувањето и квалитетот на живот на децата со цистична фиброза, релевантните психолошки аспекти, сепак, се инсуфициентни, слично како и кај другите хронични болести.

Цел на ова истражување беше да се процени емоционалното здравје: психолошките карактеристики и приспособеноста на ЦФ-децата, како и справувањето на нивните семејства.

Студијата опфаќа 25 ЦФ-деца, средна возраст 13.13 ± 2.29 години (23 машки и само две девојчиња), избрани од вкупно 60 актуелно лекувани деца со ЦФ. Децата се испитувани во период на подобрена здравствена состојба (без суперинфекција, отежнато дишење или гастроинтестинални проблеми). Добиените резултати се споредени со контролна група од 25 здрави деца на иста возраст, избрани случајно од основните училишта.

Користените психометриски инструменти беа: Косов тест, Детска чек-листа на поведение, Ајзенков прашалник за личност, Општа скала на анксиозност, Емоционален профил на личност, MMPI-201 и Хуман тест на вредности, заедно со два проективни теста на цртежи (Маховерова и Корманова).

Неочекувано добрите психолошки резултати добиени со психометриските инструменти можат да се објаснат со фактот дека ЦФ-пациентите ја прифаќаат реалната состојба и манифестираат жилавост. Сепак, нивните длабоки чувства на страв кај нив наметнуваат високо ниво на самоконтрола и отпор. Добиените резултати од CBCL ги прикажуваат ЦФ-децата како незрели, со нагласена агресивност во меѓучовечките односи. Најбитен проблем е доцнењето на пубертетските промени, што води до мало самопочитување.

Генерално, членовите на семејството релативно добро се справуваат со болеста кај децата, и покрај разликите во извештаите помеѓу мајките и децата за постоење на психопатологија. Во некои семејства постојат разводи.

Психолошката поддршка е неопходна за обете страни, децата и членовите на семејството. Нагласено е дека е неопходен холистичен приод во проценатa и лекувањето, вклучително и користење на биофидбек-техники.

Клучни зборови: цистична фиброза, хронична болест, психологија, холистичен приод

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NEW TECHNIQUE OF COMPRESSION ANASTOMOSIS IN COLORECTAL SURGERY – FIRST RESULTS IN 25 PATIENTS IN MACEDONIA

Svetozar Antovic[1], Aleksandar Mitevski[2], Aleksandar Karagozov[1], Biljana Kuzmanovska[1], Nikola Jankulovski[1]

[1] University Clinic of Digestive Surgery, Medical Faculty, Skopje, R. Macedonia
[2] Clinical Hospital, Stip, R. Macedonia

Corresponding Author: Nikola Jankulovski, University Clinic of Digestive Surgery, Medical Faculty, Skopje, R. Macedonia; E-mail: prof.jankulovski@gmail.com

Abstract

Aim: Clinical evaluation of the safety and effectiveness of compression anastomosis with ColonRing™ for large-bowel end-to-end anastomosis for rectal cancer and explanation of the procedure and the device itself since this device is used for the first time in our clinic.

Material and methods: In November, 2012, a team of surgeons from our clinic attended the Clinical practice workshop in Belgrade, Serbia which was organized by the World Congress of Compression Anastomosis (WCCA) and held by its President Prof. Dr. Steven Wexner from Cleveland Clinic in USA. On this workshop, all aspects of technical point of view were obtained and surgeons were certified for the technique. A total of 25 patients have been scheduled for elective colorectal surgery with subsequent compression anastomosis using ColonRing. All patients were operated for high and mid rectal cancers excluding the low rectal cancers, since those patients are usually diverted with decompressive ileostomy. Patients, who are diverted, are at higher risk of retaining the ring, after its dislodgement, in the ampulla of the rectum since they do not have natural excretion of stool via the anus. All patients were followed for anastomotic leak, anastomotic bleeding, stricture formation, device (ColonRing) handling in general and time of expulsion of the ring via anus.

Results: We used this technique for the first time in 2013 and since then a total of 25 patients underwent anterior resection of the rectum with subsequent colorectal compression anastomosis using ColonRing. Of all patients, 9 were female while 16 were male with median age of 64 years. All patients were operated for rectal cancers. The mean length of hospital stay was 7.4 days (range 5 to 9 days). None of the patients developed anastomotic bleeding or dehiscence. To date none of the patients developed anastomotic stricture, although some patients were followed for almost two years. The average day of expulsion from the body could not be calculated since despite, and although all patients were given instruction on how to check for ring expulsion, 21 of them did not report this event. Only 2 patients brought the ring to us. In two cases after 2 week of the initial operation, the ring was find and palpated on digital rectal examination, free in the ampulla of the rectum and was easily removed via the anus during the examination. Misfiring was reported in 1 patient (first patient) and reanastomosis was employed using another ColonRing, No perioperative mortality was observed in this patient population.

Conclusion: End-to end colorectal anastomosis with the ColonRing is feasible and safe procedure with fast learning curve. To date, this type of anastomosis is possible in left sided colon lesions where anastomosis is contemplated below the promontory. We find the device easy to use with high level of confidence. Further prospective studies including comparison between the ColonRing device and the conventional staplers evaluating long-term anastomotic complications (i.e., leak or stricture) are needed to evaluate the benefits and limitations of this device.

Keywords: Anastomosis, Colon, Rectum, Leakage, Stenosis

Introduction

The resection of part of the bowel with subsequent anastomosis (reattachment) is widely used as surgical procedure of choice for various pathologies of the colon and rectum and all colorectal surgeons are closely familiar with this procedure, striving to make it better. Anastomotic leakage is one of the most serious early complications of any intestinal anastomosis, with a reported incidence rate of 1.3% to 21% [1]. Complete understanding of the mechanism of how anastomosis heals (connecting two luminal structures) is of crucial importance for the gastrointestinal anastomosis and is imperative to reduce the incidence of complications and dehiscence. Moreover, long-term functionnal outcome in patients might be adversely affected by anastomotic leakage [2].

The process of intestinal anastomotic healing can be divided into acute inflammatory (lag) phase, proliferative phase, and, finally, remodeling or maturation phase. The most important molecule for determining the intestinal wall strength is collagen, which makes its metabolism of particular interest for understanding the anastomotic healing. The factors that influence the fate of the anastomosis are both technical and patient-dependent. Currently, there are 3 available techniques for anastomosis: hand sewn, stapling, and compression. The techniques involving sutures and staples are the most widely used techniques for making bowel anastomosis with no significant advantage proven for any of them, although staplers, where possible, seem to be the surgeon preference since their usage shortens the operation time [3]. These two techniques use the penetration of foreign material into the tissue (sutures or staples), leading to breaking of the mucosal barriers and localized inflammatory response that may facilitate bacterial growth within the anastomotic line (anastomositis), thus increasing the propensity to anastomotic-related morbidity. This is the reason why we are still far from the ideal technique of anastomosis. In this regard investigators are still working on finding the most efficient way of bowel anastomosis that will reduce to the minimum the bowel wall injury and leaving no foreign material in the body of the patient. And this is where the concept of compression anastomosis comes. This is concept that was proposed for a very long time but till now, no device made it popular or convinced the surgeons in its own safety and usability.

The guiding principle of compression anastomosis is joining together and holding together in inversion manner both bowel ends that are compressed to each other until the natural healing process creates bowel continuity, and therefore, no foreign material is left in the body of the patient [4]. Compression devices have been long ago proposed and used clinically with varying degrees of success [5]. The idea of compression anastomosis has been proposed by Felix-Nicholas Denans, in 1928 [6]. Compression anastomosis was based on two opposing rings that trap the ends of transect bowel. Denans has suggested the compression anastomosis concept by applying silver or zinc rings in canine models for constructing end to end anastomosis. Bonnier, in 1885, and Murphy, in 1892, designed the first devices for performing anastomosis, which consisted of 2 metallic rings [7]. AKA-2 is a not-absorbable device, which was designed for colorectal anastomosis by Kanschin in 1984 [8]. Valtrac biofragmentable anastomotic ring (BAR) is another device developed in 1985 by Hardy [9] and probably the best studied so far. Based on various investigations, BAR could be applied in surgeries on different parts of gastrointestinal tracts, and not only on the large bowel, but it did not gain sufficient popularity [10].

The ColonRing device (NiTi Surgical Solutions): Recently this device has attracted much attention since it is a contemporary product made of high quality materials that insure confidence. The device uses Nitinol [11] (Nickel Titanium Naval Ordinance Laboratory), an alloy of nickel and titanium, which is a temperature-dependent, shape-memory alloy (SMA) that has been used in the formation of compression anastomoses [12]. The metal is shaped under high temperatures, and when it is ice cooled (to less than 0°C), it loses its rigidity and becomes flexible. These features are absent in all the previously described compression anastomosis devices.

The ColonRing device is remarkably similar to the regular circular stapler (Fig. 1). It is comprised of 2 main parts: an applier and an implanted compression element. The compression element is composed of a plastic anvil ring

and a metal ring that bares shape memory NiTi alloy (nitinol) leaf springs (Fig. 2). The purse-string technique or any closed lumen technique (stapling technique) may be used to place anvil into the organs to be anastomosed based on the surgeon's experience or judgment. When "fired" the rings are locked together by circumferentially placed barbed points, which penetrate through the tissue (Fig. 3) and the Nitinol springs that exert the desired constant controlled pressure force (7.7 Newtons or 1.65 Pounds). The device has a circular blade that cuts the tissue within the ring, creating patent anastomosis. The tissue heals around the circular edges that are held within the ring, through simultaneous necrosis-healing process, and the device along with the compressed tissue is intended to slough off over the following 8 to 10 days, at which point the ring is expelled from the body with a later bowel movement. The result is a full circumferential, hemostatic-sealed anastomosis without any retained foreign material.

Fig. 1 – NiTi Coloring compression anastomosis device

Fig. 2 – The compression anastomosis ring

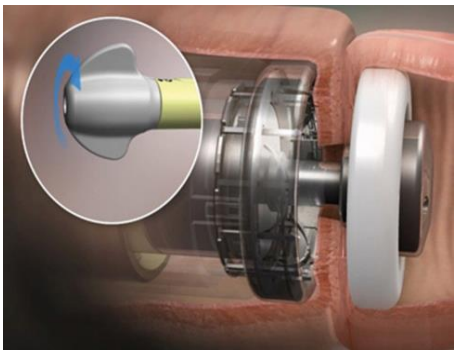


Fig. 3 – Endoluminal appearance of the two rings

The ColonRing device has been approved by the Food and Drug Administration (FDA) in 2006 for use in the colon and rectum for the creation of end-to-end and end-to side anastomoses in both open and laparoscopic colorectal surgeries. Studies have shown that the NiTi ColonRing device may overcome many limitations of the previous compression devices [13, 14] especially regarding the retained foreign material within the tissue (clips) that leads to constant inflammatory reaction that produces scar tissue and anastomotic stenosis. This is possible because of the basic nature of the compression anastomosis. The device provides for sufficient pressure for the proximal and distal tissue over a timeframe that allows for successful tissue healing, by the necrosis-healing process, by creating a zone of tissue necrosis internal to the apposed and healed tissue to allow expulsion of the ring into the lumen of the bowel followed by natural passage of the device from the body. After expulsion, no foreign material is left in the body of the patient to sustain further inflammation and there is no internal "lip" as in the stapler anastomosis, thus creating the largest possible anastomotic diameter than ever before of 27mm. (Fig. 4).

Fig. 4 – Difference between compression and stapled anastomosis

Aim

Clinical evaluation of the safety and effecttiveness of compression anastomosis with ColonRing™ for large-bowel end-to-end anastomosis for rectal cancer and explanation of the procedure and the device itself since this device is used for the first time in our clinic.

Material and methods

In November, 2012, a team of surgeons from our clinic attended the Clinical practice workshop in Belgrade, Serbia which was organized by the World Congress of Compression Anastomosis (WCCA) and held by its President Prof. Dr. Steven Wexner from Cleveland Clinic in USA. On this workshop all aspects of technical point of view were obtained and surgeons were certified for the technique. Since 2013, a total of 25 patients have been scheduled for colorectal surgery with subsequent compression anastomosis using ColonRing. All patients were planned for elective operation of high and mid rectal cancers excluding the low rectal cancers, since those patients are usually diverted with decompressive ileostomy. Patients, who are diverted, are at higher risk of retaining the ring, after its dislodgement, in the ampulla of the rectum since they do not have natural excretion of stool via the anus. All patients were followed for anastomotic leak, anastomotic bleeding, stricture formation, device (ColonRing) handling in general and time of expulsion of the ring via anus.

Results

A total of 25 patients underwent anterior resection of the rectum with subsequent colorectal compression anastomosis using ColonRing. Of all patients, 9 were female while 16 were male with median age of 64 years. All patients were operated for rectal cancers. The mean length of hospital stay was 7.4 days (range 5 to 9 days). None of the patients developed anastomotic bleeding or dehiscence. To date none of the patients developed anastomotic stricture, although some patients were followed for almost two years. The average day of expulsion from the body could not be calculated, and although all patients were given instruction on how to check for ring expulsion, 21 of them did not report this event. Only 2 patients brought the ring to us. In two cases after 2 week of the initial operation, the ring was found and palpated on digital rectal examination, free in the ampulla of the rectum and was easily removed via the anus during the examination. Misfiring was reported in 1 patient (first patient) and reanastomosis was employed using another ColonRing, No perioperative mortality was observed in this patient population.

We find that using ColonRing is surprisingly similar to the conventional stapler device. Handling and firing is practically the same. The only difference is that when you open the package, one of the rings is not attached to the device (Fig. 5) since this is the part that holds the nitinol springs.

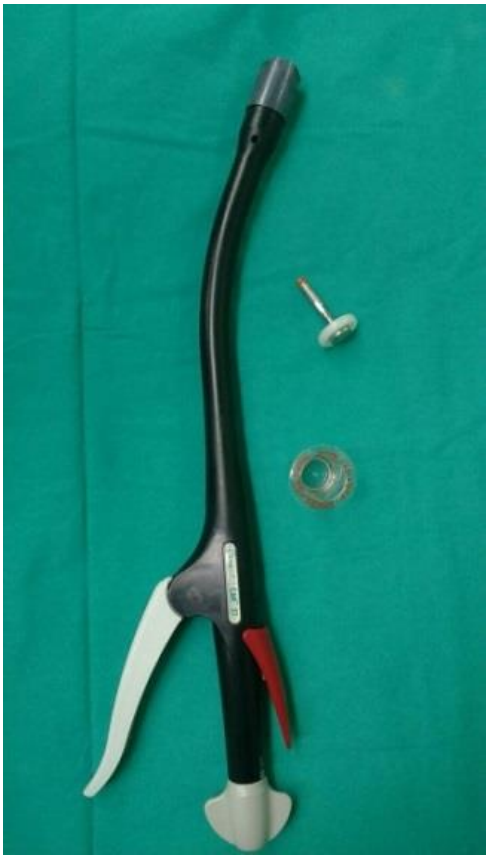


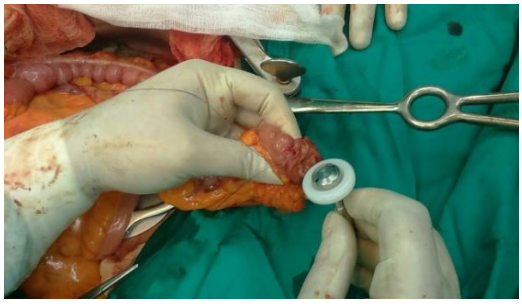
Fig. 5 – ColonRing device

Before the attachment to the device, this part is submerged in cold water below 5° (Celsius) for 5 min (Fig. 6) and then is easily attached to the device by simply pushing and rotating the plastic holder (Fig. 7 and 8)

Fig. 6 – Submerging in cold water

Fig. 7 and 8 – Placing the ring by rotation

From that point on, practically there is no difference with conventional stapler when it comes to handling. The anvil is placed in the proximal colon (Fig. 9 and 10) and the device itself is placed via the anus in the distal rectum (Fig. 11).



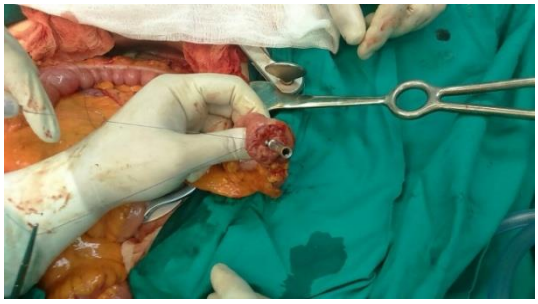


Fig. 9 and 10 – Placing the anvil in the proximal bowel lumen

Then the anvil and the device are connected and with simultaneous pressure on the handle (Fig. 12) the ring is fired. The device is retracted from the anus and the two donuts are examined for continuity (Fig. 13). Since the whole ring stays in the lumen of the rectum (Figure 14), there is no need to swing away the handle since the anvil does not pass through the anastomosis itself and it is simply retracted from the rectum much easier than the conventional stapler.

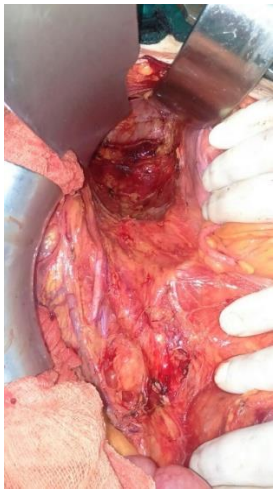


Fig. 11 – Distal transected rectum

Fig. 12 – Firing the device

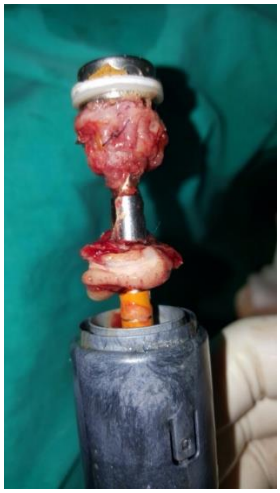


Fig. 13 – The two donuts

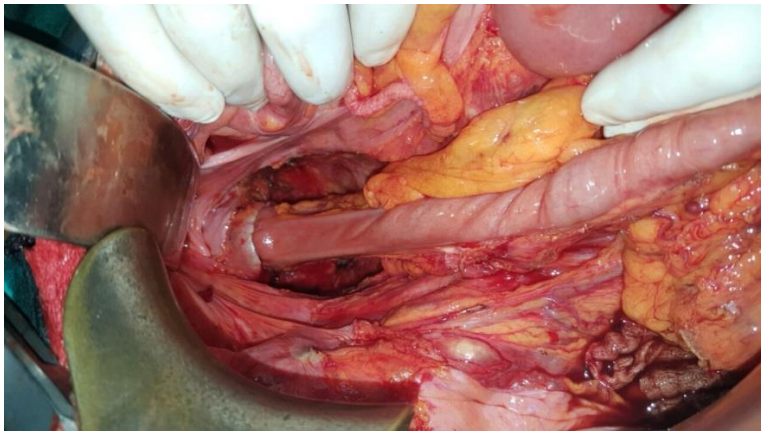


Fig. 14 – Anastomosis with compressive ring in the lumen of the bowel

In all 25 patients we did not encounter any problems with the use of the device. The scrub nurses quickly adapted to the technical aspect of the device and the attachment of the ring to the device itself.

Discussion

The incidence rate of anastomotic leakage is variable depending on the type of procedure, technique, level of anastomosis, and patient characteristics. Low anastomosis, male patients, and preoperative concomitant chemoradiotherapy have been shown to be independent risk factors for anastomotic leakage in rectal surgery [15]. By evaluating stapled versus hand-sewn methods for colorectal anastomosis, Neutzling et al. [16] in a recent systemic review study showed that the evidence was insufficient to show any superiority of stapled over hand-sewn techniques in colorectal surgery requiring anastomosis regardless of the level of anastomosis. Our study showed that the overall anastomotic leak rate using the NiTi ColonRing device was 0%, which is within the expected range for this level of anastomosis (i.e., 3% to 5%) [17]. Hence, the NiTi ColonRing device can be considered safe in both laparoscopy and open surgery.

Two separate studies looked at bursting strength of the anastomotic site using compression or double stapling technique in a porcine model. Kopelman et al. [18] measured a mean bursting strength of 247.7 mmHg (range 100-300 mmHg) in nine animals at time zero (immediately after the excision of the fashioned anastomosis). Furthermore Stewart et al. [19] revealed significantly higher bursting pressure after compression anastomosis in comparison with the conventional double stapling technique (103, 75.3 mmHg *vs* 3, 23 mmHg, respectively). Four of the nine compression anastomoses failed at the anastomotic line whereas nine of nine stapled anastomoses failed at the staple line (Fishers' exact test, $P < 0.01$). Bursting pressures measured at two weeks after the anastomosis revealed equal pressures (266, 32.2 mmHg and 230, 87.5 mmHg, respectively). Compression therefore seems to be capable of overcoming anastomotic weakness during the 'classical' lag-phase and results in equal strength after detachment of the ring [20]. Based on that experience, a study was started in May 2007 in Uppsala (Sweden) and in Leuven (Belgium) to obtain clinical data in a consecutive group of 40 patients [21]. The recruited patients had either malignant or benign (diverticular) disease requiring resection with a high colorectal anastomosis (between 10 and 15 cm from the anal verge). The preliminary results of the study showed that of the first ten patients, nine underwent high anterior resection, and left colectomy was performed in one patient. No leak occurred in this first group of patients.

In 2013, using a multinational (16 countries), multicenter (178 centers) data registry, Masoomi et al. [22] published the largest, by number of patients, review study that showed that the overall anastomotic leak rate was 3.22% (38 patients). The median length of the hospital stay was 6 days (range 2 to 21 days). The median ring expulsion time was 8 days. The earliest ring expulsion time was 6 days; however, in 1 patient, the ring did not expel. In 4 patients, the anastomosis had to be immediately recreated because of 1 misfiring and 3 incomplete anastomoses. The authors concluded that the use of the ColonRing device is feasible

and safe and could be considered an alternative technology for end-to-end colorectal anastomosis.

Although we do not have data supporting the nominal learning curve, this device functions almost identically to the current circular staplers in widespread use, facilitating the minimal learning curve. Overall, we felt that the device was easy to use and scrub nurses adopted to the technique very fast. Based on the current animal and clinical studies assessing compression anastomosis with the ColonRing device, there appears to be several potential benefits associated with its use. First, this technology delivers a constant stress plateau, which makes the ring detach from the anastomotic site at an appropriate and predictable time allowing for apposition of the bowel ends [23]. Second, the absence of foreign bodies at the anastomotic site may decrease inflammatory stimuli and formation of fibrous tissue as shown in animal studies, which may lower the risk for developing anastomotic stenosis [24]. Third, the absence of raw surface at the interface of the proximal and distal ends of the anastomosis with the ColonRing device may decrease the possibility of stricture and create a smooth and intact healing line [18].

However, our study lacked a long-term follow-up of patients, which makes it difficult to draw a conclusion on some important colorectal anastomosis-related complications, such as bowel stricture and obstruction. We did not have the ring expulsion time for all the patients; therefore, the reported ring expulsion time may not be accurate. Finally, our results report only on elective end-to-end colorectal anastomosis, and it could be different from the results of urgent surgery and/or other type of anastomoses (i.e., end-to-side and side-to-side anastomosis).

Conclusion

End-to-end colorectal anastomosis with the ColonRing is feasible and safe procedure with fast learning curve. To date, this type of anastomosis is possible in left sided colon lesions where anastomosis is contemplated below the promontory. We find the device easy to use with high level of confidence. The rate of anastomotic leak is relatively low with the use of the ColonRing for both open and laparoscopic colorectal anastomoses. Further prospective studies including comparison between the ColonRing device and conventional staplers evaluating long-term anastomotic complications (i.e., leak or stricture) are needed to evaluate the benefits and limitations of this device.

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Резиме

НОВА ТЕХНИКА НА КОМПРЕСИВНА АНАСТОМОЗА ВО КОЛОРЕКТАЛНАТА ХИРУРГИЈА – ПРВИ РЕЗУЛТАТИ КАЈ 25 ПАЦИЕНТИ ВО МАКЕДОНИЈА

Светозар Антовиќ[1], Александар Митевски[2], Александар Караѓозов[1], Билјана Кузмановска[1], Никола Јанкуловски[1]

[1] Универзитетска клиника за дигестивна хирургија, Медицински факултет, Скопје, Р. Македонија
[2] Клиничка болница, Штип, Р. Македонија

Цел: Клиничка евалуација на сигурноста и ефективноста на компресивната анастомоза со употреба на ColonRing™, како алтернативна метода за креирање на анастомоза по ресекција на дебелото црево кај карцином на ректум, како и објаснување на самата процедура и инструментот со оглед дека се употребуваат првпат во нашата клиника и земја.

Материјал и методи: Во ноември 2012 година, тим хирурзи од нашата клиника присуствуваа на работилница организирана од страна на Светското здружение за компресивни анастомози (WCCA) со кое раководеше претседателот на Здружението, проф. д-р Стивен Векснер, од клиниката Кливленд во Охајо, САД. На оваа работилница беа разгледани сите релевантни аспекти за креирање на компресивни анастомози со употреба на ColonRing и хирурзите беа сертифицирани за негова употреба. Вкупно 25 пациенти со висок или средно лоциран карцином на ректумот беа планирани за ресекциона хирургија и креирање на компресивна анастомоза. Сите пациенти беа планирани за елективен третман, додека исклучени од студијата беа итни пациенти и оние со низок карцином на ректумот кај кои вообичаено се прави ултраниска анастомоза со протективна илеостома. Сите пациенти беа следени за појава на дехисценција или крвавење на анстомозата, формирање на стриктура на местото на анастомозата, времето на експулзија на анастомозирачкиот прстен од телото на пациентите и осврт кон самата метода и инструмент.

Резултати: Првпат оваа техника беше употребена кај нас во 2013 година и оттогаш кај вкупно 25 пациенти беше направена предна ресекција на ректумот и компресивна анастомоза со употреба на ColonRing. Од пациентите, 9 беа жени, додека 16 мажи со средна возраст од 64 години. Просечното време на хоспитализација на пациентите беше 7,4 дена (5–9 дена). Кај ниеден пациент не се јави дехисценција или крвавење од анастомозата. Досега кај ниеден пациент нема стриктура на местото на анастомозата иако некои од пациентите беа следени повеќе од две години. Средното време на исфрлање на анастомозирачкиот компресивен прстен преку анусот не можеше да се пресмета бидејќи иако сите пациенти беа инструирани како да го пронајдат прстенот во столицата, сепак, само двајца пациенти успеале да го направат тоа. Кај двајца пациенти прстенот не беше спонтано исфрлен и тој, мануелно лесно, беше отстранет од страна на хирургот при контролен дигитален ректален преглед. Кај првиот пациент беше забележано неиспукување на прстенот при оперативниот зафат поради што се употреби втор ColonRing за креирање на анастомозата. Кај сите останати пациенти употребата на инструментот беше лесна.

Во оваа група пациенти немаше периоперативен морталитет.

Заклучок: Крај со крај колоректалната анастомоза, креирана со употреба на ColonRing, е лесна и едноставна процедура со брза крива на учење. Засега, оваа техника на анастомоза се препорачува за заболувања на дебелото црево каде што се планира анастомоза под промонториумот. Сметаме дека употребата на инструментот е едноставна и со големо ниво на сигурност. Понатамошни проспективни студии помеѓу ColonRing и конвенционалните степлери се неопходни за евалуација на долгорочните бенефити и лимитации на овој инструмент и на овој тип анастомоза.

Клучни зборови: анастомоза, колон, ректум, дехисценција, стеноза

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HISTORICAL NOTE

THE BRITISH MILITARY HOSPITALS IN MACEDONIA DURING THE FIRST WORLD WAR

Vladimir Cvetkovski

English Department, Faculty of Philology Blaže Koneski, Ss Cyril and Methodius University, Skopje, R. Macedonia

Corresponding Author: Vladimir Cvetkovski, English Department, Faculty of Philology Blaže Koneski, Ss Cyril and Methodius University, 10 Vodnjanska str., Skopje, R. Macedonia; E-mail: marijacvet@gmail.com

Abstract
The paper focusses its attention to the medical work of the British Military hospitals stationed in Macedonia during the First World War, the surgical work carried out under very heavy conditions in improvised operating theatres as well as the treatment of the wounded and sick solders brought from the battlefields on the Macedonian Front.

Keywords: The First World War, The Macedonian Front, Skopje, Dr. McLaren, Lady Paget, Scottish Women's Hospitals, Ostrovo.

The First World War had brought many British subjects to the Macedonian Front. Beside the soldiers who fought in the units of the allied forces, there were volunteers who came with the medical units which were sent to the Balkans by the British Red Cross, The Scottish Women's Hospitals and the Serbian Relief Fund. Field hospitals were stationed along the main battle lines in Serbia and Macedonia: Mladenovac, Kragujevac, Skopje, Bitola, Ostrovo, Voden etc.

The declaration of war on August 4th 1914 caused ferment in Britain. Recruiting offices and voluntary organizations sprang up all over the country and men, some mere boys, and women, enthusiastically enlisted. Among those were significant persons from the British cultural and public life, artists, theatre actors, writers and other persons from the high ladder of the social life. Already on the 20th August 1914 Dr. Elsie Inglis propagated the establishment of a hospital and the Scottish suffrage offices in Edinburgh became the Scottish Women's Hospitals, where not only Scottish, English, Irish and Welsh women served in their units, but many came from Australia, New Zealand and elsewhere (Krippner 1980: 30). This is particularly true of the composition of the 'America' unit, so named because of the funds raised in America stationed at Lake Ostrovo in Macedonia, where in 1917, beside the women doctors from Australia, Dr. Bennet, Dr. Hellen Saxton, Dr. De Garris and others there were orderlies and other personnel from Britain and elsewhere. Among those was the Australian writer Miles Franklin who worked as an assistant cook. She left a memorable description of the life and work at the hospital in her unpublished work *Ne mari ništa – It Matters nothing*, the manuscript of which is curated at Mitchel Library in Sydney. Many men and women were recruited by the SCWH and sent to the Front in Macedonia.

The British Red Cross was also engaged in sending medical personnel to the Balkan Front, right after the first engagements started. Among the first to arrive in Serbia was the Field Hospital led by Mrs. St. Clair Stobbard, which was stationed near Kragujevac, the activity of which is impressively described in her work *The Flaming Sword over Serbia*. In it she describes in details not only the medical work in the hospi-

tal but also the tragic Golgotha of the retreat of the Serbian Army across the frozen mountains of Albania.

By mid-November 1914 the First Serbian Relief Fund Unit commanded by Lady Leila Paget arrived in Skopje, Macedonia, to establish a fine 600 hundred bed hospital on a hill outside Skopje. The Unit had appropriated the 'Gymnasium', a fine block of buildings used primarily as a technical school and capable of accommodating three hundred beds.

A week after the arrival of Lady Paget's Unit the medical unit sent by the British Red Cross led by Dr. McLaren, Canadian Scotsman, 'the chief' as they called him, arrived in Skopje.

Very soon the Serbian Relief Fund was launched at the head of which stood Her Majesty the Queen as a Patroness, supported by distinguished public figures of the British Society: The Lord Bishop of London, President of the SRF; Vice presidents were: H. E. Cardinal Bourne, The Earl Courzon of Kedleston, The Lord Bishop of Oxford, Rt. Hon. H. H. Asquith, M. P., Rt. Hon. A. Bonar Law M. P., Rt. Hon. Austen Chamberlain, M. P., Rt. Hon. Winston Churchill, Rt. Hon. Herbert Samuel, M. P., Rt. Hon. D. Lloyd George, M. P., Madame Gruitch, Rev. G. Campbell Morgan, D. D., Rev. F. B. Mayer, D. D., Mr. Noel Baxton, M. P., Chairman: The Lord Henry Cavendish Bentinck, M. P., Vice Chairman: Mr. Glynne Williams, Committee: The Lord Charnwood, The Lord Fitzmaurice, The Lord Haversham, The Hon. Bernard Wise, Sir Edward Boyle Bart, Sir Arthur Evans LL D., F. R. S., Sir Valentine Chirol, Hon. Lady Whitehead, The Lady Rodney, Lady Boyle, Lady Grogan, Lady Paget, Mrs. Noel Baxton, Mrs Bertram Christian, Madame Christich, Mrs. James Currie, Miss Aubrie Fletcher, Mrs. Fry, Mrs. H. R. Gotto, Mrs. Arthur Harrison, Mrs. Grice Hutchinson, Dr. Elsie Inglish, Miss McQueen, Mrs. Masterman, Mrs. Scaramanga Ralli, Mrs. Seton Watson, Mrs. Carrington Wilde, Mr. W. A. Albright, Mr. P. Alden, M. P., Mr. Jas. Berry, F. R. C. S., Mr. H. N. Brailsford, Mr. R. M. Burrows, D. Litt., Mr. Bertram Cgristian, Rev., Percy Dearmer, D. D., Mr. G. Hanrahan, Mr. C. L. Graves, Mr. Jpynson-Hicks, M. P., Mr. James F. Hope, M. P., Mr. Hugh Law, M. P., Mr. R. O. Moon, M. D. F. R. C. B., Mr. H. E. Morgan, Mr. T. P. O'Connor, M. P., Mr. J. O'Grady, M. P., Mr. A. M. Scott, M. P., Mr. St. Loe Stratchey, Mr. A. G. Symonds, Mr. G. M. Trevelyan; Treasurer: Rt. Hon. The Earl of Plymonth; Hon. Secretary: Mr. R. W. Seton-Watson, D. Lit.; Hon. Financial Secretary: Mr. F. C. Lindo; General Secretary Mr. F. M. Scott; Bankers: London County & Westminster Bank Ltd.; Hon. Auditors: Messrs, Cole, Dickin & Hills.

Lady Paget's Hospital was organized according to the best medical standards capable of treating not only wounded soldiers but also sick soldiers and patients from the local population especially after the outbreak of the typhoid epidemics. In her Report entitled 'With Our Serbian Allies', Lady Paget beside the medical work carried out in the hospital she describes the political situation in the city, and the tensions that occurred when the Bulgarians and the Germans occupied the city. At the end of the Report she presents a list of the whole personnel of the hospital that elicits the structure and the solid organization of her Unit. We present it here with no changes.

LADY PAGET'S UNIT IN SKOPJE No. II
MEDICAL STAFF

Medical Director
T. GWYNNE MAITLAND, Esq., M A., B.Sc., C.M., D. Phil.

Surgeon-in-chief
FERGUS ARMSTRONG, Esq., M. D., F.R.C.S.

Assistant Surgeon.
PERCY WALLICE, Esq., M.R.C.S., L.R.C.P.

Physician.
R. M. MORISON, Esq., M.B.

Surgeon
Dr. M. SEEDORF (Danish).

Assistant Surgeon.
Dr. ERIK HIMMELSTRÜP (Danish).

Physicians
Dr. A. F. CORNELIUS (American).
Dr. ERLE D. FORREST (American).
Dr. R. V. BROKOW (American).

CHANCERY STAFF

Secretary
Mr. EDGAR DAVIES.

Treasurer
Mr. T. E. MILLIGAN GRUNDY.

Dispenser.
Mr. A. PATERSON.
Radiographer
Mr. J. LAMB.

Anesthetists
Mr. IVAN CAMPBELL.
Mr. E. P. CHAPMAN.

The following gentlemen were not official members of the Staff, but very kindly acted as voluntary workers:-

Bacteriologist.
Dr. T.H. PLOTZ (American).
Dr. GEORGE BAEHR

Sanitary Engineers.
Dr. OSBORN* (American).
C. E. FOX, Esq.*

**Member of the American Red Cross Sanitary Commission.*

LADY PAGET'S UNIT IN SKOPJE No. II
NURSING STAFF
Matron.
Miss LOUISA BALL.

Housekeeper.
Mrs. OLIVE JOURDAIN.

Masseuses.
Mrs. G. POLGREEN.
Mrs. ADA BARLOW.

SISTERS

BRITISH
Miss MABEL ATKINSON (N. Z.)
Miss MAUD BULLOCK.
Miss JEAN CALDOW.
Miss M. K. COLEMAN.
Miss T. CROMBLEHOLME.
Miss EVA E. EGERTON.
Miss I. GRAY.
Miss I. HUDSON.
Miss DORA JOHNSON.
Miss G. LLYN JONES.
Miss ALICE LEVESON.
Miss AGNES MANN.
Miss ROSALIE MANSELL.
Miss C. B. MELLIS.
Miss M.T. O'NEILL
Miss J. S. RANKIN.
Miss BEATRICE ROBINSHAW.
Miss S. A. ROUND.
Miss ETHEL SCAMMELL.
Miss M. M. SHARPIN.
Miss M. E. SKERTCHLEY.
Mrs. PERCY WALLICE

AMERICAN
Miss R. PARSONS.

DANISH
Miss HENNY GRAVESEN.
Miss JOHNNY HENDRICKSEN.
Miss THEKLA MADSEN.
Miss SIGNE MÖLLER.
Miss MINNA WIFSTRAND.

DUTCH
Miss WILLY FORBES-SCHMELTZER.
Miss MARIE VAN OYEN.
Miss VAN DER VEEN.

FRENCH
Mlle VILLEMON.

ORDERLIES

BRITISH.
Storekeeper
Mr. C. R. COOKE-TAYLOR.

Chauffeur and Carpenter
Mr. J. L. GASKING
Mr. H. C. M. HARDINGE

AMERICAN.
Mr. W. W. EATON.
Mr. F. KLEPAL.
Mr. T. B. LOGAN.
Mr. D. PETERS.
Mr. R. SHELLENS
Mr. M. A. TANCOCK.

Lady Paget and her staff remained in Skopje even after the entry of the Bulgarians and Germans and continued to treat the wounded soldiers.

… The whole hospital staff was working at high pressure, bringing the wounded and washing and dressing their wounds. The corridors and the staircases were soon full to

> excess; we therefore placed forms at the back under cover of the buildings, on which they sat with their ghastly wounds, waiting till could be attended to. During the battle we dressed the wounds of many hundreds of Serbians, but kept actually in the hospital only about two hundred. I cannot praise too highly the cool, practical way in which the whole staff went about the work.
>
> Our Austrian prisoner orderlies had always won my admiration by their loyalty and devotion to duty, but never did their behavior merit our respect than now.
>
> Lady Paget: With Our Serbian Allies, pp. 32

The British Red Cross Unit led by Dr. McLaren consisted of six doctors: Dr. J. Johnston Abraham, Dr. Barkley; the names of the others are not given, each with two orderlies. Later on to their group of doctors was added a Russian woman doctor presented in his book as The Little Red (haired) Woman. The names of only three orderlies are rendered: Anthony, Steve, James, Later on the group of orderlies was enlarged with several prisoners of war some of them Czechs, from the Austrian army.

Dr. J. Johnston Abraham, an Irishman – Britisher as he called himself is the author of a fascinating work, a kind of a diary which is more than that – *My Balkan Log* in which he describes not only the medical work and complicated operations performed in their hospital but also it renders a vivid description of the life in the city, its cultural monuments, the customs of the local ethno culture and, we may say, it is the first novel of Skopje.

Here we shall focus mainly to the work in the hospital.

> "… The hospital was stationed in three buildings all close together, which had originally been store houses for tobacco, close to the railway station. The largest 'Number One' had space accommodation for six hundred beds, the other two for approximately three hundred each. 'Number One' had three great floors, each containing two hundred beds. It was lit feebly with a few electric lights on each floor; but there was no water of any sort laid on, and absolutely no inside sanitary accommodation."
>
> *My Balkan Log*: p. 41

> '… December the first was a red-letter day in our calendar, for on that day we started a real operating theatre – the theatre for which, ever since our arrival, our Chef had been working.
>
> At any rate we had a theatre which really looked as theatre, and here the Chief, Barkley and I spent two happy months very busily until the great blight came.
>
> The next day we had a fresh convoy from the front; and now our theatre was in working order, the Serbian officials began to give us what they called more serious cases. Instead of wounds of limbs we began to get head injuries, gunshots wounds of chest, and more and more compound fractured femur. The number of abdominal injuries that passed through our hospital then and afterwards was surprisingly few.
>
> … Each new patient came, or was brought, to one of half dozen operating tables to be examined; the field dressing was taken off, and the wounds cleaned up by one or other of the assistants. Then the Major came along, and a rapid diagnosis was made. Sometimes he would pass on quickly, sometimes stop and ask a question. Every now and again he would run his fingers over an arm, leg or chest, feel a bullet or a piece of shrapnel, grip it between his fingers, and with a rapid cut of a knife turn it out without bothering about any anesthetic. It was fierce, rapid medieval surgery; and the patient stood it without even a murmur. They were all so quiet, so apathetic, so very tired.
>
> Ibid: pp. 45–46.

> … Every day now we had a fresh convoy of wounded Serbs, Austrians. The Austrian soldiers were mixed. They had Magyars (Hungarians), Czechs, Slovaks, Slovenes, Poles, Dalmatians, Croats, Jews, Romanians, Italians and Austrians proper among themselves.
>
> Soon the first cases of typhus occurred in the hospitals in Skopje and it spread throughout the city. During the winter campaign it became epidemic and we had several thousand cases through our hands in the first three months. Recurrent fever, the Serbs, following Continental nomenclature, call it Typhus Recurreus to distinguish it from Typhus Abdominalis (our Typhoid Enteric).
>
> It is caused by a spirillum and runs a very typical course. There is high fever, intense prostration, and some delirium lasting for about a week. Then comes a rapid fall of temperature, and a week when the temperature registers normal or subnormal. This is followed by a second and sometimes a third similar rise and fall till the patient is reduced to a skeleton, almost too weak to turn in bed.

> Among ourselves, at first, we label it 'Uskubitis', before we recognized the cause. Eventually, we simply called it 'IT'.
>
> Ibid: pp. 75.

> The first cases of typhus occurred in some of Skopje hospitals such as Polymesis (Polumesec – Half-moon); soon it spread throughout the city and people were dying in great numbers.
>
> Two ox-wagons lumbering along the main street, each with an armed man in front, caught my eye; and as they passed I glanced casually at the contents. There were some twenty bodies, ten in each wagon, coffinless, carelessly wrapped in blankets.
>
> Men were dying in such numbers; the carpenters could not cope with the demand for coffins. People were getting more and more frightened. Even the Tzigans began to refuse to handle the bodies.
>
> Ibid: pp. 245

The first British Field Hospital for Serbia reached Salonika on April 15, 1915. Beside the medical staff there were civilians engaged as assistants in nonmedical work, among those were Alice and Claude Askew who joined the unit as writers but 'we were prepared to turn our hands to odd jobs if called upon to do so'.

The unit consisted of some twenty six individuals all together. Surgeons, dressers, orderlies, chauffeurs, a staff of most capable nurses, a cook, a washwoman, an interpreter – and a dog.

They are the authors of *The Stricken Land* in which they described not only the medical work carried out in Skopje and later on at Pirot where they moved later on, but also the tragic retreat of the Serbian Army on their Via Dolorosa through Montenegro and Albania to Corfu where those who survived were transported to recover.

> The staff of our unit had been carefully selected by the Committee in London. The organizer of the enterprise was Dr. J. Hartnell Beavis, whose name was already known in connection with similar work in Belgium. With him – his right-hand man – was Mr. Gerald Sim who had been in Belgium too, where he had got his laurels as an expert chauffeur. Our Chief surgeon was Mr. Fergus Armstrong, F. R. C. S. who had given up an important post to join us and a more able operator or pleasant companion could not have been desired. Alas, he was not with us at Pirot, having undertaken during the slack period that preceded the Bulgarian outbreak to work for Lady Paget at Skopje. A physician we had Dr. G. Landsborough Findley, who with his wife, Lady Sybil – one of our nurses. The unit consisted of some twenty-six individuals altogether surgeons, dressers, orderlies, chauffeurs, a staff of most capable nurses, a cook a washwoman, an interpreter, and a dog.
>
> *The Stricken Land*, pp. 22–23.

The next British hospital sent to Macedonia was the one sent by the Scottish Women's Hospital known as "American Unit" because the money for the original outfit was a gift of the people of the United States. It was stationed near Lake Ostrovo - on the Macedonian Front in August 1916, where the bloodiest battle had been fought on the Mount of Kaymakchalan in 1917.

The hospital camp was situated some three hundred miles of Ostrovo and the railway station. The ward tents were twenty in number and passage ways connecting tents had been provided for them. Each tent took ten beds and the hospital for two hundred; also there were X-ray tents, a clothing store etc.

Beside these there was dressing station up the hills where the first dressing of the wound of the wounded soldiers was performed ant the patients were transported to the hospital by ambulances driven by women drivers.

The medical staffs in the hospital were all women doctors: Dr. Bennet (the C. M.), Dr. Cooper (who had joined the unit from Australia), Dr. Lewis, Dr. Scott. Dr. Mancaster.

Details about the work of this hospital, the heavy fightings in the mountains on this part of the Macedonian Front are described in the work *At the Serbian Front in Macedonia* by E. P. Stebbing, a university professor from Edinburgh who served as a transport officer – as he explains – 'the job which Dame Fortune, flung to me after two years spent in attempting to persuade generals, colonels and even majors, to give me a temporary job on active service during the few months' leisure each year which a post in a University allows one'. (Stebbing: pp. 1)

As an eye witness Stebbing gives detailed description of the heavy military engagements on this side of the Macedonian Front. So this work is also of historical value as a document of what was going on at the battlefields in Macedonia during the Great War.

Here is a detail of what he had seen when he visited the place after one of the cruelest battle was over on the Mount of Kaymakchalan fought in 1917.

> … Here the damage done was terrific and both trenches and ground behind them are littered with dead Bulgars, whilst in many cases all signs of a trench are obliterated. There is one of these lines of trenches which are particularly hideous. Here a whole line of Bulgars was either mown down or killed by grass shells and lie in every conceivable position. At a distance they look as if they were asleep, but from their tattered clothing as one approaches one knows that their sleep is the sleep of death and that the death has been a violent and bloody one.
>
> *At the Serbian Front in Macedonia*: p. 181

In the meantime the British military units arrived in Salonika as part of the Allied Forces whose soldiers were sent to the Macedonian Front; so the number of the British subjects stationed in Macedonia was considerable. Some of them never returned to their homes and remained to rest forever in their perpetual home that fate had ordained for them in the British Military Cemetery in Skopje. Inscriptions of the name, the unit in which they served and an occasional epitaph on the tombstone hide many stories of brave deeds achieved at Doyran and elsewhere on the Macedonian Front, buried there never to be told to posterity. French military cemeteries exist in Skopje and Bitola where soldiers of the French troops had been buried.

In Macedonia, particularly in Skopje crowds of English-speaking people walked in the streets of the city and its markets watching in bewilderment the medley of its population, the breath-taking beauty of the landscape, the grandeur of the old buildings, churches and monuments. On their way back home they picked up some artifacts from the place, such as pieces of folk costumes they had bought from the local peasants. Some of these might have been put aside or in attics since, for the successors, they had lost the significance and the endearment they had for the original owners who had carried them from afar as reminders of their great adventure in life. However, far more important for posterity are the works and the records they made in the years of the Great War in which they have described most vividly and in the most impressive way the major incidents of which they were not only eyewitnesses but active participants as well. Some of those narrations soar to the heights of the best examples of the English literary prose tradition which testifies to the great literary talent of their authors.

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Р е з и м е

БРИТАНСКИТЕ ВОЕНИ БОЛНИЦИ ВО МАКЕДОНИЈА ЗА ВРЕМЕ НА ПРВАТА СВЕТСКА ВОЈНА

Владимир Цветковски

Катедра за англиски јазик и книжевност,
Филолошки факултет „Блаже Конески“,
Универзитет „Св. Кирил и Методиј“,
Скопје, Р. Македонија

Статијата се осврнува на медицинската дејност на британските воени болници стационирани во Македонија за време на Првата светска војна, особено на хируршките зафати вршени под многу тешки услови во импровизирани операциони сали, како и лекувањето на ранетите и болните војници што се испраќаа од боиштата на Македонскиот фронт.

Клучни зборови: Прва светска војна, Македонски фронт, д-р Мек Ларен, Леди Пеџет, Болница на Шкотланѓанките, Остров

УПАТСТВО ЗА АВТОРИТЕ

Списанието „Прилози“ на Одделението за медицински науки на Македонската академија на науките и уметностите излегува трипати годишно и е цитирано во Index Medicus и во Medline и е достапно на www.manu.edu.mk/prilozi. Во него се објавуваат едиторијали, изворни научни трудови, научни соопштенија и прегледни статии (клинички, лабораториски и епидемиолошки искуства, прикази на случаи, куси соопштенија, писма до уредникот, историски записи и др.) од областа на медицинските науки. Трудовите не треба да ги содржат резултатите што авторите веќе ги објавиле во други публикации или списанија.

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Обемот на оригинален научен труд, вклучувајќи ги и прилозите (илустрации, графикони, табели) не смее да биде поголем од еден авторски табак (30.000 знаци, односно 16 страници од 28 реда). Обемот на кратките соопштенија не треба да биде поголем од седум страници.

На трудот, покрај името и презимето на авторот/авторите, треба да биде наведена установата или организацијата во која е изработен трудот.

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Трудот по правило треба да содржи: вовед, материјал и методи, резултати, дискусија и заклучок. Воведот мора да биде краток, со јасно дефинирана цел и со досегашно познавање на проблемот. Материјалот и методите треба да содржат доволно податоци од кои читателот ќе биде во состојба да ги повтори испитувањата без дополнителна информација. Резултатите треба да бидат напишани кратко и јасно, а дискусијата да ги објасни резултатите.

Мерните единици и другите технички податоци мораат да бидат усогласени со Sl-системот.

Илустрациите се приложуваат посебно. Графиконите и цртежите треба да бидат на паус или на бела хартија, контрастни, а ознаките и бројките во графиконите сразмерни на големината на цртежот, за да останат читливи по редуцирањето на големината на цртежот. По правило не треба да бидат повеќе од четири. Нивното место во текстот да биде означено. Сите илустрации треба да имаат легенди на англиски јазик.

Табелите можат да бидат приложени посебно, но нивното место во текстот да биде означено. Насловите на табелите треба да бидат напишани на англиски јазик.

За трудовите од областа на медицинските науки, во принцип, важат упатствата објавени во „Brit. Med. Journal“, Vol. 296, 1988, p. 101–105 („Ванкуверските правила“), односно N. Engl. J. Med., Vol. 324, 1991, p. 424–428.

Литературата се цитира во оригинал, и тоа по следниов редослед: презиме и почетна буква од името на авторот, наслов на трудот, назив на списанието, година на објавување на цитираниот труд, годиште и број, страници (од–до). Доколку се цитира книга или зборник на трудови, се наведува и издавачот и местото на издавање (пред страниците). Ако се цитира труд од повеќе од три/шест автори, по третиот/шестиот се додава „и сор.“, односно *„et al.“*. Во текстот на трудот се наведува

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Ракописите се доставуваат до Уредувачкиот одбор на сп. „Прилози“ на Одделението за медицински науки на МАНУ во два примерока (оригинал и копија) и електронска верзија на трудот.

Авторите на трудовите треба да ги покријат трошоците за печатење и за интернет-објавување на своите трудови во износ од 250 ЕУР во денарска противвредност.

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The papers submitted for publication in *Prilozi* are peer-reviewed by two experts from the respective scientific field who remain anonymous to the authors.

The papers are published in English, accompanied by a summary written in Macedonian.

The paper should be typed with double-spacing (28–30 lines), on a white paper in A4 format, with margins of 3 cm, or on a computer using Word for Windows programme enclosing the CD, or USB.

The length of the original research paper, including the annexes (illustrations, graphs, tables, etc.) should not exceed a signature or printer's sheet (30,000 signs, that is, 16 pages with 28 lines each). The length of shorter reports should not exceed seven pages.

The submitted manuscript must contain the name/s and surname/s of the author/s, the name and address of the institution and or organisation where it was prepared.

The abstract should not exceed 250 words, written in English, and a summary in Macedonian. The abstract should represent briefly the goals, methods, main results (with numerical data) and basic conclusions of the research. The most essential key words must be added to the abstract. The key words for the medical sciences are given according to M.E.S.H.

The manuscript contains an introduction, materials and methods, results, discussion and a conclusion. The introduction must be concise with a clearly defined goal and with previous knowledge of the problem. The materials and methods ought to contain sufficient data to enable the reader to repeat the investigation without seeking additional information.

The results should be presented briefly and clearly, and the discussion should explain the results.

The measuring units and other technical data should be given according to the Sl-system.

Illustrations are submitted separately. Graphs and drawings should be prepared on tracing paper or on a white sheet of paper, in contrast, while the markers and figures in the graphs must be proportional to the size of the drawing in order to remain readable after the reduction of the size of the drawing. There should not be more than four drawings and their places in the text should be clearly indicated. All illustrations must be accompanied by legends in English, and the abstract in English and in Macedonian.

Tables can be enclosed separately, but their places in the text must be indicated. The titles of the tables should be written in English.

The references used are cited in the original and as follows: surname and the first letter of the author's name, the title of the work, the title of the journal, year of publication of the work cited, publishing year and number, pages (from-to). If a book or a collection of works is cited, then the publisher and the place of publication should be given (before the pages). If the manuscript cited has more than three authors, then *et al.* is added after the third author. In the text of the paper the first author and the year are cited in brackets [ ], i.e. a number in brackets when the bibliography is numerated. When using acronym for the first time, provide the full phrase in brackets. An example: Shapiro AMJ, Lakey JRT, Ryan EA, et al. Islet Transplantation in Seven Patients with Type 1 Diabetes Mellitus Using a Glucocorticoid-Free Immunosuppressive Regimen. N Engl J Med 2000; 343(4): 230–8.

Short reports published in the journal *Prilozi* (up to 7 typed pages) do not need to have separate sections for introduction, materials and methods, discussion and conclusion, but these elements can be incorporated in the text.

The paper is to be accompanied with a letter by the corresponding author, with a statement that the paper has not been published or submitted/accepted for printing in other journal or scientific publication, and with a confirmation that the paper has been considered and approved by all co-authors, i.e. with an accompanying declaration on the possible conflict of interests by the authors. The contribution of each author in the paper is to be explained.

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The authors of the manuscripts need to cover the costs of printing and online publishing for their manuscript in total amount of Macedonian MKD equivalent to 250 EUR.

The authors of the manuscripts need to sign two documents accompanying the manuscript: Author Disclosure Form and Ethical Responsibility Form.

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Bul. Krste Misirkov 2, 1000 Skopje, Republic of Macedonia, P.O. Box 428
www.manu.edu.mk
email: manu@manu.edu.mk
tel: +389 2 32 35 400 fax: +389 2 32 35 500

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www.manu.edu.mk
email: manu@manu.edu.mk
tel: +389 2 32 35 400 fax: +389 2 32 35 500

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