ANALELE ŞTIINŢIFICE
ALE
UNIVERSITĂŢII „ALEXANDRU IOAN CUZA”
DIN IAŞI

(SERIE NOUĂ)

SECŢIUNEA II

a. GENETICĂ ŞI BIOLOGIE MOLECULARĂ

TOMUL XV, fascicula 4 **2014**

Editura Universităţii „ALEXANDRU IOAN CUZA” Iaşi

FOUNDING EDITOR

Professor Ion I. BĂRA, PhD

EDITOR IN CHIEF

Professor Vlad ARTENIE, PhD University “Alexandru Ioan Cuza”, Iaşi
vartenie@uaic.ro

ASSISTANT EDITOR

Assistant professor Lucian HRIŢCU, PhD University “Alexandru Ioan Cuza”, Iaşi
hritcu@uaic.ro

PRODUCTION EDITOR

Lecturer Eugen UNGUREANU, PhD University “Alexandru Ioan Cuza”, Iaşi
aeu@uaic.ro

EDITORS

Academician Professor Octavian POPESCU, PhD	“Babeş Bolyai” University, Cluj Napoca, Romania
Professor Roderich BRANDSCH, PhD	“Albert Ludwigs” University, Freiburg, Germany
Professor HuiGen FENG, PhD	University XingXiang, China
Professor Gogu GHIORGHIŢĂ, PhD	University Bacău, Romania
Professor Didier GUILLOCHON, PhD	Université de Sciences et Technologies de Lille, France
Professor Peter LORENZ, PhD	University of Applied Sciences, Saarbrucken, Germany
Professor LongDou LU, PhD	University XingXiang, China
Professor Toshitaka NABESHIMA, PhD	Meijo University, Nagoya, Japan
Professor Janos NEMCSOK, PhD	University Szeged, Hungary
Professor Alexander RUBTSOV, PhD	“M.V. Lomonosov” State University, Moscow, Russia
Assistant professor Costel DARIE, PhD	Clarkson University, USA
Assistant professor Mihai LESANU, PhD	State University, Chisinau, Republic of Moldova
Lecturer Harquin Simplice FOYET, PhD	University of Maroua, Cameroon
Christian GAIDDON, PhD	INSERM U1113, Strasbourg, France
Cristian ILIOAIA, PhD	Ecole Normale Supérieure, Cachan, France
Andrew Aaron PASCAL, PhD	CEA-Saclay, France

ASSOCIATE EDITORS

Professor Dumitru COJOCARU, PhD	University “Alexandru Ioan Cuza”, Iaşi
Professor Costică MISĂILĂ, PhD	University “Alexandru Ioan Cuza”, Iaşi
Professor Ovidiu TOMA, PhD	University “Alexandru Ioan Cuza”, Iaşi
Assistant professor Simona DUNCA, PhD	University “Alexandru Ioan Cuza”, Iaşi
Assistant professor Anca NEGURĂ, PhD	University “Alexandru Ioan Cuza”, Iaşi
Assistant professor Zenovia OLTEANU, PhD	University “Alexandru Ioan Cuza”, Iaşi
Lecturer Csilla Iuliana BĂRA, PhD	University “Alexandru Ioan Cuza”, Iaşi
Lecturer Elena CIORNEA, PhD	University “Alexandru Ioan Cuza”, Iaşi
Lecturer Cristian CÎMPEANU, PhD	University “Alexandru Ioan Cuza”, Iaşi
Lecturer Mirela Mihaela CÎMPEANU, PhD	University “Alexandru Ioan Cuza”, Iaşi
Lecturer Lucian GORGAN, PhD	University “Alexandru Ioan Cuza”, Iaşi
Lecturer Marius ŞTEFAN, PhD	University “Alexandru Ioan Cuza”, Iaşi
Lecturer Cristian TUDOSE, PhD	University “Alexandru Ioan Cuza”, Iaşi
Lecturer Lăcrămioara OPRICĂ, PhD	University “Alexandru Ioan Cuza”, Iaşi

SECRETARIATE BOARD

Lecturer Călin MANIU, PhD	University “Alexandru Ioan Cuza”, Iaşi
Lecturer Marius MIHĂȘAN, PhD	University “Alexandru Ioan Cuza”, Iaşi

EDITORIAL OFFICE

Universitatea „Alexandru Ioan Cuza”, Facultatea de BIOLOGIE
Laboratorul de Biochimie şi Biologie Moleculară
Bulevardul Carol I, Nr. 20A, 700506, Iaşi, România
www.gbm.bio.uaic.ro / gbmpapers@yahoo.com

CONTENT

Ioana – Miruna Balmuș, Anca Negura, Lucian Negura, Alin Ciobică, Daniel Timofte – Current status of knowledge on Alzheimer’s disease genetics 1

Elena-Loredana Sandu, Ciobica Alin, Lacramioara Oprica, Emil Anton, Daniel Timofte – The relevance body mass index on the oxidative stress status of Alzheimer’s disease pathology 9

Răzvan Boiangiu, Doina Guzun, Marius Mihăşan – Time dependent accumulation of nicotine derivatives in the culture medium of *Arthrobacter nicotinovorans* pAO1 19

Roxana Popescu, Angela Dăscălescu, Cătălin Dănăilă, Doramina Gheorghiu, Ion Antohi, Anca Goriuc, Anca-Viorica Ivanov, Doina Azoicăi – Myeloproliferative neoplasms with concurrent BCR-ABL fusion gene and JAK2V617F mutation 25

Ana Maria Holicov, Tudor Petreuş, Carmen Elena Cotrutz – Cell proliferation and migration assay on polymer surfaces 35

Anca Roxana Bivoleanu, Eduard Crauciuc, Ovidiu Toma, Dragoş Crauciuc, Mihaela Moscalu, Maria Stamatin – Complications of prematurity as risk factors for outcome 41

Răzvan V. Filimon, Roxana Filimon, Liliana Rotaru – Determination of the polyphenol oxidase activity in relationship to total phenolic and anthocyanin content of some Romanian vine varieties (*Vitis vinifera* L.) for table grapes 49

Mihaela Aurelia Ivan, Marius Nicusor Grigore, Lacramioara Oprica, Maria-Magdalena Zamfirache – Non-enzymatic antioxidants content in several species collected from salt marshes from Dobrogea 57

Corneliu Tanase, Irina Volf, Valentin I. Popa – Bioaccumulation of iron in rapeseed under natural amendments influence 65

Liviu Fărtăiş – Prospects for beech genetic resources administration in *Făgetul Secular Stuhoasa* natural area 71

INSTRUCTIONS FOR AUTHORS 77

CURRENT STATUS OF KNOWLEDGE ON ALZHEIMER'S DISEASE GENETICS

IOANA – MIRUNA BALMUŞ[1*], ANCA NEGURA[1], LUCIAN NEGURA[2], ALIN CIOBICĂ[1], DANIEL TIMOFTE[2]

Keywords: Alzheimer, genes, mutations, APOE, tau.
Abstract: As the entire human being works as a perfectly balanced whole, each and every disturbance at any level brings other disturbances, like a chain reaction to superior levels, the current researches aim for the molecular aspects of any physiological disorders. It is well possible that any physiological reaction is not the cause of a disease but an effect of molecular disturbances in biochemical and genetic mechanisms responsible for a feature or behavior exhibit. The exact causes of Alzheimer's disease are mostly unknown, excepting 1 to 5% cases notably identified with obvious genetic variance. In the scientific world, there are many hypotheses that explain the occurrence of Alzheimer's disease: amyloidal hypothesis, taupathy hypothesis, cholinergic hypothesis and so on, but from all of these it seems that the molecular/genetic hypothesis is the most studied of all, because of its relevance to the true pathological mechanism. It seems that some allelic variants and mutations of genes that encode important regulatory molecules in neuronal activity may give a certain predisposition to Alzheimer's disease or to other neurodegenerative diseases, even in young individuals. One good example is the APOE gene that encodes a surface component of triglyceride reach lipoproteins. At the neuronal level, this glycoprotein has an important role in lipid distribution during nerves growth and repair. The APOE gene exists in three allele variants present in human population in different proportions (ε2, ε3, ε4) which in different combinations give to the carrier various predispositions to cholesterol and triglycerides mechanisms disorders, Levy's bodies dementia and Alzheimer's disease (ε4 allele).

INTRODUCTION

Alzheimer's disease is a progressive neurodegenerative pathological state which occurs mostly due to aging and exhibits varying symptoms by individual, physiological, neurological, psychical, biological and molecular conditions. This process alters one's cognitive functions of brain leading to intellectual abilities and even social behavior and individual personality loss (ADA, 2007). Alzheimer's disease is characterized by heavy behavior disorders which lead the public thinking to a misused pseudonym senile dementia, from the Latin *demens*, meaning insane or demented. This condition is the most common form of mental decline in elders (ART, 2008). It is believed that it is increasingly common because of the longer sustainability of life, much longer than average life expectancy. As every part of the body, during aging, the brain is also affected, but slower than the others. As long as the drug therapies and life styles prevents body degeneration, brain degeneration often get to be visible in elder behavior (as dementia got to be called loss of mind, or doting, associated with the brain degeneration followed by functions loss – memory, cognition, poorly developed in small children) (NIA, 2011). It is also believed that during ancient and past times, this disease was very rare, death occurring from degeneration of other organs (guts, lungs, heart, muscles), this being the explanation why Alzheimer's disease was poorly described in history (Berchtold and Cotman, 1998).
In present, Alzheimer's disease is one of the most severe neurodegenerative diseases because its cause and progression are yet to be discovered. More than that, is seems that almost all elders over 85 years old exhibit Alzheimer's disease characteristic symptoms; it is highly incurable and irreversible, unstoppable and unpredictable (Mölsä *et al.*, 1986; Brookmayer *et al*., 2007, NIA, 2007). Although the main condition that precedes the neuropsychiatric symptoms is the beta-amyloid plaques (βAP) and neurofibrillary tangles (NFT) accumulation, it is possible that the molecular mechanisms behind this process might be slightly different, in molecular terms. It is highly possible that intraneuronal and extraneuronal accumulations have different origins, as a study on differential preference for small molecules to aggregate in cellular compartments shows (Brookmeyer *et al.*, 1998). The present review aims to motivate further investigations of Alzheimer's disease genetics due to its immense complexity and importance in determining the true Alzheimer's disease's etiology so that finnaly an efficient treatment can be found.

MOLECULAR MECHANISMS INVOLVED IN ALZHEIMER'S PATHOLOGY

Alzheimer's disease was firstly described as "presenile dementia" based on the observations made by the German psychiatrist Alois Alzheimer on a patient who exhibited a progressive loss of cognitive functions and died shortly. During autopsy, Alzheimer observed the brain using histological methods and described features as it follows: "Numerous small

milliary foci are found in the superior layers. They are determined by the storage of peculiar material in the cortex" (Maurer *et al*,. 1997). Alzheimer continued: "All in all we have to face a peculiar disease process. Such peculiar disease processes have been verified recently in considerable numbers". "Milliar foci, which are caused by deposition of a peculiar substance in the cortex" were lately defined as senile plaques and "very peculiar changes in the neurofibrils" as the helical tangles. These molecular aggregations Alzheimer observed back then remain the main explanation of pathogenesis of Alzheimer's disease even though other very important molecular, genetic and epidemiological hypotheses were expressed (Povova *et al.*, 2012). The main problem of the explanation of pathology of Alzheimer's disease still could be seen in the inability to identify key mechanisms that release pathologies observed in Alzheimer's disease.

Amyloid plaques (βAP) often occur in association areas cortex and are associated with cell synapse endings. Neurofibrillary tangles (NFT) are characteristic to entorhinal cortex (median part of the temporal lobe) and affect cortico-cortical projection origin cells (Hoff, 1997). The first neurons that succumb due to NFTs are cholinergic Meynert basal nuclei, entorhinal pyramidal neurons and hippocampal neurons (Morrison *et al.*, 1997). βAP are a permanent characteristic to Alzheimer's but NFTs do not always occur, meaning that it is highly possible that βAP are also a effect or a collateral damage. βAP affect sensorial, motor and association areas, but NFTs only association one, meaning that NFTs only affect long distance interactions (Gomez-Isla *et al*., 1997). As another argument, tau protein, major NFT component, is not present in dendrites and is active primarily in the distal portions of axons where it provides microtubule stabilization but also flexibility as needed. While βAP aren't always associated with neuron death, NFTs are the main cause of Alzheimer's type degenerescence (Rapaport *et al.*, 2002). More than that, it has been shown that MAPT knock down mice are resistant to βA toxicity [idem]. So it is possible that changes occurred in tau protein pathway to be caused by βA which triggers the activation of some specific kinases and phosphorilases (Rapaport and Ferreira, 2000).

The way βA and other small peptides are formed through APP cleavage can be understood through precursor protein structure. It is consisted in several active domains situated both extracellular, intracellular and intramembranar. It is thought that the small domains growth factor-like and bind to copper ions, closely related, are the key domains in cleavage promotion. There are many APP isoforms but the brain isoform shows no specific serin protease inhibitor domain probably because the brain associated mechanism is independent to kinasic regulation (De Stooper and Annaert, 2000). Although it has been shown that βA triggers a certain type of apoptosis, through a kinase cascade mechanism, this fact is important because it says that brain isoform cannot be regulated by external specific pathways' factors.

After synthesis, APP undergoes a serie of posttranslational maturation events such as glycosylation, phosphorylation and tyrosine residue sulfonation. Proteolytic action is due to a suite of proteolytic specific enzymes: α, β and γ secretases. Both α and β secretases lead to C-terminal end cleavage partly associated with apoptosis. Next to this action, γ secretase triggers transmembrane domain cleavage in βA and βA-like fragments (De Stooper and Annaert, 2000). The amyloidogenic processes are highly associated with membrane phospholipids presence. As the γ secretase activity is conditioned by the β secretase one, only after C-terminal end was cleaved, γ secretase can be activated. More than that, γ secretase is also activated only in presence of cholesterol and apoE. That led to the theory that Alzheimer's can be triggered by high concentrations of cholesterol and low activity of apoE (inheritance of disabilited apoEε4 variant) (Vetrivel *et al.*, 2004).

Tau protein is a highly soluble microtubule-associated protein (MAP) also essential in Alzheimer's pathology, the major component of NFTs. In humans, these proteins are found mostly in neurons compared to non-neuronal cells. One of tau protein's main functions is to modulate the stability of axonal microtubules. Other nervous system MAPs may perform similar functions, as suggested by tau knockout mice that did not show abnormalities in brain development - possibly because of compensation in tau deficiency by other MAPs (Harada *et al.* 1994). Tau proteins interact with tubulin to stabilize microtubules and promote tubulin assembly into microtubules.

Tau has two ways of controlling microtubule stability: isoforms and phosphorylation. Hyperphosphorylation of the tau protein can result in the self-assembly of tangles of paired helical filaments and straight filaments, which are involved mainly in the pathogenesis of Alzheimer's disease (Alonso *et al.*, 2001). All of the six tau isoforms are present in an often hyperphosphorylated state in paired helical filaments from Alzheimer's disease brain. In other neurodegenerative diseases, the deposition of aggregates enriched in certain tau isoforms has been reported. When misfolded, this otherwise very soluble protein, it can form extremely insoluble aggregates that contribute to a number of neurodegenerative diseases (Hall, 2011; Saman and Hall, 2011).

GENES INVOLVED IN ALZHEIMER'S PATHOLOGY

Regarding the effects and the course of the molecular mechanisms discussed, the genes involved in Alzheimer's pathology are to be described according to the encoded product's role. Therefore there are genes that encode the substrate proteins (APP or tau protein), the enzymes that cut them (PSEN1 and PSEN2 as domains of the γ secretase complex) or other genes that encode for proteins or receptors encountered alongside (APOE, TREM2). Thus the genes involved directly in Alzheimer's pathology are generically called deterministic genes and their mutations cause early onset

Alzheimer's disease, a dominant autosomal inherited Alzheimer's type, familial and extremely rare. The other genes involved in Alzheimer's pathology but not directly are called risk factors and their mutations cause a vulnerability of their carrier to developing Alzheimer's pathology. Whether they have deficits in cholesterol metabolism, whether 'brain maintenance cells' get a disability in 'cleaning', risk factors give the opportunity to certain mechanisms to be disturbed and the effects to disseminate like a chain reaction until they reach the mechanisms involved in Alzheimer's pathology.

APP and PSEN1 and 2 genes

APP and PSEN genes are involved directly in Alzheimer's pathology by being the main genes that encode the substrate and the enzymes that lead to the main product of which accumulation cause plaques and Alzheimer's symptoms: the amyloid precursor protein's gene and the presenilin 1 and 2 genes, key domains of the γ secretase complex.

APP gene, a highly conserved ancestral gene, is localized on the 21st chromosome's long arm encodes the protein that by its cleavage leads to amyloid synthesis – a membrane protein classified as a endogenous ligand (Yoshikai *et al.*, 1990; Sarkar and Tharp, 2013). Along its 18 exons, it carries coding informations regarding the structure and the functions of APP and more importantly, its variants arose by alternative splicing of the transcript (Lamb *et al*., 1993). In human, alternative splicing of APP gene transcript is tissue characteristic arising certain protein isoforms specific to brain, medulla and other tissues. It is believed that certain changes in isoforms proportions lead to Alzheimer's disease (Zheng and Koo, 2006). It also seems that APP binds to surface proteins in order to regulate cell adhesivity. Studies show that in brain APP regulates neuron migration during early ontogenesis.

In spite of its high gene sequence conservation, it has been shown that the amino acids sequence of the intramembrane domain is highly variable (Goate *et al.*, 1991). The mutations which occur in βA generating domain and other important APP domains cause familial Alzheimer's disease characterized by highly dense and thick amyloid plaques (Murrell *et al.,* 1991). The mutations in the regulating or intronic gene sequences are associated with high amounts of APP and βA synthesized (Chartier-Harlin *et al.*, 1991).

The most common APP gene mutation is a substitution that leads to protein structure change (Val717Ile) (Talarico *et al.*, 2010) and it is associated with early onset Alzheimer's disease. Some mutations cause an aberrant protein synthesis, longer and highly adhesive. When the high amounts of normal or aberrant APP are being cleaved and excreted outside the cells, they accumulate and block mostly synaptic gaps. Also a highly toxic protein fragment can appear that can trigger an apoptotic mechanism.

Nevertheless it has been shown that there can appear protective mutations. It is the case of the A673T mutation, a substitution contiguous to β secretase cleavage site that cause a decrease by 40% of in vitro βA formation (Citron *et al.*, 1992).

It has been proven that any variation in transcription promoting sequences can alter gene expression and therefore alter the normal mechanisms which can give a susceptibility to many neurodegenerative diseases. These promoter sequences are located in the immediate vicinity of the main regulation sequence and of the upstream 5' region.

The PSEN genes encode two important proteins, presenilin 1 and presenilin 2 – components of the secretasic complex, and have a remarkable sequence similarity. They are localized on the 14th and 1st chromosomes, respectively. Studies show that both nitrogenous base gene sequence and amino acids expression product successions are very similar for both PSEN1 and PSEN2. Also, the splicing mechanism, functions, structure but not regulating sequence, are almost identical. Each gene is consisted of 13 exons of which 10 encode the proteins' sequences. Regulating sequences are consisted by the first three exons of the PSEN1 gene and the last three of PSEN2. By the fact that intron-exon jonctions are also almost virtually identical, it is believed that these two genes derive from the same ancestral gene which was duplicated during evolution and speciation, equivalent to *Coenorhabditis elaegans* sel-12 gene (Smialowska and Baumeister, 2006).

It seems that the presenilins are major component of the nine region transmembrane domain γ secretasic complex. On the other hand, it has been shown that they interact with an intramembrane domain involved in calcium ions homeostasis which regulates the neurotransmitter releasing mechanism on presynaptic level [idem]. These proteins function are highly dependent on a phosphorylation mechanism acetylcholine dependent for presenilin 1. The way that they interact with the apoptosis pathway is yet to be revealed (Smialowska and Baumeister, 2006).

PSEN1 and 2 gene expression is important for neurons and glia. PSEN1 is equally expressed in all body tissues but PSEN2 expresses tissue dependent (brain, heart, and pancreas). Both gene expression products are stored in neuron soma and dendrites (Spasic *et al.*, 2006).

There have been described over 40 PSEN1 mutations and only two PSEN2 mutations all of which are missense mutations of highly conserved residues. There is one exception, a 9th exon splicing site PSEN1 mutation which cause an amino acid substitution correlated in young with paraplegia (S290C). No other mutation has been shown to neither alter nor block presenilin cell processing and it has been postulated that not the presenilins blockage undergoes Alzheimer's occurrence but the aberrant N-terminal and C-terminal faulty cleavage fragments increase γ secretasic complex action and amyloid synthesis. More than that some say that because there are no records of nonsense or frame shift mutations it is highly possible that these genes might not be determinant for Alzheimer's. A more relevant explanation is that the mutations might alter presenilins functions or that the aberrant protein product might block the wild-type protein functions through a dominant-negative mechanism (Brouwers *et al.*, 2008).

Tau protein gene (MAPT gene)
Tau protein is encoded by MAPT (microtubule-associated protein tau) gene. As it is called, this protein is important in neurons' cytoskeleton integrity as the main microtubules component. This gene is expressed in neuron nucleus and then the protein is carried to axons where it exhibits its functions. MAPT gene is localized on the 17th chromosome and by its expression leads to six protein isoforms synthesis through three exons alternative splicing. Because of the phosphorylation activation mechanism, it is thought that any error occurred both in MAPT gene and enzymes involved in this pathway can cause tau protein instability or isoforms proportion disturbance. However only some errors can lead to an Alzheimer's disease predisposition but all of them lead to neurological or neurodegenerative pathologies (Avila *et al.*, 2004).
It is known that MAPT gene has two haplotypes, H1 and H2, in which it seems that the gene is expressed in reverse order. The H2 haplogroup is common only to Europeans and populations with European inheritance. The H1 haplogroup is associated with a high probability of neurodegenerative diseases occurrence such as dementia and Alzheimer's disease (Shaw-Smith *et al.*, 2006).
Tau protein gene variations and tau protein disorders are mostly specific to frontotemporal dementia with Parkinsonism rather than Alzheimer's. There isn't yet an explanation of this fact but it is thought that MAPT mutations are associated with progranulin gene mutations to cause pathological conditions. MAPT mutations do not cause familial AD, but can certainly cause frontotemporal dementia (FTD). The pathogenic mutations, either exonic or intronic, generally alter the relative production of tau isoforms and can lead to disturbances in microtubule assembly mechanisms or tau protein adhesivity.
There have been described three MAPT gene mutations. The R5H mutation, a missense single nucleotide substitution alongside 1st exon of the gene, reduces tau protein's ability to promote microtubule assembly that leads to neuronal loss in the frontal and temporal lobes, tau protein deposits in glia and aberrant insoluble tau protein synthesis. This mutation is characteristic to frontotemporal dementia (Hayashi *et al.*, 2002). The non-coding 10th intron C>T mutation has no pathological implications found yet for both FDT not AD (De Silva, 2009). K280del 10th exon mutation causes a three nucleotide deletion but has an unclear pathogenicity for AD and FDT. The K280del variant inhibits 10th exon inclusion and leads to unusual tau transcripts. It also has been shown that it reduces the ability of tau protein to promote microtubule assembly. These effects cause variable pathogenicity, mainly severe atrophy of the frontal and temporal cortex and alternatively, tangles, neuritic amyloid plaques, Lewy bodies, or atherosclerosis (Roks *et al.*, 1999; Rizzu *et al.*, 1999). The latter mutation is very rare and associated with early onset Alzheimer's. There are no animal models for none of these three mutations.

RISK FACTORS GENES

Apolipoprotein E gene
APOE gene is localized on the 19th chromosome in a gene cluster with APOC1 and APOC2. Consisting in four exons and three introns, APOE gene expression is regulated by cholesterol, fatty acids and glucose homeostasis hepatic receptors and peroxisomal proliferating receptor (Chawla *et al.*, 1999). The most important aspect to Alzheimer's is that APOE is a three allele polymorphic gene: APOEε2 (cys112, cys158), APOEε3 (cys112, arg158) and APOEε4 (arg112, arg158). These minor differences in protein structure can slightly change apolipoprotein E functions and abilities.
The ε2 allele has a lower than 7% frequencies and the APOEε2 protein variant exhibits certain deficiencies regarding surface receptors binding. It seems that homozygote ε2 individuals exhibit lower alimentary fats metabolizing capacity; therefore they have a higher risk for atherosclerosis. The ε3 allele is considered the neuter genotype and the most frequent (79%). In contrast, ε4 allele has an intermediate frequency (14%) and the worst fenotypical impact. The carriers exhibit high risk to develop Alzheimer's disease, but also atherosclerosis, multiple sclerosis, fast telomere shortage and others. There is not a valuable explanation of the so high impact mechanism. The only explanation has been found to the increased allele frequency in spite its aggressive effects is that this allele promotes high levels of vitamin D to carriers (Eisenberg *et al.*, 2010).
It has been shown that the ε4 allele inheritance is an autosomal dominant manner of Alzheimer's disease inherited risk development. More than that it seems that the age of early Alzheimer's symptoms occurrence lowers as the ε4 allele is more present. However, it has been shown that ε4 APOE allele inheritance is an insufficient risk for disease development. More than that, APOEε4 has been associated with other neurodegenerative disorders so APOE might pay a common role in neuronal response to injury (Murrell *et al.*, 1991).

TREM2 gene
TREM2 gene encodes an important cellular receptor protein (triggering receptor expressed on myeloid cells 2). It is localized on the 6th chromosome and its homozygous state mutant can cause rare forms of dementia especially Alzheimer's. The R47H exon 2 mutation generates a substitution which also causes an amino acid substitution. As

TREM2 exhibits a anti-inflammatory function in brain cells, this mutation interferes with the local leukocytes' ability to find and destroy amyloid plaques (Jonsson *et al*., 2012; Lambert, 2013). This mutation has been reported present to many Alzheimer's patients older than 85.

In addition to these genes, it is considered that the Alzheimer's genetics spectrum is consisted in at least 10 other genes involved directly or triggering vulnerabilities that lead to Alzheimer's development. The molecular mechanisms from which βAP and NFT accrue are still partly unknown, but are intensely studied through medicine and psychiatry, analytical and clinical biochemistry, molecular genetics, proteomics, genomics, biostatistics and biomodeling techniques using both human subject and animal models.

CONCLUSIONS

In conclusion, Alzheimer's disease can be considered a highly complex disease that exhibits symptoms in all organisms' levels from behavioral changes to subcellular discrepancies. It has been shown that besides external factors that lead to Alzheimer's development such as life style, alimentary habits or prolonged life sustainability there are certain internal factors uncontrollable but predictable. Alzheimer's genetics, roughly overlooked, seems to pay an important role in understanding this complex disease. As every visible effect has a molecular background, even emotions, memories, thoughts and feeling, it is highly possible that pathologies has molecular causes too. There are many genes and molecular factors involved in Alzheimer's development worth to be discussed and considered as starting points in Alzheimer's pathology, so it would be helpful to consider the molecular level in further Alzheimer's disease and other neurodegenerative diseases research.

REFERENCES

Alonso, A., Zaidi, T., Novak, M., Grundke-Iqbal, I., Iqbal, K. (2001): *Hyperphosphorylation induces self-assembly of tau into tangles of paired helical filaments/straight filaments,* Proc. Natl. Acad. Sci. U.S.A. 98 (12): 6923–8;

Alzheimer's Disease Association (2007): *What is Alzheimer's disease?*, alzheimers.org.uk;

Alzheimer's Research Trust (2008): *Alzheimer's diagnosis of AD*;

Avila, J., Lucas, J., Pérez, M., Hernández, M. (2004): *Role of Tau Protein in Both Physiological and Pathological Conditions*, Physiological Reviews, Vol. 84 no. 2, 361-384;

Berchtold, N.C., Cotman, C.W. (1998): *Evolution in the Conceptualization of Dementia and Alzheimer's Disease: Greco-Roman Period to the 1960s*, Neurobiology of Aging 1998;19(3):173–89;

Brookmeyer, R., Gray, S., Kawas, C. (1998): *Projections of Alzheimer's Disease in the United States and the Public Health Impact of Delaying Disease Onset,* American Journal of Public Health. 1998;88(9):1337–42;

Brookmeyer, R., Johnson, E., Ziegler-Graham, K., Arrighi, H.M. (2007): *Forecasting the global burden of Alzheimer's disease*, Alzheimer's & Dementia 3(3):186–91;

Brouwers, N., Sleegers, K., Van Broeckhoven, C. (2008): *Molecular genetics of Alzheimer's disease: an update,* Ann Med 40 (8): 562–83;

Chartier-Harlin, M.C., Crawford, F., Houlden, H., Warren, A., Hughes, D., Fidani, L., Goate, A., Rossor, M., Roques, P., Hardy, J. (1991):*Early-onset Alzheimer's disease caused by mutations at codon 717 of the beta-amyloid precursor protein gene,* Nature 353(6347):844–6;

Chawla, A., Boisvert, W.A., Lee, C.H., Laffitte, B.A., Barak, Y., Joseph, S.B., Liao, D., Nagy, L., Edwards, P.A., Curtiss, L.K., Evans, R.M., Tontonoz, P. (2001): *A PPAR gamma-LXR-ABCA1 pathway in macrophages is involved in cholesterol efflux and atherogenesis,* Mol Cell 7 (1): 161–71;

Citron, M., Oltersdorf, T., Haass, C., McConlogue, L., Hung, A.Y., Seubert, P., Vigo-Pelfrey, C., Lieberburg, I., Selkoe, D.J. (1992):*Mutation of the beta-amyloid precursor protein in familial Alzheimer's disease increases beta-protein production,* Nature 360 (6405): 672–4;

De Silva, R. (2009): *Clinical and pathological features of an Alzheimer's disease patient with the MAPT Delta K280 mutation,* Neurobiol Aging. 2009 Mar;30(3):388-93;

De Strooper, B., Annaert, W. (2000): *Proteolytic processing and cell biological functions of the amyloid precursor protein,* J. Cell. Sci. (Pt 11): 1857–70;

Eisenberg, D.T., Kuzawa, C.W., Hayes, M.G. (2010): *Worldwide allele frequencies of the human apoliprotein E (APOE) gene: climate, local adaptations and evolutionary history,* American Journal of Physical Anthropology 143 (1): 100–111;

Goate, A., Chartier-Harlin, M.C., Mullan, M., Brown, J., Crawford, F., Fidani, L., Giuffra, L., Haynes, A., Irving, N., James, L. (1991): *Segregation of a missense mutation in the amyloid precursor protein gene with familial Alzheimer's disease,* Nature 349 (6311): 704–6;

Gómez-Isla, T., Hollister, R., West, H., Mui, S., Growdon, J.H., Petersen, R.C., Parisi, J.E., Hyman, B.T. (1997): *Neuronal loss correlates with but exceeds neurofibrillary tangles in Alzheimer's disease*, Ann Neurol. 1997 Jan;41(1):17-24;

Hall, G.F. (2011): *Tau misprocessing leads to non-classical tau secretion via vesicle release – implications for the spreading of tau lesions in AD*, Int Conf. Alz Dis. meeting Paris, France;

Harada, A., Oguchi, K., Okabe, S., Kuno, J., Terada, S., Ohshima, T., Sato-Yoshitake, R., Takei, Y., Noda, T., Hirokawa, N. (1994): *Altered microtubule organization in small-calibre axons of mice lacking tau protein,* Nature 369 (6480): 488–91;

Hayashi, S., Toyoshima, Y., Hasegawa, M., Umeda, Y., Wakabayashi, K., Tokiguchi, S., Iwatsubo, T., Takahashi, H. (2002): *Late-onset frontotemporal dementia with a novel exon 1 (Arg5His) tau gene mutation,* Ann Neurol. 2002 Apr;51(4):525-30;

Hoff, P.R. (1997): *Morphology and Neurochemical Characteristics of the Vulnerable Neurons in Brain Aging and Alzheimer's Disease*, Eur Neurol 1997;37:71–81;

Jonsson, T., Stefansson, H., Steinberg, S., Jonsdottir, I. *et al.* (2012): *Variant of TREM2 associated with the risk of Alzheimer's disease,* The New England Journal of Medicine. 2012;368(2):107–16.

Lamb, B.T., Sisodia, S.S., Lawler, A.M., Slunt, H.H., Kitt, C.A., Kearns, W.G., Pearson, P.L., Price, D.L., Gearhart, J.D. (1993): *Introduction and expression of the 400 kilobase amyloid precursor protein gene in transgenic mice,* Nat. Genet. 5 (1): 22–30;

Lambert, J.C. (2013): *Meta-analysis of 74,046 individuals identifies 11 new susceptibility loci for Alzheimer's disease,* Nature Genetics 45 (12): 1452–8;

Maurer, K., Volk, S., Gerbaldo, H. (1997): *Auguste D and Alzheimer's disease,* The Lancet 1997; 349: 1546-1549;

Mölsä, P.K., Marttila, R.J., Rinne, U.K. (1986): *Survival and Cause of Death in Alzheimer's Disease and Multi-Infarct Dementia,* Acta Neurologica Scandinavica 74(2):103–7;

Morrison, J.H., Hof, P.R. (1997): *Life and death of neurons in the aging brain*, Science 1997 Oct 17;278(5337):412-9;

Murrell, J., Farlow, M., Ghetti, B., Benson, M.D. (1991): *A mutation in the amyloid precursor protein associated with hereditary Alzheimer's disease,* Science 254 (5028): 97–9;

National Institute on Aging (2007): *World population prospects: the 2006 revision, highlights,* Annual Report;

National Institute on Aging (2011): *About Alzheimer's Disease: Symptoms*;

P**ovova, J., Ambroz, P., Bar, M., Pavukova, V., Sery, O., Tomaskova, H., Ja Nout, V. (2012):** *Epidemiological of and risk factors for Alzheimer's disease: A review,* Biomed Pap Med Fac Univ Palacky Olomouc Czech Repub 2012; 156: 108-114;

Rapoport, M. and Ferreira, A. (2000): *Prevents Neurite Degeneration Induced by Fibrillar β-Amyloid in Mature Hippocampal Neurons*, Journal of Neurochemistry, volume 74, Issue 1, pages 125–133;

Rapoport, M., Dawson, H.N., Binder, L.I., Vitek, M.P., Ferreira, A. (2002): *Tau is essential to β-amyloid-induced neurotoxicity*, Proceedings Of The National Academy Of Sciences (Usa); Rapoport,M ; 99(9):6364-6369;

Rizzu, P., Van Swieten, J.C., Joosse, M., Hasegawa, M., Stevens, M., *et al*. (1999): *High prevalence of mutations in the microtubule-associated protein tau in a population study of frontotemporal dementia in the Netherlands,* Am J Hum Genet. 1999 Feb;64(2):414-21;

Roks, G., Dermaut, B., Heutink, P., Julliams, A., Backhovens, H., Van de Broeck, M., Serneels, S., Hofman, A., Van Broeckhoven, C., van Duijn, C.M., Cruts, M. (1999): *Mutation screening of the tau gene in patients with early-onset Alzheimer's disease,* Neurosci Lett. 1999 Dec 24;277(2):137-9;

Saman, S. and Hall, G. F. (2011): *Tau secretion from M1C human neuroblastoma cells occurs via the release of exosomes,* Keystone Meeting on Neurodegenerative diseases;

Sarkar, I.N., Tharp, G. (2013): *Origins of amyloid-beta,* BMC Genomics 14 (1): 290;

Shaw-Smith, C., Pittman, A.M., Willatt, L., Martin, H., Rickman, L., Gribble, S., *et al.* (2006): *Microdeletion encompassing MAPT at chromosome 17q21.3 is associated with developmental delay and learning disability,* Nat. Genet. 38 (9): 1032–7;

Smialowska, A., Baumeister, R. (2006): *Presenilin function in Caenorhabditis elegans,* Neurodegener Dis 3 (4–5): 227–32;

Spasic, D., Tolia, A., Dillen, K., Baert, V., De Strooper, B., Vrijens, S., Annaert, W. (2006): *Presenilin-1 maintains a nine-transmembrane topology throughout the secretory pathway,* J. Biol. Chem. 281 (36): 26569–77;

Talarico, G., Piscopo, P., Gasparini, M., Salati, E., Pignatelli, M., Pietracupa, S., *et al*., (2010): *The London APP mutation (Val717Ile) associated with early shifting abilities and behavioral changes in two Italian families with early-onset Alzheimer's disease*, Dement Geriatr Cogn Disord. 2010;29(6):484-90;

Vetrivel, K.S., Cheng, H., Lin, W., Sakurai, T., Li, T., Nukina, N., Wong, P.C., Xu, H., Thinakaran, G. (2004): *Association of gamma-secretase with lipid rafts in post-Golgi and endosome membranes*, J. Biol. Chem. 279 (43): 44945–54;

Yoshikai, S., Sasaki, H., Dohura, K., Furuya, H., Sakaki, Y. (1990): *Genomic organization of the human amyloid beta-protein precursor gene,* Gene 87 (2): 257–63;
Zheng, H., Koo, E.H. (2006): *The amyloid precursor protein: beyond amyloid,* Mol Neurodegener 1 (1): 5;

The institutional affiliation of authors. [1]Faculty of Biology, University of “Alexandru Ioan Cuza” Iași, Romania, [2]University of Medicine and Pharmacy "Gr. T. Popa" Iași.
Corresponding address. [*] Balmuş Ioana – Miruna, +40729338788, balmus.ioanamiruna@yahoo.com.

THE RELEVANCE BODY MASS INDEX ON THE OXIDATIVE STRESS STATUS OF ALZHEIMER'S DISEASE PATHOLOGY

ELENA-LOREDANA SANDU[1*], CIOBICA ALIN[1], LACRAMIOARA OPRICA[1], EMIL ANTON[2], DANIEL TIMOFTE[2]

Keywords: Body mass index, Alzheimer's disease, oxidative stress

Abstract

Introduction: While dementia affects 6-10% of persons 65 years or older, industrialized countries have witnessed an alarming rise in obesity. Obesity affects over 500 million people worldwide, and has far reached negative health effects. In addition, oxidative stress is a risk factor for metabolic diseases and was previously shown to be independently associated with obesity.

Current status of research: Researchers investigated the relationship between body mass index (BMI), age and oxidative stress. In this way, convincing evidences demonstrated that oxidative stress is a prominent feature in Alzheimer disease and links oxidative stress to the development of neuronal death and neural dysfunction, which suggests a key pathogenic role for oxidative stress in AD. Moreover, the disease progression is enhanced by oxidative stress. Also, while many hypotheses have been provided as the causes of the disease, the exact mechanisms remain elusive and difficult to verify. Results demonstrate that oxidative stress increases with the increasing of BMI and age, as a sequel to an impaired antioxidant status, an increase of peroxides and uric acid and a disadvantaged lipid profile.

Conclusions: Future studies are needed to understand optimal weight and biological mechanisms. Oxidative stress and inflammation are implicated in the pathogenesis of obesity and its related complications.

INTRODUCTION

Obesity affects over 500 million people worldwide, and has far reaching negative health effects. Oxidative stress is a risk factor for chronic diseases and was previously shown to be independently associated with obesity.

Obesity is a chronic disease of multifactorial origin that develops from the interaction of social, behavioral, psychological, metabolic, cellular, and molecular factors (Kaufer et al., 2001). It is the condition under which adipose tissue is increased and can be defined as an increase in body weight that results in excessive fat accumulation. The World Health Organization (WHO) defines obesity as a body mass index (BMI) > 30 and defines overweight as with a BMI of 25 (Sikaris et al., 2004).

Adiposity, commonly measured as body mass index (BMI), may influence or be influenced by brain structures and functions involved in dementia processes. Adipose tissue changes in degree and intensity over the lifespan, and has been shown to influence brain development in relationship to early and late measures of cognitive function, intelligence, and disorders of cognition such as dementia. A lower BMI is associated with prevalent dementia, potentially due to underlying brain pathologies and correspondingly greater rates of BMI or weight decline observed during the years immediately preceding clinical dementia onset. However, high BMI during mid-life or at least approximately 5-10 years preceding clinical dementia onset may increase risk. The interplay of adiposity and the brain occurring over the course of the lifespan will be discussed in relationship to developmental origins, mid-life sequelae, disruptions in brain structure and function, and late-life changes in cognition and dementia. Characterizing the life course of adiposity among those who do and do not become demented enhances understanding of biological underpinnings relevant for understanding the etiologies of both dementia and obesity and their co-existence.

Researchers investigated the relationship between body mass index (BMI), age and oxidative stress. In this way, convincing evidences demonstrated that oxidative stress is a prominent feature in Alzheimer disease and links oxidative stress to the development of neuronal death and neural dysfunction, which suggests a key pathogenic role for oxidative stress in AD. There is increasing evidence for the role of total adiposity, usually measured clinically as body mass index (BMI), and central adiposity, measured in AD. This topic is of enormous public health importance given the global epidemic of high adiposity and its consequences.

Alzheimer's disease (AD), the most common form of dementia among the elderly, is characterized by the progressive decline of cognitive function and has a detrimental impact globally. The number of AD cases worldwide was estimated at 36 million in 2010 and is predicted to triple by 2050.

Oxidative stress is a major factor in the aging process and in the course of various diseases associated with aging, such as AD. Oxidative stress, the direct result of the imbalance between the production of reactive oxygen species (ROS) and reactive nitrogen species (RNS), and intracellular antioxidant defenses, is invariably involved in the onset of diabetes and neurological pathologies such as Alzheimer's disease (AD), Parkinson's disease, and amyotrophic lateral sclerosis. Diabetes, in many cases, usually results in further complications such as cardiovascular disease, atherosclerosis,

and diabetic nephropathy. Thus, the hypothesis of diabetes leading, in part, to the onset of AD is an appealing topic and is hotly debated in the current literature.

OBJECTIVE

This review comprehensively examines the current knowledge on the relationship between body mass index (BMI), oxidative stress and dementia. Understanding the life-course changes in BMI and their influence on dementia risk, cognitive prognosis and mortality after diagnosis may provide new insights into the underlying pathophysiology of dementia and shape possible intervention and treatment strategies. These observations provide a strong base for addressing biological mechanisms underlying this complex association.

CURRENT STATUS OF RESEARCH ON THE OXIDATIVE STRESS OF ALZHEIMER'S DISEASE

With the emergence of disease-modifying strategies for the treatment of AD, impetus to diagnose the condition in its early 'preclinical' stages – before significant brain damage occurs – has intensified. Fortunately, advances in technology and in our perspective on what defines AD may soon make such antecedent diagnosis possible.

Dementia is an acquired syndrome in which there is a decline in memory and thinking that is sufficient to interfere with everyday performance. Some individuals demonstrate deficits either in memory alone or in memory and other cognitive domains that are indicative of an abnormality but are not yet severe enough to be termed "dementia". Most people who go on to develop dementia go through a transitional stage that some term very mild dementia and others term mild cognitive impairment (MCI) or 'cognitively impaired no dementia' (CIND). There are many different entities which can lead to cognitive impairment and dementia, including a variety of neurodegenerative disorders, vascular damage, infections, tumors, and other causes. AD is the most common cause of cognitive impairment and dementia in people over the age of 65. Determination that acquired cognitive impairment or dementia is present, and diagnosis of its likely cause, is based on clinical history (especially from a reliable informant), neurological and psychiatric examinations, and certain laboratory tests.

Recent advances indicate that dementia risk is modified by perinatal events, education status, nutritional intake, degree of physical activity and cognitive and social engagement (Borenstein et al., 2006). Several of these factors impact on adult-onset vascular disorders including strokes, hypertension, atherosclerotic disease, atrial fibrillation, diabetes mellitus, dyslipidemia, hyperinsulinaemia, hyperglycaemia, hyperhomocysteinemia, and obesity (Luchsinger et al., 2005). It is increasingly recognized that factors which increase cardiovascular disease or brain vascular pathology exacerbate the onset or progression of late-onset dementias. While data vary in individual studies that may relate to the clinical diagnosis of dementia, carriers of the apolioprotein E ε4 allele in general appear at greater at risk in the presence of vascular disease. However, the evidence for single clinically defined cardiovascular risk factors being associated with incident AD is inconsistent (Purnel et al., 2009). Some genetic influences or environmental factors may modify the progressive changes which define the final phenotype and burden of brain pathology (Premkumar et al., 1996). While randomized or controlled trials of risk factor modification (with multiple simultaneous interventions) are lacking it is encouraging that interventions of cognitive and physical activity were shown to improve cognitive performance and slow cognitive decline (Middleton et al., 2009).

As with other age-related diseases (cardiovascular disease, diabetes, cancers, etc), there are likely to be behavioral, dietary and other environmental factors that may affect the risk of AD. However, this area of research has not yet matured to a point where clear recommendations can be made. Epidemiological findings suggest that a low education level, history of head trauma, consumption of high calorie – high fat diets and a sedentary lifestyle may each increase the risk of AD (Mattson, 2004).

Changes in lifestyle and diet have resulted in an increase in the number of obese subjects; obesity has been regarded as an important factor in causing various health problems. The central nervous system (CNS), by means of signals, regulates appetite, energy intake, and weight gain; obesity can result from a failure of these signaling pathways (Sikaris, 2004).

Obesity is considered the largest public health problem worldwide, especially in industrialized countries (Bravo et al., 2006). Obesity increases mortality and the prevalence of cardiovascular diseases, diabetes, and colon cancer (Amiekhizi et al., 2007). Substantial literature has emerged that shows that overweight and obesity are major causes of co-morbidities, including T2DM, cardiovascular diseases, various cancers, and other health problems, which can lead to further morbidity and mortality. The related health-care costs are also substantial. Therefore, a public health approach to

develop population-based strategies for the prevention of excess weight gain is of great importance. However, public health intervention programs have had limited success in tackling the rising prevalence of obesity.

Alzheimer's disease (AD) is a complex, multifactorial, heterogeneous mental illness, which is characterized by an age-dependent loss of memory and an impairment of multiple cognitive functions. AD is associated with the presence of intracellular neurofibrillary tangles (NFTs) and extracellular amyloid beta (Aβ) plaques, the loss of neuronal subpopulations, mitochondrial oxidative damage, synaptic loss, and the proliferation of reactive astrocytes and microglia (Selkoe, 2001). With the life span of humans increasing and with decreasing cognitive function in elderly individuals with AD-related dementia, AD has become a major health problem in society. Therapeutic interventions are urgently needed to minimize the ill effects of this devastating disease. Genetic mutations are responsible for causing early onset “familial” AD (constituting only 2% of AD cases), but the causal factor(s) for the vast majority of late-onset “sporadic” AD cases is still unknown.

Histological, pathological, molecular, cellular, and gene expression studies of AD have revealed that multiple cellular pathways are involved in AD progression (Mattson, 2004). Pathologically, there are no differences between early- and lateonset AD (Anekonda et al., 2005). Several factors are known to be involved in the development of late-onset AD, with two of the major ones being aging (Selkoe, 2001) and mitochondrial abnormalities (Reddy, 2006; Zhu et al., 2005; Sullivan et al., 2005). Other contributing factors are the ApoE genotype (Raber et al., 2004), insulin-dependent diabetes (de la Monte et al., 2005), and environmental conditions, including diet (Kitozawa et al., 2004).

Recent cellular and animal model studies revealed that AD progression involves such cellular changes as inflammatory responses, mitochondrial oxidative damage, synaptic failure, and hyperphosphorylation of tau, all of which are directly related to aging and Aβ production (Kitozawa et al., 2004; Reddy et al., 2004, 2005; Manczak et al., 2004).

Oxidative stress is a major factor associated with the development and progression of AD and other forms of dementia. A large body of data suggests that free radical oxidative damage—particularly of neuronal lipids (Markesbery et al., 1999), nucleic acids (Manczak et al., 2004; Pappolla et al., 1996, 1999), and proteins (Pappolla et al., 1996, 1999; Butterfield et al., 2001) is extensive in the brains of AD patients. Increased oxidative stress is thought to result in the generation of free radicals and ROS, which is reported to be released by microglia activated by Aβ (Qin et al., 2002). Compared to other organs, the brain has been found to be more vulnerable to oxidative stress due to its high lipid content, its relatively high oxygen metabolism, and its low level of antioxidant defenses (Butterfield et al., 2001;). Markers of oxidative stress, such as 8-hydroxyguanosine and hemeoxygenase, have been localized to pathologic lesions in the brains of AD patients (Smith et al., 1994).

The free radical theory of normal aging proposes that the slow generation of oxygen free radicals, an unavoidable consequence of life in an aerobic environment, results in cumulative damage to critical cellular components, and eventually leads to age-related pathology (Harman et al., 1994). Free radical-mediated damage to neuronal membrane components have been implicated in the etiology of many neurodegenerative disorders, especially Alzheimer disease (Butterfield et al., 1994; Hensley et al., 1996), in which one of the most dominant risk factors is age.

According to the free radical theory of aging, reactive oxygen species cause oxidative damage, proposed to be an underlying factor of the aging process. There is considerable literature to suggest that free radical scavengers can be used to prevent free radical damage in a variety of systems.

Oxidative stress occurs due to an imbalance in the levels of antioxidant defense systems and production of reactive oxygen/reactive nitrogen species. Oxidative stress has reported to be important in the pathophysiology of a number of age-related diseases, including Alzheimer disease (AD). AD is characterized by the presence of three principal pathological hallmarks: synapse loss, extracellular senile plaques (SP), and intracellular neurofibrillary tangles (NFTs). The major component of SP is amyloid β-peptide (Aβ), a 40–42 amino acid peptide that is derived from proteolytic cleavage of an integral membrane protein (Duyckaerts et al., 2009).

ROS occur under physiological conditions and in many diseases and cause direct or indirect damage in different organs; thus, it is known that oxidative stress (OS) is involved in pathological processes such as obesity, diabetes, cardiovascular disease, and atherogenic processes. It has been reported that obesity may induce systemic OS and, in turn, OS is associated with an irregular production of adipokines, which contributes to the development of the metabolic syndrome (Esposito et al., 2006). The sensitivity of CRP and other biomarkers of oxidative damage are higher in individuals with obesity and correlate directly with BMI and the percentage of body fat, LDL oxidation, and TG levels (Pihl et al., 2006); in contrast, antioxidant defense markers are lower according to the amount of body fat and central obesity (Chrysohoou et al., 2007; Hartwich et al., 2007). A research showed that a diet high in fat and carbohydrates induces a significant increase in OS stress and inflammation in persons with obesity (Patel et al., 2007).

Several epidemiological show that a history of adult onset diabetes mellitus increases the risk of cognitive impairment and dementia in the elderly (Ott et al., 1996). Risk for AD and particularly VaD was reported to be 2–2.5 fold greater among type II diabetics, irrespective of age at which diabetes occurs. Several scenarios including impaired insulin signalling induced neurodegenerative changes, advanced glycation of neuronal components, oxidative stress and inflammatory mechanisms have been proposed.

Features of the insulin resistance syndrome have also been associated with low cognitive function (Kalmijin et al., 2000) and with AD (Kuusisto et al., 1997). The risk is even higher in individuals expressing components of the metabolic syndrome including high blood pressure, increased triglycerides, high blood glucose, low LDL cholesterol and obesity (Whitmer et al., 2007). Moreover, obesity and overweight in midlife, measured by body mass index and skin-fold thickness, are strongly associated with an increased risk of both AD and VaD, independent of the development of diabetes or other cardiovascular-related morbidities. Conversely, higher baseline body mass index and slower declining body mass in late life appear to reduce risk of dementia (Hughes et al., 2000). This suggests that a faster decline in body mass index in late life is a preclinical indicator of an underlying dementing illness, especially for those who were initially overweight (Hughes et al., 2000).

The increase in obesity-associated OS is probably due to the presence of excessive adipose tissue itself, because adipocytes and preadipocytes have been identified as a source of proinflammatory cytokines, including TNF-α, IL-1, and IL-6; thus, obesity is considered a state of chronic inflammation. These cytokines are potent stimulators for the production of reactive oxygen and nitrogen by macrophages and monocytes; therefore, a rise in the concentration of cytokines could be responsible for increased OS. Obesity increases the mechanical load and myocardial metabolism; therefore, oxygen consumption is increased. One negative consequence of increased oxygen consumption is the production of ROS as superoxide, hydroxyl radical, and hydrogen peroxide derived from the increase in mitochondrial respiration and, of course, from the loss of electrons produced in the electron transport chain, resulting in the formation of superoxide radical (Khan et al., 2006).

Mitochondria provide the energy required for nearly all cellular processes that ultimately permit the carrying out of physiological functions; additionally, they play a central role in cell death by the mechanism of apoptosis. Mitochondrial dysfunction has been implicated in a variety of diseases ranging from neurodegenerative diseases to diabetes and aging. Obesity takes place in disorders that affect mitochondrial metabolism, which favors ROS generation and the development of OS. On the other hand, another mechanism has been proposed that involves an effect of high triglyceride (TG) on the functioning of the mitochondrial respiratory chain, in which intracellular TG, which is also high, inhibits translocation of adenine nucleotides and promotes the generation of superoxide (Monteiro et al., 2010).

The mitochondrial process of oxidative phosphorylation is very efficient, but a small percentage of electrons may prematurely reduce oxygen, forming potentially toxic free radicals, impairing mitochondrial function. Beyond that, under certain conditions, protons can be reintroduced into the mitochondrial matrix through different uncoupling proteins, affecting the control of free radical production in mitochondria (Martinez et al., 2006).

In AD, oxidative stress can alter the functions of TCA enzymes as well as induce intracellular accumulation of calcium, which may lead to cellular death (Fu et al., 1998;). Dysfunction of TCA enzymes may compromise cellular energetics and elevate oxidative stress further (Smith et al., 2000). If not intervened, this feed-forward loop may accelerate the progression of the disease. Various studies have proposed using antioxidant therapeutics to prolong the time of onset of AD or retard the rate of its progression (Opii et al., 2008; Behl et al., 2002;). In the same study, co-treatment of cells with N-acetyl cysteine showed much better protection from oxidative stress than lipoic acid or N-acetyl cysteine alone (Moreira et al., 2007). Apart from acting as an antioxidant itself, lipoic acid in the reduced form can also reinforce other water-or lipid-soluble antioxidants such as glutathione, ascorbate, and vitamin E by scavenging their radicals (Kagan et al., 1992).

Using PC 12 cells and Aβ (25–35) peptide, Bozner et al. studied the connection between Aβ and mitochondrial DNA damage. They exposed PC 12 cells to an Aβ (25–35) in frame and scrambled at 50 mM concentration for 24 hours to 50 hours. Oxidative damage of mitochondrial DNA was assessed using a Southern blot technique and a mitochondrial DNA-specific probe recognizing a 13.5-kilobase restriction fragment. PC 12 cells exposed to Aβ exhibited marked oxidative damage of mitochondrial DNA as evidenced by characteristic changes on Southern blots, but not in cells exposed to the scrambled Aβ peptide, suggesting that Aβ peptide is responsible for mitochondrial DNA damage, and ultimately leading to mitochondrial dysfunction in AD (Bozner et al., 1997).

Further, evidence from a recent gene expression study (Reddy et al., 2004) suggests that mutant APP or Aβ may generate free radicals and promote mitochondrial dysfunction, one or both of which may lead to oxidative damage. Altered levels of mitochondrial enzymes have been found to be directly responsible for a decrease in energy production in the brains of late-stage AD patients (Reddy et al., 2006). Soluble or insoluble forms of Aβ have been suggested to impair ATP production by generating defects in mitochondrial energy metabolism and oxidative stress (Behl et al., 2005). Taken together, these results suggest that oxidative stress is a key event in AD pathogenesis.

The major species responsible for oxidative stress is the overproduction or ROS and RNS, a major source of which is mitochondrial dysfunction (Zhu et al., 2007). ROS, which include superoxide radical anion and hydroxyl radicals are involved in the damage of lipids, DNA, and protein modifications. Minor modifications of the nucleic acid bases are repaired through base excision repair involving DNA glycosylase and AP endonuclease, which are located in nuclei and mitochondria. The progression of AD is associated with the diminished expression of these DNA repair enzymes (Nakabeppu et al., 2004). The accumulation of the oxidatively damaged nucleic acids and proteins likely exceed the limit of cellular repair and detoxification mechanisms and leads to the onset or progression of diabetic and

neurological pathologies. In general, accumulation of oxidatively damaged proteins, lipids, and nucleic acids correlate with the onset of age-related diseases, especially in diabetes and AD (Stadman et al., 2001), indicative of one and the same common pathological mechanisms.

Increasing evidence suggests that mitochondrial dysfunction and oxidative stress play a crucial role in the majority of neurodegenerative diseases. Mitochondria are a major source of intracellular reactive oxygen species (ROS) and are particularly vulnerable to oxidative stress. Oxidative damage to mitochondria has been shown to impair mitochondrial function and lead to cell death via apoptosis and necrosis. Because dysfunctional mitochondria will produce more ROS, a feed-forward loop is set up whereby ROS-mediated oxidative damage to mitochondria favors more ROS generation, resulting in a vicious cycle. It is now appreciated that reduction of mitochondrial oxidative stress may prevent or slow down the progression of these neurodegenerative disorders. However, if mitochondria are the major source of intracellular ROS and mitochondria are most vulnerable to oxidative damage, then it would be ideal to deliver the antioxidant therapy to mitochondria.

In the literature on AD, the terms "oxidative stress" or "oxidative damage" are commonly used to explain the balance between the production of oxidants and the endogenous antioxidant defenses in neuronal cells. In general, cells undergo apoptotic death when there is an imbalance between oxidants and antioxidants (more oxidants than antioxidant defenses). This oxidative damage mainly occurs via the mitochondrial ETC (Reddy et al., 2006).

There is mounting evidence to suggest that in late-onset AD, age-related free radicals, which are generated in the mitochondria, are carried to the cytoplasm where they activate beta secretase and facilitate the cleavage of the APP molecule (Reddy et al., 2006). The cleaved APP molecule (ie, Aβ) further generates free radicals, leading to the disruption of the ETC and enzyme activities, the inhibition of ATP, and the subsequent oxidation of both nuclear and mitochondrial DNA proteins. The damage caused by mitochondria ultimately leads to neuronal damage, neurodegeneration, and cognitive decline in AD patients (Reddy et al., 2006).

When obesity persists for a long time, antioxidant sources can be depleted, decreasing the activity of enzymes such as superoxide dismutase (SOD) and catalase (CAT). The activity of SOD and glutathione peroxidase (GPx) in individuals with obesity is significantly lower compared with that in healthy persons, having implications for the development of obesity-related health problems (Ozata et al., 2002). A study in rats showed that the liver concentration of vitamin A having antioxidant activity was significantly lower in rats with obesity compared with those without obesity; the concentration of vitamin A in rats with obesity probably indicates the dilution of this fat-soluble vitamin in high liver lipid storage (Capel et al., 1984). In addition to vitamin A, levels of serum antioxidants, such as vitamin E, vitamin C, and β-carotene, as well as glutathione, are decreased in obesity (Vincent et al., 2005). In addition to this, ROS decrease the expression of adiponectin, suggesting that treatment with antioxidants or ROS inhibitors could restore the regulation of adipokines (Furukawa et al., 2004). Thus, supplementation with antioxidants would reduce the risk of complications related with obesity and OS (Higdon et al., 2003).

Findings from clinical and experimental studies show that chronic accumulation of reactive oxygen species in older brains may exhaust antioxidant capacity and trigger neurodegenerative processes as characterized in AD. Thus dietary supplementation with fruit or vegetable extracts high in antioxidants help to decrease the enhanced vulnerability to oxidative stress and improve neuronal communication via increases in neuronal signaling and animal behavior (Joseph et al., 2009). Onset of AD was significantly delayed by use of antioxidant vitamins and polyphenols derived from fruits and vegetables (Luchsinger et al., 2007). Results from the Kame Project suggest that drinking fruit juices which are high in polyphenolic compounds, was associated with lower risk of incident AD (Luchsinger et al., 2007). Congruent with the notion that vascular health is key to maintaining cognitive function polyphenols from wine, cocoa, coffee, grape seed, blueberries, strawberries, tea, curcumin, pomegranate and green leafy vegetables also have beneficial effects on endothelial function and cardiovascular performance (Luchsinger et al., 2007). The beneficial actions of resveratrol have been implicated in anti-oxidant defence, regulation of the cell cycle, mitochondrial energy production, vascular reactivity, oncogene suppression and activation of sirtuins (silent information regulator-related enzymes), as anti-ageing inhibitors (Markus et al., 2008).

Accumulating evidence suggests changes in lifestyle factors such as increasing physical activity will decrease the risk of developing dementia in later life (Flicker et al., 2009). Most studies (Rovio et al., 2008) studies show reduced rate of age-related cognitive decline, decreased risk of incident dementia or AD in individuals who exercise regularly.

There are no known curative or preventive measures for most types of dementia. Diet and lifestyle could influence risk, and studies suggest that midlife history of disorders that affect the vascular system, such as hypertension, type 2 diabetes, and obesity, increase the risk for dementia including Alzheimer's disease (AD) (Luchsinger et al., 2004; Whitmer et al., 2005). Increased trends in demographic transition and urbanisation within many developing countries are predicted to lead to lifestyle changes (Ineichen et al., 1998). Delaying of onset, by modifying risk or lifestyle, decreases the prevalence and public health burden of dementia; a delay in onset of 1 year would translate to almost a million fewer prevalent cases in the USA (Brookmeter et al., 1998). However, this in turn might increase demands on health services and costs for older populations (Brayne et al., 2007).

Surprisingly, countries in Latin America, such as Venezuela and Argentina, bear a higher burden of over 5% prevalence of dementia. By contrast, a systematic analysis of six Indian studies suggests low prevalence (2–3%) of all dementias, with marginally fewer cases in urban compared with rural areas and in the northern versus southern states (Das et al., 2006). Pooled analysis of 25 Chinese studies by Dong and colleagues, (Dong et al., 1980-2004) comprising a total population of more than 76 000, suggested that the overall prevalence of dementia was 3.1%, indicating a significant rise from 1980 to 2004. However, a recent survey of over 34 807 Han Chinese residents aged at least 55 years in 79 rural and 58 urban communities of four distant areas reported a crude prevalence estimate of 5.0%, and 6.8% after adjustment for negative screening (Zhang et al., 2006). Higher prevalence was apparent in northern regions compared with the south, but no difference was evident among urban and rural Chinese residents (Zhang et al., 2006). In the Upper Assiut region along the Nile, age-adjusted dementia prevalence in people aged 65 years and older was 5.9% (Farrag et al., 1998). In the Yoruba (Niger-Kordofanian people) of Nigeria, dementia prevalence was low (2.3%) compared with an African American population in Indiana, USA (8.2%) (Hendrie et al., 1995). Among Arabs living in Wadi Ara, a community south of Haifa in Israel, the crude prevalence estimate for all dementias was 21% in those aged over 60 years (Bowirrat et al., 2001). Consanguinity among families was suggested as a reason for this high prevalence (Bowirrat et al., 2001). Studies from developing countries in Eastern Europe have assessed some risk factors, but prevalence or incidence data in these communities are unknown (Suhanov et al., 2006).

Multiple epidemiological studies have demonstrated a remarkable overlap among risk factors for cerebrovascular disorder and sporadic, late-onset AD (Jellinger, 2010; Kalaria, 2010). For example, mid-life diabetes (Knopman and Roberts 2010), hypertension (Iadecola and Davisson, 2008), and obesity (Whitmer et al., 2008) have all been shown to increase the risk for both AD and vascular dementia. It is now generally acknowledged that most AD cases have mixed vascular pathology and small-vessel disease (Jellinger, 2010). Moreover, reduced brain blood perfusion (Ruitenberg et al. 2005), silent infarcts (Vermeer et al., 2003), and the presence of one or more infarctions (Snowdon et al., 1997) all increase the risk of AD.

On the one hand, illiteracy or low educational achievement has been shown to be a robust risk factor for dementia (Borenstein et al., 2006). On the other hand, intellectually stimulating, socially engaging, or physical activities might lower the risk of dementia (Fratiglioni et al., 2004). The situation is not different in developing countries, where surveys have consistently identified low education as a risk factor for dementia (Ampil et al., 2005). However, in some communities, level of education, indexed by years of primary schooling, might not necessarily contribute to low prevalence (Hendrie et al., 2006). Low literacy is often linked to poverty or lower socioeconomic status, which is also associated with poorer health, lower access to health care, and increased risk of dementia (Keskinoglu et al., 2006.)

CONCLUSIONS

Alzheimer's disease is the most common cause of dementia late in life, affecting approximately 8 percent of people who are 65 years of age or older. Increasing frequency of vascular disease and global trends in modernisation will add to the burden of AD within developing countries. Harmonisation of screening methods worldwide could help to define risks and to devise novel approaches for dementia prevention. The impact of dementia in developing countries deserves further epidemiological and implementation research to enable early detection, widespread adequate treatment, and caregiver support. Such efforts will no doubt promote greater awareness, refine the policy agenda, and lead to a call for concerted action.

Recent advances in molecular, cellular, and animal model studies have revealed that mitochondria are the major source of free radical generation and of oxidative damage in aging and age-related neurodegenerative diseases. It is possible that age-related mitochondrial abnormalities and oxidative damage are major contributing factors for late-onset AD. To stop or delay the progression of late-onset AD, and also to reduce disease symptoms, several therapeutic strategies have been developed, including anti-inflammatory, antioxidant, and antiamyloid approaches. Among these, mitochondrial antioxidant therapy reduces AD pathology more than any other approach.

Adipose tissue is a secretory organ of great importance for the organism because the substances that it secretes meet the requirements for specific biological functions. As obesity is characterized by excessive storage of adipose tissue, adipokine secretion is increased; therefore, the effects produced in the body are altered, and resistance to its effect can be generated, as in the case of leptin. In addition to adipokines, we also found an overproduction of ROS, which damage cellular structures and trigger, together with underproduction of NO, progressive accumulation of fat and, eventually, the development of other pathologies. On the other hand, it was observed that the decrease in body fat reflected in weight improves oxidation markers and increases antioxidant activity, which was impaired with obesity. Therefore, weight loss through nutritional and pharmacological treatment, in addition to supplementation with antioxidant nutrients such as vitamins E, A, and C, flavonoids, among others, may be the key to reducing the risk of developing other pathologies related with OS and obesity.

Obesity is a condition that is epidemic and that has increased in recent decades. Parallel to the increase of this disease, the study of obesity has undergone considerable development. This has been accomplished thanks to research in

various fields of knowledge that have broken down multiple archetypes, allowing changes in views on overweight, adipose tissue function, and the pathophysiology of the disease that prevail at present. The breakdown of old paradigms and the new knowledge platform provide a solid foundation for understanding the disease and for developing strategies for prevention and treatment.

REFERENCES

Amirkhizi F., Siassi F., Minaie S., Djalali M., Rahimi A., Chamari M., (2007): Is obesity associated with increased plasma lipid peroxidación and oxidative stress in women. ARYA Atheroscler. J. 2:189–192.

Ampil E.R., (2005): Fook-Chong S, Sodagar SN, et al. Ethnic variability in dementia: results from Singapore. Alzheimer Dis Assoc Disord. 19:184–85.

Anekonda T.S., Reddy P.H., (2005): Can herbs provide a new generation of drugs for treating Alzheimer's disease? Brain Research. Brain Research Reviews. 50(2):361–376.

Behl C., Moosmann B., (2002): Antioxidant neuroprotection in Alzheimer's disease as preventive and therapeutic approach. Free Radical Biology and Medicine. 33(2):182–191.

Behl C., (2005): Oxidative stress in Alzheimer's disease: implications for prevention and therapy.Sub-Cellular Biochemistry. 38:65–78.

Borenstein A.R., Copenhaver C.I., Mortimer J.A., (2006): Early-life risk factors for Alzheimer disease. Alzheimer Dis Assoc Disord. 20:63–72.

Bowirrat A., Treves T.A., Friedland R.P., Korczyn A.D., (2001): Prevalence of Alzheimer's type dementia in an elderly Arab population. Eur J Neurol. 8:119–23.

Bozner P., Grishko V., LeDoux S.P., Wilson G.L., Chyan Y.C., Pappolla M.A., (1997):The amyloid beta protein induces oxidative damage of mitochondrial DNA. Journal of Neuropathology and Experimental Neurology. 56(12):1356–1362.

Bravo P., Morse S., Borne D., Aguílar E., Reisin E., (2006): Leptin and hypertension in obesity.Vasc. Health Risk Manage. 2:163–169.

Brayne C., (2007): The elephant in the room—healthy brains in later life, epidemiology and public health. Nat Rev Neurosci. 8:233–39.

Brookmeyer R., Gray S., Kawas C., (1998): Projections of Alzheimer's disease in the United States and the public health impact of delaying disease onset. Am J Public Health.88:1337–42.

Butterfield D.A., Hensley K., Harris M., Mattson M., Carney J., (1994): Biochem Biophys Res Commun. 200:710–715.

Butterfield D.A., (1996): Alzheimer’s Disease Rev. 1:68–70.

Butterfield D.A., Drake J., Pocernich C., Castegna A., (2001): Evidence of oxidative damage in Alzheimer's disease brain: central role for amyloid beta-peptide. Trends in Molecular Medicine. 7(12):548–554.

Capel I., Dorrell H., (1984): Abnormal antioxidant defense in some tissues of congenitally obese mice. Biochemistry. 219:41–49.

Chrysohoou C., Panagiotakos D.B., Pitsavos C., Skoumas I., Papademetriou L., Economou M., Stefanadis C., (2007):The implication of obesity on total antioxidant capacity apparently healthy men and women: The ATTICA study. Nutr. Metab. Cardiovasc. Dis. 17:590–597.

Das S.K., Biswas A., Roy T., et al., (2006): A random sample survey for prevalence of major neurological disorders in Kolkata. Indian J Med Res. 124:163–72.

de la Monte S.M., Wands J.R., (2005): Review of insulin and insulin-like growth factor expression, signaling, and malfunction in the central nervous system: relevance to Alzheimer's disease.Journal of Alzheimer's Disease. 7(1):45–61

Dong M.J., Peng B., Lin X.T., et al. (2007): The prevalence of dementia in the People's Republic of China: a systematic analysis of 1980–2004 studies. Age Ageing. 36:619–24.

Duyckaerts C., Delatour B., Potier M.C., (2009): Classification and basic pathology of Alzheimer disease. Acta Neuropathology. 118(1):5–36.

Esposito K., Ciotola M., Giugliano D., (2006): Oxidative stress in the Metabolic Syndrome. J. Endocrinol. Invest. 29:791–795.

Farrag A., Farwiz H.M., Khedr E.H., et al., (1998): Prevalence of Alzheimer's disease and other dementing disorders: Assiut-Upper Egypt study. Dement Geriatr Cogn Disord. 9:323–28.

Flicker L., (2009): Life style interventions to reduce the risk of dementia. Maturitas. 63:319–322.

Fratiglioni L., Paillard-Borg S., Winblad B., (2004): An active and socially integrated lifestyle in late life might protect against dementia. Lancet Neurol. 3:343–53. 135.

Fu W., Luo H., Parthasarathy S., Mattson M.P., (1998): Catecholamines potentiate amyloid beta-peptide neurotoxicity: involvement of oxidative stress, mitochondrial dysfunction, and perturbed calcium homeostasis. Neurobiology of Disease. 5(4):229–243.

Furukawa S., Fujita T., Shimabukuro M., Iwaki M., Yamada Y., Nakajima Y., Nakayama O., Makishima M., Matsuda M., Shimomura I., (2004): Increased oxidative stress in obesity and its impact on metabolic syndrome. J. Clin. Invest. 114:1752–1761.

Hartwich J., Goralska J., Siedlecka D., Gruca A., Trzos M., Dembinska-Kiec A., (2007):Effect of supplementation with vitamin E and C on plasma hsCPR level and cobalt-albumin binding score as markers of plasma oxidative stress in obesity. Genes Nutr. 2:151–154.

Hendrie H.C., Osuntokun B.O., Hall K.S., et al., (1995): Prevalence of Alzheimer's disease and dementia in two communities: Nigerian Africans and African Americans. Am J Psychiatry.152:1485–92.

Hendrie H.C., Murrell J., Gao S., et al., (2006): International studies in dementia with particular emphasis on populations of African origin. Alzheimer Dis Assoc Disord. 20:S42–46.

Hensley K., Butterfield D.A., Hall N., Cole P., Subramaniam R., Mark R., Mattson M.P., Markesbery W.R., Harris M.E., Aksenov M., Aksenova M., Wu J.F., Carney J.M., (1996): Ann NY Acad Sci. 786:120–134. **Higdon J., Frei B.,** (2003): Obesity and oxidative stress: A direct link to CVD? Arterioscler. Tromb. Vasc. Biol. 23:365–367.

Hughes T.F., Borenstein A.R., Schofield E., Wu Y., Larson E.B., (2009): Association between late-life body mass index and dementia: The Kame Project. Neurology. 72:1741–1746.

Iadecola C., Davisson R.L., (2008): Hypertension and cerebrovascular dysfunction. Cell Metab 7: 476–484

Ineichen B., (1998): Influences on the care of demented elderly people in the People's Republic of China. Int J Geriatr Psychiatry. 13:122–26.

Jellinger K.A., (2010): Prevalence and impact of cerebrovascular lesions in Alzheimer and lewy body diseases. Neurodegen Dis 7: 112–115

Joseph J.A., Shukitt-Hale B., Willis L.M., (2009): Grape juice, berries, and walnuts affect brain aging and behavior. J Nutr. 139:1813S–1817S.

Kagan V.E., Shvedova A., Serbinova E., Khan S., Swanson C., Powell R. (1992): Dihydrolipoic acid—a universal antioxidant both in the membrane and in the aqueous phase: reduction of peroxyl, ascorbyl and chromanoxyl radicals. Biochemical Pharmacology. 44(8):1637–1649.

Kalaria R.N., (2010): Vascular basis for brain degeneration: Faltering controls and risk factors for dementia. Nutr Rev 68: S74–S87

Kalmijn S., Foley D., White L., et al., (2000): Metabolic cardiovascular syndrome and risk of dementia in Japanese-American elderly men. The Honolulu-Asia aging study. Arterioscler Thromb Vasc Biol. 20:2255–2260.

Kaufer M., Tavano L., Ávila H., (2001): Obesidad en el adulto. In: Casanueva E, Kaufer M, Pérez A, Arroyo P, editors. Nutriología Médica. 1st ed. Editorial Médica Panamericana; México, México.

Keskinoglu P., Giray H., Picakciefe M., et al., (2006): The prevalence and risk factors of dementia in the elderly population in a low socioeconomic region of Izmir, Turkey. Arch Gerontol Geriatr. 43:93–100.

Khan N., Naz L., Yasmeen G., (2006): Obesity An independent risk factor systemic oxidative stress. Park. J. Pharm. Sci. 19:62–69.

Kitazawa M., Yamasaki T.R., LaFerla F.M., (2004): Microglia as a Potential Bridge between the Amyloid {beta}-Peptide and Tau. Annals of the New York Academy of Sciences.1035:85–103.

Knopman D.S., Roberts R., (2010) :Vascular risk factors: Imaging and neuropathologic correlates. J Alzheimers Dis 20: 699–709

Kuusisto J., Koivisto K., Mykkanen L., et al., (1997): Association between features of the insulin resistance syndrome and Alzheimer's disease independently of apolipoprotein E4 phenotype: cross sectional population based study. BMJ. 315:1045–1049.

Luchsinger J., Mayeux R., (2004): Cardiovascular risk factors and Alzheimer's disease. Curr Atheroscler Rep. 6:261–66.

Luchsinger J.A., Reitz C., Honig L.S., Tang M.X., Shea S., (2005): Mayeux R. Aggregation of vascular risk factors and risk of incident Alzheimer disease. Neurology. 65:545–551.

Luchsinger J.A., Noble J.M., Scarmeas N., (2007): Diet and Alzheimer's disease. Curr Neurol Neurosci Rep. 7:366–372.

Manczak M., Park B.S., Jung Y., Reddy P.H., (2004): Differential expression of oxidative phosphorylation genes in patients with Alzheimer's disease:implications for early mitochondrial dysfunction and oxidative damage. Neuromolecular Medicine. 5(2):147–162.

Mark P. Mattson, (2004): Pathways Towards and Away from Alzheimer's Disease, 430:631-639

Markesbery W.R., Carney J.M., (1999): Oxidative alterations in Alzheimer's disease. Brain Pathology. 9(1):133–146.

Markus M.A., Morris B.J., (2008): Resveratrol in prevention and treatment of common clinical conditions of aging. Clin Interv Aging. 3:331–339.

Martínez J., (2006): Mitocondrial oxidative stress and inflammation: A slalom to obesity and insulin resistance. J. Physiol. Biochem. 62:303–306.

Mattson M.P., (2004): Pathways towards and away from Alzheimer's disease. Nature. 430(7000):631–639

Middleton L.E., Yaffe K., (2009): Promising strategies for the prevention of dementia. Arch Neurol. 66:1210–1215.

Monteiro R., Azevedo I., (2010): Chronic inflammation in obesity and the metabolic syndrome.Mediators. Inflamm. 2010:289645.

Moreira P.I., Harris P.L.R., Zhu X., Santos M.S., Oliveira C.R., Smith M.A., (2007): Lipoic acid and N-acetyl cysteine decrease mitochondrial-related oxidative stress in Alzheimer disease patient fibroblasts. Journal of Alzheimer Disease. 12(2):195–206.

Nakabeppu Y., Tsuchimoto D., Ichinoe A., Ohno M., Ide Y., Hirano S., Yoshimura D., Tominaga Y., Furuichi M., Sakumi K., (2004): Biological significance of the defense mechanisms against oxidative damage in nucleic acids caused by reactive oxygen species: from mitochondria to nuclei. Ann N Y Acad Sci.1011:101–111.

Opii W.O., Joshi G., Head E., Milgram N.W., Muggenburg B.A., Klein J.B., (2008): Proteomic identification of brain proteins in the canine model of human aging following a long-term treatment with antioxidants and a program of behavioral enrichment: relevance to Alzheimer’s disease. Neurobiology of Aging. 29(1):51–70.

Ozata M., Mergen M., Oktenli C., Aydin A., Sanisoglu S.Y., Bolu E., Yilmaz M.I., Sayal A., Isimer A., Ozdemir I.C., (2002): Increased oxidative stress and hypozincemia in male obesity. Clin. Biochem. 35:627–631.

Pappolla M.A., Omar R.A., Kim K.S., Robakis N.K., (1996): Immunohistochemical evidence of oxidative [corrected] stress in Alzheimer's disease. The American Journal of Pathology.1992;140(3):621–628. Erratum in: The American Journal of Pathology.149(5):1770.

Pappolla M.A., Chyan Y.J., Poeggeler B., et al., (1999): Alzheimer beta protein mediated oxidative damage of mitochondrial DNA: prevention by melatonin. Journal of Pineal Research. 27(4):226–229.

Patel C., Ghanim H., Ravishankar S., Sia C.L., Viswanathan P., Mohantym P., Dandona P., (2007): Prolonged reactive oxygen species generation and Nuclear Factor- kB activation after a high-fat, high-carbohydrate meal in the obese. J. Clin. Endocrinol. Metab. 92:4476–4479.

Pihl E., Zilmer K., Kullisaar T., Kairane C., Magi A., Zilmer M., (2006): Atherogenic inflammatory and oxidative stress markers in relation to overweight values in male former athletes. Int. J. Obesity. 30:141–146.

Premkumar D.R., Cohen D.L., Hedera P., Friedland R.P., Kalaria R.N., (1996):Apolipoprotein E-epsilon4 alleles in cerebral amyloid angiopathy and cerebrovascular pathology associated with Alzheimer's disease. Am J Pathol.148:2083–2095.

Purnell C., Gao S., Callahan C.M., Hendrie H.C., (2009): Cardiovascular risk factors and incident Alzheimer disease: a systematic review of the literature. Alzheimer Dis Assoc Disord. 23:1–10.

Qin L., Liu Y., Cooper C., Liu B., Wilson B., Hong J.S., (2002): Microglia enhance beta-amyloid peptide-induced toxicity in cortical and mesencephalic neurons by producing reactive oxygen species. Journal of Neurochemistry. 83(4):973–983.

Raber J., Huang Y., Ashford J.W., (2004): ApoE genotype accounts for the vast majority of AD risk and AD pathology. Neurobiology of Aging. 25(5):641–650.

Reddy P.H., McWeeney S., Park B.S., et al., (2004): Gene expression profiles of transcripts in amyloid precursor protein transgenic mice: up-regulation of mitochondrial metabolism and apoptotic genes is an early cellular change in Alzheimer's disease. Human Molecular Genetics. 13(12):1225–1240.

Reddy P.H., Beal M.F., (2005): Are mitochondria critical in the pathogenesis of Alzheimer's disease?Brain Research. Brain Research Reviews. 49(3):618–632.

Reddy P.H., Mani G., Park B.S., et al., (2005): Differential loss of synaptic proteins in Alzheimer's disease: implications for synaptic dysfunction. Journal of Alzheimer's Disease. 7(2):103–117. discussion 173-80.

Reddy P.H., (2006): Amyloid precursor protein-mediated free radicals and oxidative damage: implications for the development and progression of Alzheimer's disease. Journal of Neurochemistry. 96(1):1–13.

Rovio S., Spulber G., Nieminen L.J., et al., (2008): The effect of midlife physical activity on structural brain changes in the elderly. Neurobiol Aging.

Ruitenberg A., den Heijer T., Bakker S.L., van Swieten J.C., Koudstaal P.J., Hofman A., Breteler M.M., (2005): Cerebral hypoperfusion and clinical onset of dementia: The Rotterdam Study. Ann Neurol 57: 789–794

Selkoe D.J., (2001): Alzheimer's disease: genes, proteins, and therapy. Physiological Reviews.81(2):741–766.

Sikaris K., (2004): The clinical biochemistry of obesity. Clin. Biochem. Rev. 25:165–181.

Smith M.A., Kutty R.K., Richey P.L., et al., (1994): Heme oxygenase-1 is associated with the neurofibrillary pathology of Alzheimer's disease. The American Journal of Pathology. 145(1):42–47.

Smith M.A., Perry G., Richey P.L., et al. (1996): Oxidative damage in Alzheimer's. Nature. 382(6587):120–121. **Smith M.A., Rottkamp C.A., Nunomura A., Raina A.K., Perry G.** (2000): Oxidative stress in Alzheimer's disease. Biochimica Biophysica Acta. 1502(1):139–144.

Snowdon D.A., Greiner L.H., Mortimer J.A., Riley K.P., Greiner P.A., Markesbery W.R., (1997):Brain infarction and the clinical expression of Alzheimer disease. The Nun Study.JAMA 277: 813–817

Stadtman ER., (2001): Protein oxidation in aging and age-related diseases. Ann N Y Acad Sci.928:22–38.

Suhanov A.V., Pilipenko P.I., Korczyn A.D., et al., (2006): Risk factors for Alzheimer's disease in Russia: a case-control study. Eur J Neurol. 13:990–95.

Sullivan P.G., Brown M.R., (2005): Mitochondrial aging and dysfunction in Alzheimer's disease.Progress in Neuro-Psychopharmacology & Biological Psychiatry. 29(3):407–410.

Vermeer S.E., Prins N.D., den Heijer T., Hofman A., Koudstaal P.J., Breteler M.M., (2003):Silent brain infarcts and the risk of dementia and cognitive decline. New Engl J Med 348: 1215–1222

Whitmer R.A., Gunderson E.P., Barrett-Connor E., et al., (2005): Obesity in middle age and future risk of dementia: a 27 year longitudinal population based study. BMJ. 330:1360.

Whitmer R.A., Gustafson D.R., Barrett-Connor E., Haan M.N., Gunderson E.P., Yaffe K., (2008): Central obesity and increased risk of dementia more than three decades later.Neurology 71: 1057–1064

Whitmer R.A., Gunderson E.P., Quesenberry C.P., Jr., Zhou J., Yaffe K., (2007): Body mass index in midlife and risk of Alzheimer disease and vascular dementia. Curr Alzheimer Res. 4:103–109.

Zhang Z.X., Zahner G.E., Roman G.C., et al., (2005): Dementia subtypes in China: prevalence in Beijing, Xian, Shanghai, and Chengdu. Arch Neurol. 62:447–53.

Zhang Z.X., Zahner G.E., Roman G.C., et al., (2006): Socio-demographic variation of dementia subtypes in China: methodology and results of a prevalence study in Beijing, Chengdu, Shanghai, and Xian. Neuroepidemiology. 27:177–87.

Zhu X., Lee H.G., Casadesus G., (2006): Oxidative imbalance in Alzheimer's disease.Molecular Neurobiology. 31(1–3):205–217.

Zhu X., Su B., Wang X., Smith M.A., Perry G., (2007): Causes of oxidative stress in Alzheimer disease. Cell Mol Life Sci. 64:2202–2210.

[1]Faculty of Biology, University of "Alexandru Ioan Cuza" Iași, Romania,
[2]University of Medicine and Pharmacy "Gr.T.Popa" Iasi
****ELENA-LOREDANA SANDU*** "Alexandru Ioan Cuza" University of Iasi, Faculty of Biology
0755 254 796; e-mail: lori_chem89@yahoo.com

TIME DEPENDENT ACCUMULATION OF NICOTINE DERIVATIVES IN THE CULTURE MEDIUM OF *ARTHROBACTER NICOTINOVORANS* pAO1

BOIANGIU RĂZVAN[1], GUZUN DOINA[1], MIHĂŞAN MARIUS[1*]

Keywords: *Arthrobacter, catabolic megaplasmid, nicotine, 6-hydroxi-L-nicotine, HPLC, biotechnology.*

Abstract: Previous studies have shown that the metabolic intermediate 6-hidroxy-D-nicotine (6HNic) found in the *Arthrobacter nicotinovorans* pAO1+ nicotine catabolic pathway has the ability to bind nicotinic acetylcholine receptors and to sustain spatial memory in rats. These properties make 6HNic a valuable compound with some potential for medical applications, thereby a suitable, simple and efficient method for producing 6-hidroxy-D-nicotine is necessary. Here, we focus on identifying the best moment for harvesting *A. nicotinovorans* cells in order to directly convert nicotine to 6HNic with the best yield. The growth of *A. nicotinovorans* pAO1+ was monitored and the correlation between the growth phases and nicotine metabolism was established. After about 5 hours of lag, the strain entered the log phase and was fully grown after 10 hours. The nicotine concentration began to drop dramatically as the pAO1+ culture reached saturation and was depleted in 5 hours. As the nicotine concentration dropped, 6HNic began to accumulate, reaching the maximum levels after about 11 hours of growth. Two other products could be detected by HPLC, one which was identified as the nicotine-blue (NB) pigment and a second a still unknown end-product.

INTRODUCTION

Tobacco is reasonably cultivated worldwide, with a global production reaching 6.7 million tons per year. The main tobacco producer is China with 39.6% from the global production, followed by India (8.3%), Brazil (7.0%) and USA (4.6%). The tobacco processing industry produces annually about 3 million tons of waste (Gurusamy and Natarajan, 2013), with a mean nicotine content of about 18g / kg dry weight. EPA (The Environmental Protection Agency) and the UE regulations have designated these wastes as “toxic and hazardous” when the nicotine content is more than 0.05% (w/w). The waste containing nicotine is not only an unused source of aromatic compounds, but also a potential danger for the environment and human health.

Our previous studies have shown that 6-hidroxy-D-nicotine (6-HNic) has the ability to bind nicotinic acetylcholine receptors (nAChR) and thus, to sustain spatial memory in rats (Mihasan et al., 2013, Hritcu et al., 2011). These properties make 6HNic a valuable compound with high potential for medical applications, especially in the therapy of neurodegenerative disorders such as such as Alzheimer's disease (AD). Taking into account that the Alzheimer's Organization estimates the direct costs to American society of caring for those with Alzheimer's will total $214 billion for 2014 and a staggering $1.2 trillion by 2050 (Hebert et al., 2013), the ability to use an “toxic and hazardous” waste in order to produce active compounds with high impact on the medical sector is very appealing.

6-Hidroxy-D-nicotine is a metabolic intermediate found in the *Arthrobacter nicotinovorans* pAO1+ nicotine catabolic pathway. It is formed by a hydroxylation reaction catalyzed by the heterotrimeric enzyme nicotine-dehydrogenase. The three genes encoding the subunits of the active enzyme have been previously cloned (Sachelaru et al., 2006, Andrei and Mihasan, 2013) and expressed (Sachelaru et al., 2006), but the amount of purified enzyme is very low, making this approach unusable for production of 6HNic. Thereby, the current work makes the first efforts into developing a method of producing 6HNic by directly employing the wild type *A. nicotinovorans* pAO1 strain. Here, we focus on identifying the best moment for harvesting *A. nicotinovorans* cells in order to directly convert nicotine to 6HNic with the best yield.

MATERIAL AND METHODS

Chemicals. All chemicals were purchased from well-known suppliers and were of greatest purity available. 6-Hydroxy-nicotine was produce by chemical synthesis and is a kind gift from Prof. Dr. Roderich Brandsch - Institute of Biochemistry and Molecular Biology, Albert-Ludwigs University of Freiburg, Germany.

Strains and growth conditions. *Arthrobacter nicotinovorans* (strain ATCC 4991) harboring (pAO1+) or not harboring (pAO1-) the pAO1 megaplasmid were grown on citrate medium supplemented with 0,05% nicotine and 0,005% minerals solution (Eberwein et al., 1961) on a rotary shaker at 28^{0}C/190 rpm. The growth of the culture was followed at 660 nm. Samples from the growth medium were taken at specific time points and analyzed by UV-VIS spectroscopy and HPLC.

Absorption spectra's were recorded on a Beckman Coulter DU 730 Life Science spectrophotometer using citrate medium as blank. **HPLC analysis** was performed on a Bischoff system equipped with 2 pumps, a DAAD detector and a Machery-Nagel Nucleodur RP C18 ec column (150x4.6 mm, particle size 3µm). 20 µL of sample was injected and isocratic elution at room temperature was used for separation. The mobile phase was a mixture of 1mM H_2SO_4 and methanol at various ratios.

RESULTS AND DISCUSSIONS

Separation on nicotine from 6HNic. Despite the fact that the same reverse-phase principle is applied to all the described methods for separation of nicotine intermediates in complex mixtures, different authors employ various conditions in terms of mobile phase concentration (Table 1).

Table 1. Separation conditions employed for resolving nicotine derivatives.

Column	Mobile phase	Ratio	Flow rate/ temperature	Detection	Reference
Kromasil KR100-5C18, 150mm×4.6mm; particle size 5µm	Methanol:1 mM H_2SO_4	15:85	0,5 ml/ min / 30°C	210 nm	(Wang et al., 2005)
Kromasil KR100-5C18, 150mm×4.6mm; particle size 5µm	Methanol:1 mM H_2SO_4	25:75	0,5 ml/ min / 30°C	207 nm	(Tang et al., 2008)
Kromasil KR100-5C18, 150mm×4.6mm; particle size 5µm	Water:Methanol	75:25	0,5 ml/ min / 30°C	210 nm	(Tang et al., 2009)
Grace Alltima C18, 4.6×250 mm; particle size 5µm	Methanol:Water	10:90	0,5 ml/ min / 30°C	263 nm; 232 nm; 307 nm	(Ma et al., 2014)
Eclipse XDB-C18, 250×64.6 mm; particle size 5µm	Methanol:1 mM H_2SO_4	5:95	0,5 ml/ min / 30°C	-	(Liu et al., 2014)

In order to identify the best separating condition the complex mixture and system used in our experiment, pure nicotine and 6-hydroxy-nicotine were injected in the HPLC system and eluted at different methanol concentrations. The mobile phase mixture that gave the best results in terms of differences between retention time for nicotine and 6-hydroxy-nicotine was found to be 1mN H_2SO_4 : methanol 75:25 (Figure 1, A).

Growth of *Arthrobacter nicotinovorans* strains on nicotine containing medium. As 6HNic is a metabolic intermediate, one can clearly expect that its half-life is rather low as it is further processed by the nicotine-degradation machinery (Brandsch, 2005).

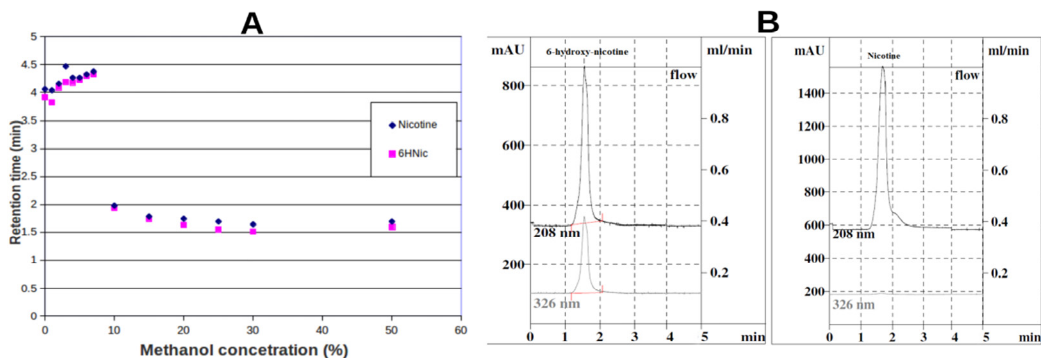
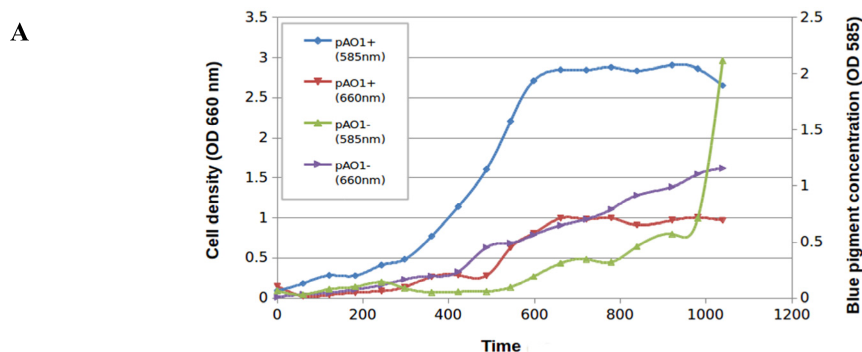


Figure 1. **A.** Dependence of nicotine and 6-hydroxy-nicotine retention times on the methanol concentration. Largest differences in retention times were obtained at a methanol concentration of 25%. **B.** Typical HPLC run for separation of nicotine and 6-hydroxy-nicotine using 1mN H_2SO_4 : methanol 75:25 as mobile phase.

Still, primary reports on nicotine metabolism in this strain indicate that 6HNic is present in the growth medium in small amounts (Hochstein and Rittenberg, 1959). In order to identify the best moment for harvesting *A. nicotinovorans* pAO1+ cells and obtain largest amounts of 6HNic a strict correlation between the age of the culture and the level of nicotine consumption is required. Thereby, the pAO1+ and pAO1- strains growth on liquid citrate medium was measured at 660 nm and was at first correlated with an primary indicator of nicotine depletion in the medium: the accumulation of the final end-product (Nicotine-blue pigment, NB), followed at 585 nm. After about 5 hours of lag, both the pAO+ and pAO1- strains entered the log phase and were fully grown after 10 hours. As the lag phase ended, the characteristic blue-pigment appeared in the medium of the pAO1+ strain (Figure 2, A).

6-Hydroxy-nicotine is excreted in the *Arthrobacter nicotinovorans* growth medium. When grown on citrate medium supplemented with nicotine, the *Arthrobacter nicotinovor*ans pAO1 strain is able to use nicotine as a carbon and nitrogen source (Baitsch et al., 2001; Igloi and Brandsch, 2003). The HPLC analysis of the growth medium has allowed to strictly correlate the main growth phases of the bacterial culture with the first steps of the nicotine catabolic pathway. As shown in figure 2, B, the nicotine concentration began to drop dramatically as the pAO1+ culture reached saturation and was depleted within 5 hours. As the nicotine concentration dropped, 6HNic started to be exported into the medium and began to accumulate, reaching the maximum levels after about 11 hours of growth. Two other products could be detected by HPLC, one which was identified as the nicotine-blue pigment and a second a still unknown aromatic ring containing end-product.

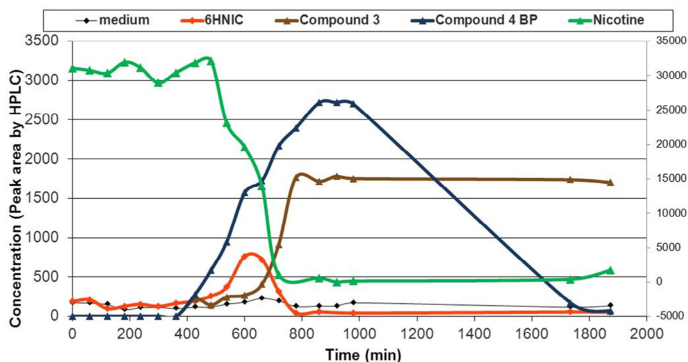
A



B

Figure 2. Dynamics of *Arthrobacter nicotinovorans* cultures on citrate medium supplemted with nicotine **A.** Growth curve and the time-dependent accumulation of nicotine-blue pigment accumulation **B.** Dynamics of nicotine and nicotine metabolites concentration during growth.

As it can be inferred from Figure 2, B, the levels of 6HNic dropped quite rapidly when the nicotine levels were low. This fact has two major practical implications. On one hand, it proves that there is a bottleneck (Liu et al., 2002) in the catabolism of nicotine due probably to different catalytic efficiencies of the involved enzymes. It seems that nicotine-dehydrogenase is able to process its substrate much faster then the fallowing enzyme 6-hidroxy-L-nicotine-oxidase



(6HLNO). This leads to the accumulation of 6HNic in the medium, but as nicotine is depleted, 6HLNO is able to catch up and starts using the excreted 6HNic. On the other hand, the small interval of lag between these two enzyme gives a one hour time frame on which the bacteria could be harvest and for the isolation of 6HNic. Still, as the accumulation levels of this compound are quite low, the yield of the process would be low also.

CONCLUSION

The direct utilization of A. nicotinovorans pAO1+ cells for transformation of nicotine into the biotechnological valuable product 6-hydroxy-nicotine is feasible, although with a rather low yield. The best moment for cells harvesting is after 11 hours of growth on citrate medium supplemented with 0,05% nicotine.

REFERENCES

Andrei A, Mihasan M. 2013. Molecular gene cloning of nicotine-dehidrogenase from the pAO1 megaplasmid of Arthrobacter nicotinovorans. Analele Stiintifice ale Universitatii "Alexandru Ioan Cuza" din Iasi Sec. II a. Genetica si Biologie Moleculara 14(3):15-19 Vă rog sa scrieți denumirea revistei fără prescurtări.

Baitsch D, Sandu C, Brandsch R, Igloi GL. 2001. Gene cluster on pAO1 of Arthrobacter nicotinovorans involved in degradation of the plant alkaloid nicotine: cloning, purification, and characterization of 2,6-dihydroxypyridine 3-hydroxylase. J Bacteriol 183:5262–5267.

Brandsch R. 2005. Microbiology and biochemistry of nicotine degradation. Appl. Microbiol. Biotechnol. 69:493–498.

Eberwein H, Gries FA, Decker K. 1961. (On the decomposition of nicotine by bacterial enzymes. II. Isolation and characterization of a nicotine-splitting soil bacterium). Hoppe Seylers Z Physiol Chem 323:236–248.

Gurusamy R, Natarajan S. 2013. Current status on biochemistry and molecular biology of microbial degradation of

nicotine. Scientific World Journal. 2013:125385.
Hebert LE, Weuve J, Scherr PA, Evans DA. 2013. Alzheimer disease in the United States (2010-2050) estimated using the 2010 census. Neurology 80:1778–83.
Hochstein Li, Rittenberg Sc. 1959. The bacterial oxidation of nicotine. II. The isolation of the first oxidative product and its identification as (1)-6-hydroxynicotine. J. Biol. Chem. 234:156–60.
Hritcu L, Stefan M, Brandsch R, Mihasan M. 2011. 6-Hydroxy-L-nicotine from Arthrobacter nicotinovorans sustain spatial memory formation by decreasing brain oxidative stress in rats. In: . J. Physiol. Biochem. Vol. 69, p. 25–34.
Igloi GL, Brandsch R. 2003. Sequence of the 165-kilobase catabolic plasmid pAO1 from Arthrobacter nicotinovorans and identification of a pAO1-dependent nicotine uptake system. J Bacteriol 185:1976–1986.
Liu C-J, Blount JW, Steele CL, Dixon RA. 2002. Bottlenecks for metabolic engineering of isoflavone glycoconjugates in Arabidopsis. Proc. Natl. Acad. Sci. U. S. A. 99:14578–83.
Liu Y, Wang L, Huang K, Wang W, Nie X, Jiang Y, Li P, Liu S, Xu P, Tang H. 2014. Physiological and Biochemical Characterization of a Novel Nicotine-Degrading Bacterium Pseudomonas geniculata N1. . PLoS One 9:e84399.
Ma Y, Wei Y, Qiu J, Wen R, Hong J, Liu W. 2014. Isolation, transposon mutagenesis, and characterization of the novel nicotine-degrading strain Shinella sp. HZN7. Appl. Microbiol. Biotechnol. 98:2625–36.
Mihasan M, Capatina L, Neagu E, Stefan M, Hritcu L. 2013. In-Silico Identification of 6-Hydroxy-L-Nicotine as a Novel Neuroprotective Drug. Rom. Biotechnol. Lett. 18:8333–8340.
Sachelaru P, Schiltz E, Brandsch R. 2006. A functional mobA gene for molybdopterin cytosine dinucleotide cofactor biosynthesis is required for activity and holoenzyme assembly of the heterotrimeric nicotine dehydrogenases of Arthrobacter nicotinovorans. Appl. Environ. Microbiol. 72:5126–31.
Tang H, Wang L, Meng X, Ma L, Wang S, He X, Wu G, Xu P. 2009. Novel nicotine oxidoreductase-encoding gene involved in nicotine degradation by Pseudomonas putida strain S16. Appl. Environ. Microbiol. 75:772–8.
Tang H, Wang S, Ma L, Meng X, Deng Z, Zhang D, Ma C, Xu P. 2008. A novel gene, encoding 6-hydroxy-3-succinoylpyridine hydroxylase, involved in nicotine degradation by Pseudomonas putida strain S16. Appl. Environ. Microbiol. 74:1567–74.
Wang SN, Xu P, Tang HZ, Meng J, Liu XL, Qing C. 2005. “Green” route to 6-hydroxy-3-succinoyl-pyridine from (S)-nicotine of tobacco waste by whole cells of a Pseudomonas sp. Environ. Sci. Technol. 39:6877–80.

1. “Alexandru Ioan Cuza” University of Iasi, Romania. BR and GD equally contributed to the practical work involved in this work; MM was involved in experimental design, practical work and manuscript writing.
* marius.mihasan@uaic.ro

ACKNOWLEDGEMENTS
This work was supported by the strategic grant POSDRU/159/1.5/S/133652, co-financed by the European Social Fund within the Sectorial Operational Program Human Resources Development 2007 – 2013

MYELOPROLIFERATIVE NEOPLASMS WITH CONCURRENT BCR-ABL FUSION GENE AND JAK2V617F MUTATION

ROXANA POPESCU[1], ANGELA DĂSCĂLESCU[1]*, CĂTĂLIN DĂNĂILĂ[1], DORAMINA GHEORGHIU[2], ION ANTOHI[1], ANCA GORIUC[1], ANCA-VIORICA IVANOV[1], DOINA AZOICĂI[1]

Keywords: Myeloproliferative neoplasms, BCR-ABL fusion gene, JAK2V617F mutation

Abstract: This study investigates the occurrence of BCR-ABL fusion gene and JAK2V617F mutation in myeloproliferative neoplasms (MPN) patients at diagnosis, in order to evaluate the clinical features, and compare them to the literature data. The study was conducted between January 2012 and February 2014 and included 190 cases of MPN from Regional Institute of Oncology Iasi. Molecular evaluation of BCR-ABL transcript and JAK2V617F mutation by RT-PCR, were assessed for diagnosis and monitorization. Only 2 patients showed simultaneous occurrence of both the JAK2V617F mutation and the BCR/ABL translocation. Pacient 1 presented a complex clinical picture with clinical signs of CML and essential thrombocythemia, while the second patient had a clinical picture suggestive of polycythemia vera. The screening for the JAK2V617F mutation and BCR-ABL should be considered at MPN diagnosis. Following these patients up might provide new data regarding the long term evolution of such cases.

INTRODUCTION

Myeloproliferative neoplasms (MPNs) are a group of diseases characterized by the cloning neoplastic proliferation of hematopoietic stem cells with the expansion of one or more myeloid lines. These diseases display typically increased number of medullar cells, maturation of cellular lines and organomegaly and are classified according to phenotypic and clinical characteristics and genetic abnormalities.

According to the data provided by the World Health Organization (WHO) in 2008, a classification of the major entities includes chronic myeloid leukemia (CML), polycythemia vera (PV), essential thrombocythemia (ET), and primary myelofibrosis (PMF) (Vardiman et al., 2009).

CML is specifically associated with Philadelphia (Ph) chromosome resulting from the reciprocal translocation between chromosomes 9 and 22, t(9;22)(q34;q11) which lead to the formation of the fusion gene *BCR-ABL.*

JAK2 is a tyrosine kinase that has a major role in the signaling pathways of many hematopoietic growth factors receptors. In patients with BCR-ABL-negative MPN, it was identified a point mutation in the *JAK*2 gene causing a substitution of a valine to phenylalanine at codon 617 which leads to a constitutive activation of JAK2 kinase and induces cellular proliferation and resistance to apoptosis. The discovery of the JAK2V617F mutation in 90% of PV and 50-60% of ET and PMF was the molecular evidence for the common pathogenesis of PV, ET and MF (Levine et al., 2005).

It was initially thought that *BCR-ABL* fusion gene and JAK2V617F mutation are mutually exclusive (Jelinek et al., 2005). Yet, in the last few years, some rare cases of patients with the coexistence of these two anomalies have been reported in the literature (Hussein et al., 2007, Inami et al., 2007), raising questions about their phenotypic and prognosis relevance.

This study aims to investigate the occurrence of this double mutated phenotype in MPN patients at diagnosis, to evaluate the clinical features, and compare them to data provided by literature.

MATERIALS AND METHODS

This study was conducted between January 2012 and February 2014 and included 190 MPN cases from Regional Institute of Oncology Iasi. Hematological parameters including complete blood picture and differential count were taken from patients' records. Bone marrow morphology, cytogenetic, molecular evaluation of BCR-ABL transcript and JAK2V617F mutation were assessed at diagnosis. Informed consent was signed by the patients under the protocol approved by the Ethics Committee of the University of Medicine and Pharmacy, Iasi.

Methods for quantitative assessment of BCR-ABL transcripts

The peripheral blood lymphocytes were isolated using red blood lysis solution (Promega Inc, Madison, WI, USA), then washed with phosphate buffer saline solution, and resuspended at a concentration of $2x10^7$cells in 1 mL of Guanidin Thiocianate reagent (RNAzol® RT, Sigma-Aldrich, US). RNA was afterwards isolated according to the

manufacturer's instructions by alcohol precipitation, washing and solubilization. RNA was quantified using a NanoDrop 2000 (Thermo Fischer Scientific) spectrophotometer.

Reverse transcription

2 µg of RNA (4 µl of total RNA 500ng/µl) was used in a reverse transcription reaction using a RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific). The annealing, extension, and enzyme inactivation parameters were 25°C/ 5 min, 42°C/ one hour, and 70°C/ 5 min, respectively. cDNA was then diluted 1/5 (with 80 µl nuclease free water) in order to be used in PCR amplification.

Quantitative assessment of BCR-ABL expression

The assessment of BCR-ABL p210 transcript (b2a2, b3a2) was performed by quantitative Taqman Real Time PCR using Translocation Kit t(9;22)/M-BCR-RQ (Experteam, Italy) according to the manufacturer's instructions.

ABL gene transcripts were used as internal control and for the calculation of BCR-ABL/ABL ratio. Expression of BCR-ABL transcript was calculated as a ratio of BCR-ABL transcripts over ABL transcripts. Samples showing a Ct > 28 for ABL were excluded from the analysis.

The assessment of JAK2V617F mutation

DNA was extracted from peripheral blood using a Wizard Genomic DNA Purification kit (Promega, USA). The assessment of JAK2V617F mutation consisted of Taqman Real-Time PCR genotyping, using Clonit qualitative detection kit (Milano, Italy).

The 5µL DNA was amplified in a 25 µL reaction volume (12.5 µl Amplification mix and 7.5 µl JAK2 Probe mix - fluorescence FAM for wild type and VIC for mutant) using the Stratagene Mx3005P thermocycler (Agilent Technologies, Inc., USA). The allelic discrimination was performing making a scatter plot of mutated allele's fluorescence (y axis) versus wild type alleles' fluorescence (x axis) and discriminating this way the tree possible genotypes: homozygote wild-type (100% WT DNA), homozygote mutated (100% JAK2V617F DNA) and heterozygote mutated (reference control, limit of detection 2% JAK2V617F DNA).

RESULTS AND DISCUSSIONS

Out of the 190 patients in which both the *BCR-ABL* fusion gene and the JAK2 mutation were evaluated, 94 patients (49.4%) displayed JAK2V617F mutation (79 heterozygote, 15 mutant) without the presence of BCR-ABL gene, 68 patients (35.8%) displayed wild type JAK2 genotype and were negative for BCR-ABL and 26 patients (13.7%) tested negative for the JAK2 mutation (wild type genotype) and positive for BCR-ABL. Only 2 patients showed simultaneous occurrence of both the JAK2V617F mutation and the BCR/ABL translocation, accounting for 1.05% of the total number.

Case 1 was a 62 year old female who was investigated in April 2013 for thrombocytosis after a thrombotic stroke. The first diagnosis assumption was essential thrombocythemia. The patient's laboratory findings at admission were as follows: hemoglobin - 14.2 g/dL; platelets- 579×10^9/L; white blood cells- 11.4×10^9/L with a differential blood count revealing 79% segmented neutrophils, 4% eosinophils, 1% basophils, 12% lymphocytes, and 4% monocytes. The peripheral blood smear revealed erythrocyte anisocytosis, rare elliptocytes, platelet anisocytosis and enlarged (macro) platelets. Splenomegaly (160 mm) was detected by ultrasound at diagnosis.

The patient's GTG karyotype revealed the presence of Ph chromosome in all of twenty analyzed metaphase cells. Real Time PCR for the detection of BCR/ABL transcript proved positive for b2a2 type transcript.. The BCR-ABL/ABL ratio was 99.54% at diagnosis. The patient was found positive also for JAK2V617F heterozygous mutation. On the basis of these findings, the patient was diagnosed with JAK2V617F- and Ph-positive CML.

Starting with August 2013 treatment was initiated, consisting of Imatinib mesylat (IM) 400mg/day. After one month a complete hematologic response (CHR) was obtained, while a complete molecular response (CMR) was recorded after three months.

In February 2014, CHR was maintained, while the repeated cytogenetic examination revealed the occurrence of Ph chromosome in three of twenty analyzed metaphase cells

(46,XX[17]/46,XX,t(9;22)(q34;q11)[3]) and molecular analysis showed an increased level of BCR–ABL transcript (BCR–ABL/ABL ratio: 8.4%). The JAK2V617F heterozygous mutation remained present. The patient continued the treatment with IM 400mg/day.

In September 2014 (one year after the diagnosis) a complete blood count revealed hemoglobin 13.5g/L, normal white blood cells (7.74×10^9/L) and slight thrombocytosis (450×10^9/L). The differential blood count showed 73% segmented neutrophils, 3% eosinophils, 15% lymphocytes, 6% monocytes and 1% basophils. The first patient's evolution is indicated in figure 1.

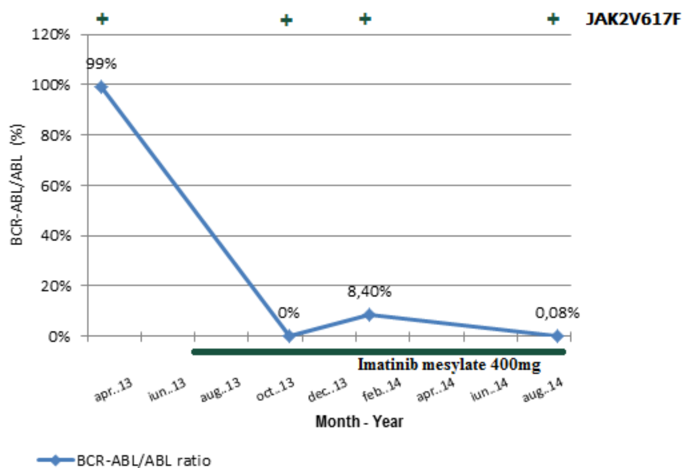


Figure 1. Evolution of BCR-ABL/ABL ratio and of the JAK2V617F mutation in Case 1;

Repeated molecular analyses indicated a major molecular response - MMR (BCR-ABL/ABL ratio 0.08%). The evaluation of the JAK2 mutation indicated that the mutation is still present as a heterozygous status (Fig. 2).

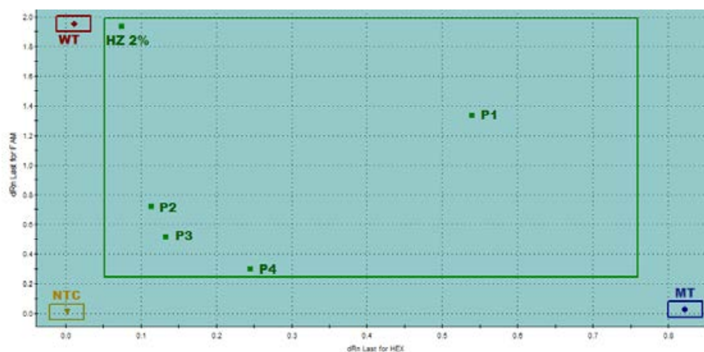


Figure 2. Real Time PCR aspect, with a scatter plot graphic obtained during the interpretation of the JAK2V617F mutation status for case 1; WT - homozygote wild-type, MT - homozygote mutated, HZ 2% - heterozygote mutated 2%, P1-P4 – Case 1 evaluations

Case 2 was a 45 year old male patient who presented dyspnea on moderate efforts and palpitations. A complete blood count revealed polycythemia (hemoglobin 19.8 g/L), normal white blood cells (8.8×10^9/L) and normal platelet counts ($314x10^9$/L). The differential blood count showed 87% segmented neutrophils, 3% eosinophils, 8% lymphocytes and 2% monocytes. The patient was found positive for the JAK2V617F heterozygous mutation and the BCR-ABL/ABL ratio was 2.6% at diagnosis. The patient was diagnosed with Polycythemia vera with a major BCR/ABL fusion transcript. No further data were available.

In this study, the occurrence of cases displaying BCR-ABL fusion gene and the JAK2V617F mutation was 1.05% (2/190). A similar result was reported by Cappetta et al., who analyzed 1320 cases of MPN suspicion and identified 5 patients (0.37%) with atypical MPN, who displayed both anomalies (Cappetta et al., 2013). A low frequency of the occurrence of JAK2V617F mutation was reported in Ph+ CML patients by Pieri et al. (2.25%, X/314) (Pieri et al., 2011) and Campiotti et al. (7.7%, 1/13) (Campiotti et al., 2009). A higher frequency of cases with both mutations was reported by two studies which included CML patients: 26.7% (12/45) (Pahore et al.) and 44% (11/25) (Tabassum et al., 2014, Pahore et al., 2011). The higher frequency reported in the two studies could be explained by means of the evaluated population and the different inclusion criteria. In order to determine the real frequency and the role the two anomalies play in determining the phenotype, further studies are required.

Patients can display variable, unspecific phenotype (leukocytosis, splenomegaly, as well as increased Ht, thrombocytosis), the evaluation of bone marrow biopsy being a valuable test in MPN differentiation. Initially they can display a suggestive phenotype for Ph- MPN, most frequently PV (Mirza et al., 2007, Bocchia et al., 2007, Pingali et al., 2009, Ursuleac et al., 2013) or PMF (Bornhauser et al., 2007, Jallades et al., 2008) and more seldom ET (Curtin et al 2005), associated with BCR-ABL positive CML throughout the evolution of the disease. Another category is represented by those who display specific CML phenotype and later develop Ph- MPN, PV (Tefferi et al., 2010, Inami et al., 2007, Cambier et al., 2008), PMF (Hussein et al., 2007, Kramer et al., 2007) or TE (Pastore et al., 2013, Veronese et al., 2010, Lee et al., 2013) (Supplemental material 1). There is no connection between the treatment administered prior to diagnosis and the occurrence of CML or Ph- MPN clinical signs in these patients.

From the perspective of the two anomalies, the JAK2V617F mutation might precede the acquisition of the BCR-ABL fusion gene (Bocchia et al., 2007, Jallades et al., 2008, Bornhauser et al., 2007, Mirza et al., 2007), it can follow it (Tefferi et al., 2010, Nadali et al., 2009) or, even more frequently, the two anomalies might appear at the same time (Inami et al., 2007, Cambier et al., 2008, Hussein et al., 2008, Kramer et al., 2007, Hussein et al., 2007, Pahore et al., 2011). The retrospective evaluation of the JAK2V617F mutation, at the CML diagnosis, has proved its presence, concomitantly with the BCR-ABL fusion gene, in 8 cases reported in the literature (Supplemental material 1).

Generally, the occurrence of the BCR-ABL fusion gene changes the phenotype in CML, which sustains the hypothesis of an advantage of this clone in determining the phenotype. During the evolution there seems to be a phenomenon of competition between the proliferations of the two abnormal clones, so that the mutant JAK2V617F clone decreases or disappears when the BCR-ABL transcript increases and reappears possibly in a dominant position, with clinical manifestations, when the BCR-ABL+ clone is inhibited by the TKI treatment (Inami et al., 2007, Cambier et al., 2008, Hussein et al., 2008, Kramer et al., 2007).

The two patients presented in detail in this study displayed both anomalies concomitantly at diagnosis, yet had different phenotypes.

Patient 1 displayed a phenotype which was untypical for CML (unspecific blood smear, discrete leukocytosis without left deviation in the leukocyte formula, without myeloblasts, without basophilia, despite displaying a high level of expression of the BCR-ABL at diagnosis) associating specific TE elements. Cases associating CML and TE phenotype are rare. Payande et al. reported one case of co-existence of the JAK2V617F mutation and the BCR-ABL fusion gene at diagnosis, the patient displaying intricate phenotype of CML and TE (leukocytosis, thrombocytosis, anemia, high LDH level) (Payande et al., 2011). Lee et al. reported two cases diagnosed with CML, where the phenotype evolved towards TE after 119 and respectively 30 months after the occurrence of the JAK2V617F mutation. Other two cases are reported with CML diagnosis, which displayed low levels of the JAK2 mutation at diagnosis (0.12 and 0.2%, respectively), the TE phenotype appearing at 67 and respectively 6 months, after the levels of the JAK2V617F mutation increased up to 10% and 9% respectively (Supplemental material 1) (Veronese et al., 2010, Pastore et al., 2013).

As far as response to treatment is concerned, in most reported patients the suppression of the positive BCR-ABL clone was obtained under TKI treatment (complete cytogenetic response - CCyR) or MMR/CMR), yet the hematologic response was partial or medullar fibrosis progression was reported. In most cases, IM did not affect the co-existence or the acquisition of the JAK2V617F clone (Inami et al., 2007, Hussein et al., 2007, Kramer et al., 2007, Veronese et al., 2010, Cambier et al., 2008, Pardini et al., 2008). In the case of the first patient presented in this study, the response to the treatment with IM 400 mg/day was very good, CHR and CMR being achieved within three months. A slight increase of the transcript was recorded afterwards, MMR (BCR-ABL/ABL 0.08%) being maintained after 9 months. Hematologic parameters kept within normal limits except a slight thrombocytosis.

Case 2 was diagnosed with PV without phenotypic alterations specific to CML, the BCR-ABL/ABL ratio being as reduced as 2.6%, which provides an explanation for the lack of CML clinical manifestations. Only two reported cases display the BCR-ABL fusion gene (with reduced levels of expression) without clinical signs of CML throughout the Ph- MPN evolution (Park et al., 2013, Bornhauser et al., 2007). Unfortunately, there is no data available about the further evolution of the patient. Another case of BCR-ABL and JAK2V617F co-existence was described in our country in a patient diagnosed with PV, who developed a CML phenotype after 7 years of disease progression. The JAK2V617F mutation was identified after 24 months of IM treatment, when the Ph+ clone was no longer detectable (Ursuleac et al., 2013).

Two hypotheses were proposed for explaining the presence of JAK2V617F and the BCR-ABL fusion in the same patient.

The first hypothesis states that Ph- MPN and CML are two separate diseases, developed from different clones of the cellular progenitors, the phenotype being determined by the dominant clone. This hypothesis is supported by both the phenotypic heterogeneity of the patients and the fact that the JAK2V617F mutation stays positive, constant or it increases at the suppression of the BCR-ABL+ clone (Hussein et al., 2008, Xu et al., 2014, Veronese et al., 2010, Bee et al., 2010, Pastore et al., 2013). We do not exclude the possibility that the two anomalies superpose independently over an unknown founding mutation capable of generating genomic instability at the hematopoietic stem cell level (Tefferi et al., 2010, Hussein et al., 2007, Inokuchi et al., 2012, Nadali et al., 2009).

The second hypothesis supposes the existence of a single sub-clone of hematopoietic progenitor cells that acquire the two anomalies within the same cell, at different moments. The concomitant presence of the two anomalies at the moment of CML diagnosis and the

maintenance of a constant level of the JAK2V617F mutation arguments in favor of the occurrence of the JAK2V617F mutation as an initial step, a sub-clone acquiring the BCR-ABL translocation afterwards, the latter providing a proliferative advantage to the double mutant clone. The suppression of the BCR-ABL bearing clone after the TKI treatment allows the proliferation of positive JAK2V617F cells, allowing for the clinical manifestation of the Ph-MPN. (Bornhauser et al., 2007, Bocchia et al., 2007, Jallades et al., 2008, Kramer et al., 2007, Hussein et al., 2008, Inami et al., 2007). This hypothesis is supported by a few studies on cellular cultures from patients presenting the two anomalies. The JAK2V617F mutation is identified in myeloid progenitor cells, mostly BFU-E (erythroid burst-forming units) and CFU-GM (colony-forming unit granulocyte macrophage) alone or together with the BCR-ABL transcript, yet none of the colonies display the BCR-ABL gene in isolation (Bocchia et al., 2007, Wang et al., 2013). After the IM treatment, when MMR is achieved, most colonies display JAK2V617F and none of them display BCR-ABL (Bocchia et al., 2007). Bornhauser et al. notes both mutations in a limited number of granulocyte colonies, while the JAK2 mutation is identified in both types of colonies (erythroid and granulocyte). This aspect does not exclude the existence of the initial mutation that leads to the acquisition of several genetic anomalies (Kramer et al., 2007, Bocchia et al., 2007).

In both cases reported in this study, the JAK2V617F mutation was detectable in heterozygous status, at the diagnosis, together with the *BCR-ABL* fusion gene and remained positive during the whole evolution of the disease in patient 1. Unfortunately there is no data regarding the evolution of the second patient. Clinically, the first patient displayed intricate CML and TE signs, while the second patient's phenotype was PV (decreased expression of BCR-ABL). This fact supports the second hypothesis, namely of the co-existence of the two mutations in the same clone, with the occurrence of BCR-ABL in a sub-clone of positive JAK2V617F cells, the latter gaining proliferative grounds. However, we cannot exclude the possibility of the existence of two different mutant clones.

The evolution of CML does not seem to be influenced by the presence of the JAK2V617F mutation, but it can be a rare cause of the lack of hematologic response to ITK treatment (Xu et al., 2014). The differentiation between relapse, disease progression or IM resistance and the superposition of a MPN Ph- can be done by means of evaluating both anomalies in these patients. Similarly, the occurrence of clinical signs of CML in patients displaying mutant JAK2V617F indicates the evaluation of the presence of BCR-ABL fusion gene, in order to initiate the TKI treatment.

CONCLUSIONS

Besides other cases described in literature, the cases presented by us support the co-existence of the *BCR-ABL* gene and the JAK2V617F mutation in the same patient. The screening for the JAK2V617F mutation and BCR-ABL should be considered at the very first suspicion of a MPN diagnosis, as well as in the case of patients with a CML diagnosis who keep developing myeloproliferations despite obtaining MMR.

Following up patients with JAK2V617F mutation and BCR-ABL in clinical trials might provide new data regarding the long term evolution of such cases.

REFERENCES

Bee, P. C., Gan, G. G., Nadarajan, V. S., Latiff, N. A., et al., (2010): *A man with concomitant polycythaemia vera and chronic myeloid leukemia: The dynamics of the two disorders*. Int J Hematol, 91(1)**,** 136-139.

Bocchia, M., Vannucchi, A. M., Gozzetti, A., Guglielmelli, P., et al., (2007): *Insights into jak2-v617f mutation in cml*. Lancet Oncol, 8(10)**,** 864-866.

Bornhauser, M., Mohr, B., Oelschlaegel, U., Bornhauser, P., et al., (2007): *Concurrent jak2(v617f) mutation and bcr-abl translocation within committed myeloid progenitors in myelofibrosis*. Leukemia, 21(8)**,** 1824-1826.

Cambier, N., Renneville, A., Cazaentre, T., Soenen, V., et al., (2008): *Jak2v617f-positive polycythemia vera and philadelphia chromosome-positive chronic myeloid leukemia: One patient with two distinct myeloproliferative disorders*. Leukemia, 22(7)**,** 1454-1455.

Campiotti, L., Appio, L., Solbiati, F., Ageno, W., et al., (2009): *Jak2-v617f mutation and philadelphia positive chronic myeloid leukemia*. Leuk Res, 33(11)**,** e212-213.

Cappetta, M., Perez, V., Zubillaga, M. N., Elizondo, V., et al., (2013): *Concomitant detection of bcr-abl translocation and jak2 v617f mutation in five patients with myeloproliferative neoplasm at diagnosis*. Int J Lab Hematol, 35(1)**,** e4-5.

Hussein, K., Bock, O., Seegers, A., Flasshove, M., et al., (2007): *Myelofibrosis evolving during imatinib treatment of a chronic myeloproliferative disease with coexisting bcr-abl translocation and jak2v617f mutation*. Blood, 109(9)**,** 4106-4107.

Hussein, K., Bock, O., Theophile, K., Seegers, A., et al., (2008): *Chronic myeloproliferative diseases with concurrent bcr-abl junction and jak2v617f mutation*. Leukemia, 22(5)**,** 1059-1062.

Inami, M., Inokuchi, K., Okabe, M., Kosaka, F., et al., (2007): *Polycythemia associated with the jak2v617f mutation emerged during treatment of chronic myelogenous leukemia*. Leukemia, 21(5)**,** 1103-1104.

Inokuchi, K., Yamaguchi, H., Tamai, H., Dan, K., (2012): *Disappearance of both the bcr/abl1 fusion gene and the jak2v617f mutation with dasatinib therapy in a patient with imatinib-resistant chronic myelogenous leukemia*. J Clin Exp Hematop, 52(2)**,** 145-147.

Jallades, L., Hayette, S., Tigaud, I., Johnston, A., et al., (2008): *Emergence of therapy-unrelated cml on a background of bcr-abl-negative jak2v617f-positive chronic idiopathic myelofibrosis*. Leuk Res, 32(10)**,** 1608-1610.

Jelinek, J., Oki, Y., Gharibyan, V., Bueso-Ramos, C., et al., (2005): *Jak2 mutation 1849g>t is rare in acute leukemias but can be found in cmml, philadelphia chromosome-negative cml, and megakaryocytic leukemia*. Blood, 106(10)**,** 3370-3373.

Kramer, A., Reiter, A., Kruth, J., Erben, P., et al., (2007): *Jak2-v617f mutation in a patient with philadelphia-chromosome-positive chronic myeloid leukaemia*. Lancet Oncol, 8(7)**,** 658-660.

Lee, Y. J., Moon, J. H., Shin, H. C., Seo, J. W., et al., (2013): *Two cml patients who subsequently developed features of essential thrombocythemia with jak2-v617f mutation while in complete cytogenetic remission after treatment with imatinib mesylate*. Int J Hematol, 97(6)**,** 804-807.

Levine, R. L., Wadleigh, M., Cools, J., Ebert, B. L., et al., (2005): *Activating mutation in the tyrosine kinase jak2 in polycythemia vera, essential thrombocythemia, and myeloid metaplasia with myelofibrosis*. Cancer Cell, 7(4)**,** 387-397.

Mirza, I., Frantz, C., Clarke, G., Voth, A. J., et al., (2007): *Transformation of polycythemia vera to chronic myelogenous leukemia*. Arch Pathol Lab Med, 131(11)**,** 1719-1724.

Nadali, F., Ferdowsi, S., Karimzadeh, P., Chahardouli, B., et al., (2009): *Jak2-v617f mutation and philadelphia positive chronic myeloid leukemia*. IJHOSCR, 3(3)**,** 43-45.

Pahore, Z. A., Shamsi, T. S., Taj, M., Farzana, T., et al., (2011): *Jak2v617f mutation in chronic myeloid leukemia predicts early disease progression*. J Coll Physicians Surg Pak, 21(8)**,** 472-475.

Pardini, S., Fozza, C., Contini, S., Rimini, E., et al., (2008): *A case of coexistence between jak2v617f and bcr /abl*. Eur J Haematol, 81(1)**,** 75-76.

Park, S. H., Chi, H. S., Cho, Y. U., Jang, S., et al., (2013): *Two cases of myeloproliferative neoplasm with a concurrent jak2 (v617f) mutation and bcr/abl translocation without chronic myelogenous leukemia phenotype acquisition during hydroxyurea treatment*. Ann Lab Med, 33(3)**,** 229-232.

Pastore, F., Schneider, S., Christ, O., Hiddemann, W., et al., (2013): *Impressive thrombocytosis evolving in a patient with a bcr-abl positive cml in major molecular response during dasatinib treatment unmasks an additional jak2v617f*. Exp Hematol Oncol, 2(1)**,** 24.

Payande, M., Zare, M. E., Shure, S. G. H., Zadeh, F. S., (2011): *Jak2-v617f mutation combined with philadelphia chromosome-positive chronic myeloid leukaemia: A case report*. International Journal of Hematology-Oncology and Stem Cell Research, 5(2)**,** 34-37.

Pieri, L., Spolverini, A., Scappini, B., Occhini, U., et al., (2011): *Concomitant occurrence of bcr-abl and jak2v617f mutation*. Blood, 118(12)**,** 3445-3446.

Pingali, S. R., Mathiason, M. A., Lovrich, S. D., Go, R. S., (2009): *Emergence of chronic myelogenous leukemia from a background of myeloproliferative disorder: Jak2v617f as a potential risk factor for bcr-abl translocation*. Clin Lymphoma Myeloma, 9(5)**,** E25-29.
Tabassum, N., Saboor, M., Ghani, R., Moinuddin, M., (2014): *Frequency of jak2 v617f mutation in patients with philadelphia positive chronic myeloid leukemia in pakistan*. Pak J Med Sci, 30(1)**,** 185-188.
Tefferi, A., Levitt, R., Lasho, T. L., Knudson, R. A., et al., (2010): *Postimatinib therapy emergence of a new jak2v617f clone and subsequent development of overt polycythemia vera in a patient with chronic myelogenous leukaemia*. Eur J Haematol, 85(1)**,** 86-87.
Ursuleac, I., Colita, A., Adam, T., Jardan, C., et al., (2013): *The concomitant occurrence of jak2v617f mutation and bcr/abl transcript with phenotypic expression - an overlapping myeloproliferative disorder or two distinct diseases? - case report*. J Med Life, 6(1)**,** 34-37.
Vardiman, J. W., Thiele, J., Arber, D. A., Brunning, R. D., et al., (2009): *The 2008 revision of the world health organization (who) classification of myeloid neoplasms and acute leukemia: Rationale and important changes*. Blood, 114(5)**,** 937-951.
Veronese, L., Tchirkov, A., Richard-Pebrel, C., Ledoux-Pilon, A., et al., (2010): *A thrombocytosis occurring in philadelphia positive cml in molecular response to imatinib can reveal an underlying jak2(v617f) myeloproliferative neoplasm*. Leuk Res, 34(4)**,** e94-96.
Wang, X., Tripodi, J., Kremyanskaya, M., Blouin, A., et al., (2013): *Bcr-abl1 is a secondary event after jak2v617f in patients with polycythemia vera who develop chronic myeloid leukemia*. Blood, 121(7)**,** 1238-1239.
Xu, W., Chen, B., Tong, X., (2014): *Chronic myeloid leukemia patient with co-occurrence of bcr-abl junction and jak2 v617f mutation*. Int J Hematol, 99(1)**,** 87-90.

1. University of Medicine and Pharmacy „Grigore T. Popa , Iasi
2. Regional Institute of Oncology, Iasi, Hematology and Oncology Unit
***** e-mail: angdascalescu@yahoo.com; Phone: 0740157774, Universitatii Street No. 16, Iasi, Romania

Supplemental material 1: Characteristics of reported cases with JAK2V617F MPN discovered in initially diagnosed Ph+ CML.

Authors	Age (years) /sex	MPN Ph- Second diagnosis (time to Ph- MPN-mo)	JAK2V617F mutated allele		Chronology of molecular findings	Treatment	Evolution of BCR-ABL and JAK2 V617F mutation
			At CML diagnosis	At MPN diagnosis			
Veronese et al. 2010	82/F	PMF (12)	0.5%	92%	concomitant (retrospective)	HUR, IM , hydroxycarbamide, salicylic acid	MMR/NA
	62/M	ET (6)	0.2%	9%	concomitant	IM, salicylic acid	MMR/NA
Conchon et al. 2008	52/F	PMF (0)	High levels	High levels	concomitant	HUR, IM	Good response/positive
Cambier et al. 2008	64/M	PV (0)	High levels	High levels	concomitant	IM, phlebotomy	MMR/positive
Inami et al. 2007 Inokuchi et al. 2012	43/M	PV(>6a)	20%	20%	concomitant (retrospective)	INFα, HUR, IM flebotomy, dasatinib	CCyR/positive CMR/negative
Hussein et al. 2008	32/M	Ph- MPN (44)	-	21	BCR then JAK2	anagrelide , HUR,IM	CMR/positive
	58F/	PMF (12)	ND	23	JAK2 ND at diagnosis	HUR, INFα	NA/positive
	45/M	No Ph- MPN (0)	43	43	concomitant	NA	CMR/positive
Hussein et al.2007	55/M	PMF	5%	23%	concomitant (retrospective)	IM	CMR/positive
Krämer et al. 2007	50/M	PMF (>3a)	35%	≈ 50%	concomitant (retrospective)	Hydroxycarbamide, IM	CMR/positive
Caocci et al 2010	70/M	Ph- MPN (84)	ND	+	JAK2 ND at diagnosis	INFα, IM	CCyR/positive
Lee et al. 2013	53/M	TE (119)	-	+	BCR then JAK2	HUR, INFα,IM; anagrelide and HUR,	MMR/positive
	60/F	TE (30)	-	+	BCR then JAK2	IM, nilotinib, anagrelide	MMR/positive
Payande et al.2011	82/M	TE (0)	+	+	concomitant	HUR, IM, INFα	NA
Nadali et al. 2009	39/M	No MPN Ph-(48)	ND	+	BCR then JAK2	HUR,IM	NA
Campiotti et al. 2009	na	No MPN Ph- (0)	12%	-	concomitant	IM	PCyR/negative
Pastore et al. 2013	42/F	TE(67)	0.122	>10	concomitant (retrospective)	HUR, IM, dasatinib	MMR/positive
Pardini et al. 2008	67/M	PMF	HZ	+	concomitant (retrospective)	IM,HUR	MMR/positive
Xu et al. 2014	21/F	No MPN Ph- (0)	+	+	concomitent	IM	CMR/positive
Kim et al. 2008	49/M	PMF (6)	77.3%	>50%	concomitant (retrospective)	IM, dasatinib	CMR/positive
	64/M	PMF (0)	61%	61%	concomitant (retrospective)	IM, nilotinib	CCyR /positive

MPN - myeloproliferative neoplasms; PV - polycythemia vera, ET - essential thrombocythemia, PMF - primary myelofibrosis, NA - not available, ND – not determined; IM- imatinib mesylate, HUR- hydroxiureea, INFα- Interferon alfa,, CMR- complete molecular response, MMR- major molecular response, CCyR- complete cytogenetic response

CELL PROLIFERATION AND MIGRATION ASSAY ON POLYMER SURFACES

ANA MARIA HOLICOV, TUDOR PETREUŞ*, CARMEN ELENA COTRUTZ

Keywords: scratch assay, cell migration assay, osteoblast-like cells
Abstract. Cell proliferation and migration on phosphonated polymer surfaces may impair cell adherence and migration. The aim of the present study was to evaluate the ability of osteoblast-like cells to migrate at the surface of the phosphonated chitosan pellicle compared to normal chitosan pellicle. Results. Cells proliferated and migrated at different rates according to surface charge. Negative surfaces showed lower adherence and the migration difference could be quantified. Conclusions. Cell migration on polymer surfaces can be successfully evaluated by improved scratch test performed directly on polymer pellicles. Migration assay can be quantified by measuring the scratch size following separator removal. At the same time, viability assays are applicable following this improved scratch test while with no cells are damaged by mechanical means mechanically damage.

INTRODUCTION

Cell proliferation and migration on polymer surfaces may quantify not only survival rate but also cell adherence according to various surface properties (Bhattacharyya, et al., 2010, Choi, et al., 2012, Guidoin, et al., 2005). Polymer phosphorylation by phosphorous acid, due to extensive negative charges determined by phosphite groups, may impair cell adherence and migration.
One of the most abundant natural biopolymer is represented by chitin which is extracted from crustaceans, fungal cell walls and insects (Francesko and Tzanov, 2011, Jayakumar, et al., 2011, Mir, et al., 2008). This natural biopolymer can be deacetylated to obtain chitosan. Together with other natural polymers, as polyvinyl alcohol and cellulose, chitosan is part of a new class of biomaterials with various functions due to their biological activity, excellent biocompatibility, and complete biodegradability together with low toxicity (Jayakumar, et al., 2008, Wang and Liu, 2014).
As chitosan represents a main element in osteoconductive and drug delivery systems and while chitosan functionalization by phosphorylation may play an important role in cell migration in various scaffolds used in regenerative medicine, the aim of the present study was to evaluate the ability of osteoblast-like cells to migrate at the surface of the phosphorylated chitosan pellicle compared to normal chitosan pellicle.

MATERIAL AND METHODS

Chistosan blends and membranes
1% chitosan solution was obtained by chitosan powder (Fluka) dissolution in 1% aqueous acetic acid solution at room temperature. Chitosan pellicles were obtained by pipetting 0.2 mL of chitosan solution on 96-well tissue culture plate, and then by drying in a dry incubator at 40°C for 48h.
2% collagen solution was added to chitosan solved in 1% acetic acid, to improve cell adherence. Differences in cell adherence were observed between simple chitosan solution and phosphorylated chitosan.
Prior to cell incubation, coated plates were sterilized by UV action in a transilluminator for 30 minutes. Following UV sterilization, each well was washed three times by sterile PBS then dried again in the dry incubator, in sterile conditions.
Cell cultures
For the present study, MNNG-human osteosarcoma cell line – HOS (CLS, Germany) was used. These cells have a similar phenotypic profile with osteoblast cells and are usually used in most assays regarding biocompatibility, cell proliferation and viability in contact with polymer or metallic biomaterials.
Complete medium used was composed by alpha MEM supplemented by 10% FCS, 1% Glutamin and 1% Antibiotic/antimycotic (penicillin /streptomycin). Cells at passage 5 were thawed for 90 sec at 37°C in a waterbath, washed in 10 ml complete culture media and centrifuged for 5 min at 400 G in a 15 ml centrifuge tube. The media was removed and the pellet resuspended in 1 ml warm fresh complete media. Cells were counted and viability evaluated by a Countess system using trypan blue (Invitrogen). Cell count showed values of $1x10^6$ with 80% viability. Resuspended cells were distributed in two 75 cm^2 flasks with 20 ml fresh warm medium each. Cells were placed in an incubator at 37°C with 5%CO_2. Cell subconfluence was obtained at 2 days from the initial passage. Cells were washed by PBS to remove traits of FCS and then detached by 1x TrypLE (Invitrogen). Cells were subsequently dispersed in 96 well plate, with the well’s bottom coated by chitosan-collagen and phosphorylated chitosan-collagen pellicles, and with separators glued to the plate lid (figure 1).

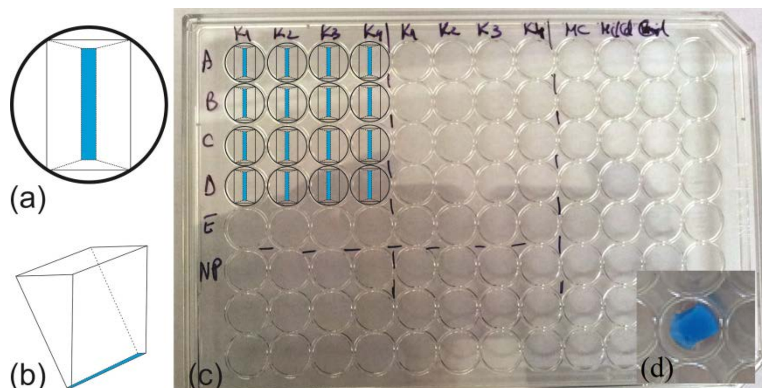


Figure 1. Silicone separator attached to a 96 well plate lid to simulate scratch test without impairing polymer surface. (a) top-view; (b) side-view; (c) 96-well plate lid with glued separators (draw); (d) Silicone separator attached to the lid of a 96 well plate

Prior to lid placement, each well was filled by 70µl complete media with $1x10^5$cells. Plates were incubated for 24 h for the cells to allow attachment and proliferation, and then the lid with separators was removed and replaced by a new one, with no separators. Plates were incubated again for another 48 hours to allow cells proliferate on the polymer pellicle side that was partially covered by the separator on the bottom of each well.

Plates were imaged at 24, 48 and 72 h for 3D cultures with a Nikon D-5000 camera adapted by a C-tube to a Nikon T-3000 reversed phase contrast microscope.

Viability assay - MTT

Cell viability by MTT assay (Mosmann, 1983) is a versatile method used to evaluate the cell survival following incubation with extraction liquid (LEx) from the investigated samples. It is a colorimetric method that uses a tetrazolium salt (MTT) which is transformed by mitochondrial dehydrogenases in purple formazan granules. MTT is a yellow hydrosoluble powder that can be metabolized by active (viable) cells, generating the formazan granules that can be subsequently dissolved by DMSO. Results are interpreted on spectrophotometric reading plates at 570-590 nm

Following medium removal, cells in 24, 48 and 72 wells were incubated with MTT solution (1 mg/ml in PBS) for 3 h and the resulting formazan was dissolved by DMSO (100 µl). Absorbance was measured at 590 nm using an automated multiplate reader (Pharmacia LKB Ultrospec Plus). Cell viability was expressed as percent compared to control lanes (blank - culture medium without cells; control – culture medium with cells) according to the formula CV=100x (ODs-ODb)/(ODc-ODb), where ODs represent the optical density (in units) for the sample, ODb – the optical density for the blank wells and and ODc – the optical density for the control wells. Assays for each extract were carried out in three replicates, including untreated cell control and the blank cell-free control.

RESULTS

Following cell spreading in the 96 well plates, in each well at 24 hours, there were observed aspects of cell proliferation, with an improved better spreading and adherence for the blend containing chitosan and collagen and compared with a lower adherence and proliferation rate for to the phosphorylated chitosan and collagen blend (figure 2). The starting point for simulated scratch filling was at 24 hours from initial cell spreading into the wells. Thus, further observations were performed at 48 and 72 hours respectively. At 48h, cells spread on chitosan-collagen membrane had a more obvious trend to close the scratch than on the phosphorylated polymer surface. Supplementary negative charges at the phosphorylated chitosan membrane surface influences also cell proliferation and adhesion. Thus, it appears that a relationship could be observed between lower surface adherence on such polymer blends and cell proliferation. Results are more obvious at 72 hours, cell proliferation producing wound closure at this

incubation time for the simple blend. At the same time, on the phosphorylated polymers surface, cell proliferation was somehow poor, with reduced cell density and lack of real confluence. The simulated wound was not closed completely at 72 hours for the phosphorylated chitosan-collagen blend (figure 2). While visual results and wound healing measurements were reproducible but gave no indication related to cell viability, an MTT test was performed in a second 96-well plate, incubated at the same time with that submitted to simulated scratch test.

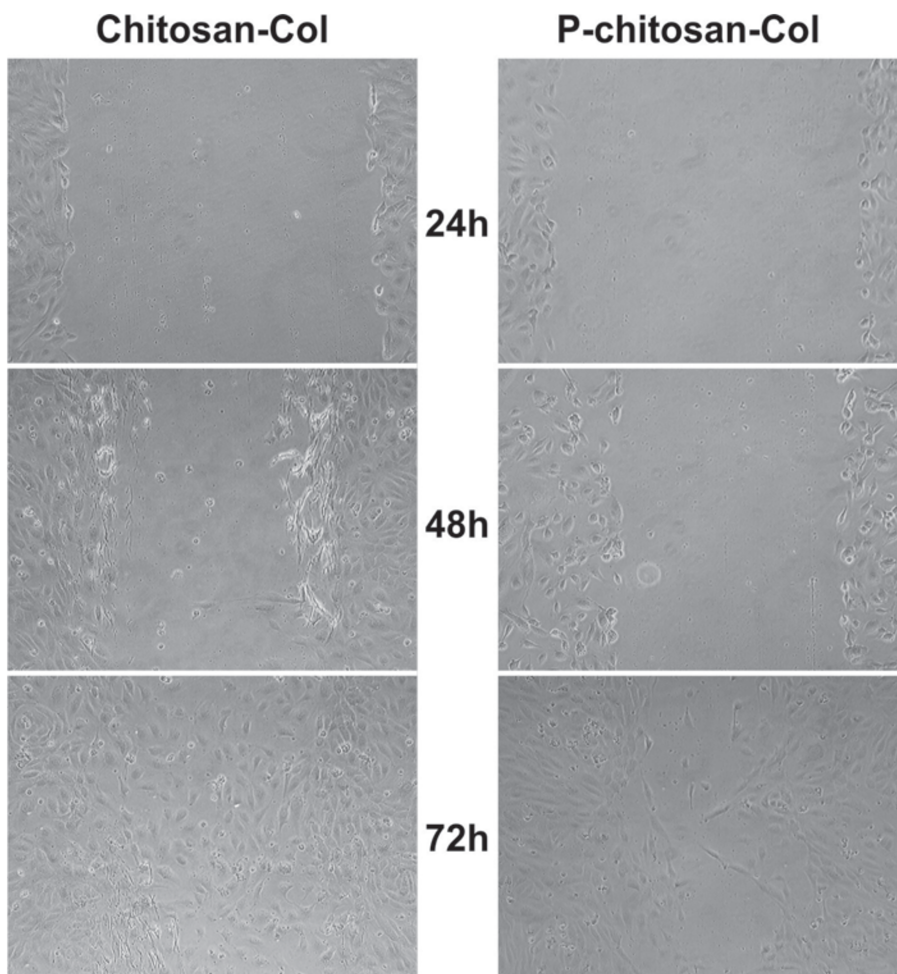


Figure 2. Scratch test using silicon separator on 2 chitosan membranes (left – chitosan-collagen and right phosphorylated chitosan-collagen)

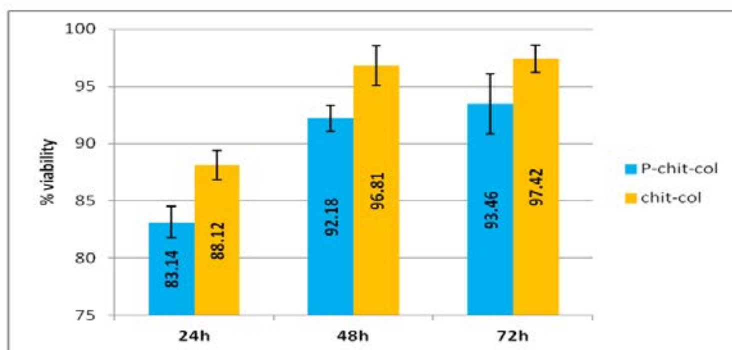


Figure 3. HOS cell viability at different times following dispersion on chitosan and chitosan-phosphate-collagen blend membranes. Error bars – 95% confidence interval.

At 24 hours, viability was over 80% for cells proliferating on both membranes (figure 3). Cell viability improves at 48 an 72 hours but the difference between simple chitosan-collagen and phosphorylated chitosan-collagen membranes are still visible. We may interpret this result as a consequence of lower adherence of the phosphorylated polymer membrane on cell viability and proliferation rate. However, cell viability was good overall, and in order to appreciate polymer surface effects on cell behavior, one should explore integrin expression or other adhesion proteins presence at cell-polymer interface and also caspase activity in proliferating cells.

CONCLUSIONS

Cell migration on polymer surfaces can be successfully evaluated by improved scratch test performed directly on polymer pellicles. Customized scratch test on polymer surface with no pellicle/membrane impairment is a cheap and feasible procedure to evaluate cell behavior on sensitive interfaces (as cell polymer one). Simulated scratch test allows the evaluation of cell adhesion, proliferation and survival at various polymer interfaces. Considering that cell viability remained very good following this assay, we may also recommend it as a preliminary test regarding adhesion proteins expression on various polymer surfaces following scratch assay. Also cells from this test may be further fixed and stained or submitted to DNA/RNA extraction for molecular analysis of adhesion protein expression or caspase activity (flow cytometry).

REFERENCES

Bhattacharyya, D., Xu, H., Deshmukh, R. R., Timmons, R. B., & Nguyen, K. T. (2010): *Surface chemistry and polymer film thickness effects on endothelial cell adhesion and proliferation*. J Biomed Mater Res A, 94(2), 640-648.

Choi, J., Konno, T., Takai, M., & Ishihara, K. (2012): *Regulation of cell proliferation by multi-layered phospholipid polymer hydrogel coatings through controlled release of paclitaxel*. Biomaterials, 33(3), 954-961.

Francesko, A., & Tzanov, T. (2011): *Chitin, chitosan and derivatives for wound healing and tissue engineering*. Adv Biochem Eng Biotechnol, 125, 1-27.

Guidoin, M. F., Guidoin, R., Frayssinet, P., Legrand, A. P., & How, T. (2005): *Poly-L-lactide surfaces subjected to long-term cell cultures: cell proliferation and polymer degradation*. Artif Cells Blood Substit Immobil Biotechnol, 33(4), 411-422.

Jayakumar, R., Prabaharan, M., Sudheesh Kumar, P. T., Nair, S. V., & Tamura, H. (2011): *Biomaterials based on chitin and chitosan in wound dressing applications*. Biotechnol Adv, 29(3), 322-337.

Jayakumar, R., Selvamurugan, N., Nair, S. V., Tokura, S., & Tamura, H. (2008): *Preparative methods of phosphorylated chitin and chitosan--an overview*. Int J Biol Macromol, 43(3), 221-225.
Mir, V. G., Heinamaki, J., Antikainen, O., Revoredo, O. B., Colarte, A. I., Nieto, O. M., & Yliruusi, J. (2008): *Direct compression properties of chitin and chitosan*. Eur J Pharm Biopharm, 69(3), 964-968.
Mosmann, T. (1983): *Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays*. J Immunol Methods, 65(1-2), 55-63.
Wang, K., & Liu, Q. (2014): *Chemical structure analyses of phosphorylated chitosan*. Carbohydr Res, 386, 48-56.
Mosmann, T. (1983): *Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays*. J Immunol Methods 65(1-2): 55-63.
Wang, K. and Q. Liu (2014): *Chemical structure analyses of phosphorylated chitosan*. Carbohydr Res 386: 48-56.

Institutional affiliation for all authors
DEPARTMENT OF CELL AND MOLECULAR BIOLOGY
FACULTY OF MEDICINE, „GR.T.POPA” UNIVERSITY OF MEDICINE AND PHARMACY, IAȘI, ROMANIA

***Corresponding author:**
Tudor Petreus, MD, PhD
Department of Cell and Molecular Biology
Faculty of Medicine, „Gr.T.Popa” University of Medicine and Pharmacy, Iasi, Romania
16, Universitatii str., 700115, Iasi, Romania
biotudor@gmail.com

COMPLICATIONS OF PREMATURITY AS RISK FACTORS FOR OUTCOME

ANCA ROXANA BIVOLEANU[1,2*], EDUARD CRAUCIUC[2], OVIDIU TOMA[3], DRAGOŞ CRAUCIUC[2], MIHAELA MOSCALU[2], MARIA STAMATIN[2]

Keywords: prematurity, follow-up, neurodevelopmental

Abstract. The increased rate of prematurity has been accompanied by high rates of neonatal complications and neurodevelopment sequels. Objective: The study was design to evaluate whether identifying certain complications of prematurity may be useful in predicting neurodevelopment outcome.
Observational longitudinal study, over six years, of a cohort of premature infants. The risk factors quantified were: gestational age, birth weight, birth asphyxia, necessity of mechanical ventilation , specific pathology (respiratory distress syndrome, intraventricular hemorrhage, periventricular leukomalacia) ,results of Amiel –Tison, neurologic exam at discharge, total numbers of evaluations, final results of evaluations, acording to Bayley Scales of Infant Development, 2nd Edition at two years corrected age. The data were analyzed using SPSS Statistics version 18.0. From January 1, 2005 to December 31, 2011 the incidence of prematurity in our maternity was 13.65%. 33.03% (n=2182) were prematures and from these, 1845 were enrolled in this study. Complications of prematurity, developped by included patients were : respiratory distress syndrome (70.51%), apneea of prematurity (40.76%), perinatal asphyxia (22.82%), intraventricular hemorrhage (17.01%), periventricular leukomalacia (5.42%). Birth weight less than 1500g, type of mechanical ventilation , intraventricular hemorrhage, results of Amiel-Tison evaluations and adherence of patients to program are predictive factors for results of final evaluation. Gestational age remains the leading factor in including premature infants in a specific group of risk ($F=69.65$, $p<<0.01$, 95%CI). We speculate that early identification of the degree of risk may facilitate early interventions with the potential to improve the neurological outcome of these patients.

INTRODUCTION

Advances in antenatal medicine and neonatal intensive care, have resulted in improved survival rates of preterm infants (Fanaroff AA et al, 2003; Hintz SR et al, 2005). Prematurity is defined as a birth that occurs before 37 completed weeks (259 days or less) of gestation (AAP, 2004; AAP and ACOG 2002; Berg AT, 1991). Internationally, the rate of preterm birth ranges from 5% to 18% of babies born, with a dramatic survival gap for prematures, depending on where they are born (Howson CP et al, 2012; Beck S et al, 2010). In Romania the rate of preterm birth varies from 8- 12.5%. For our maternity, preterm delivery increases from 6.19% in 2000, to 12.90% in 2013, due to advances technologies in this field. Similar to mortality, neonatal morbidity is inversely related to gestational age. The most immature infants commonly suffer from multiple and interacting medical conditions, which may lead to permanent impairments.Because among the generation of survivors of preterm birth, cognitive, behavioral, neurological sequels are not uncommon (Laroque B et al, 2008; Hack M, 2009; Bhutta AT et al, 2002), were organized neonatal follow-up services with an important role in early identification of neuro-developmental problems (Hack M, 2012). The purpose of present study is to evaluate degree of risk for neurologic sequels in a cohort of premature infants included in follow-up program.

MATERIALS AND METHODS

Observational longitudinal study conducted from January 1, 2005 to December 31, 2011 at Neonatal Intensive Care Unit, Cuza-Voda Maternity Hospital, Iasi. Inclusion criteria were in accordance with our national guide for follow-up of high-risk neonates (Mâtu E, 2010). Infants with major congenital anomalies and infants needing major surgery were excluded. Perinatal data were collected prospectively during admission, stored in the NICU database, and retrospectively retrieved for data analysis. Parental informed consent was obtained for participation in the follow-up program. The neonatal neurological examination was performed at discharge, as described by Amiel-Tison (Amiel-Tison C, 2001) and the Bayley Scales of Infant Development, 2nd Edition (BSID II) (Bayley N, 1993; Bayley N, 2006) during regular evaluations. The BSID II includes 2 subscales: a motor scale and a mental scale, with 11-13 items based on age level. The total number of items failed, places the infant in a category of low, moderate or severe risk for developmental delay.
In order to examine correlation between risk factors /complications presented by premature, frequency of evaluations and degree of neurodevelopmental risk, we selected next parameters:

- gestational age (GA) who plays the most important rol in development of complications of prematurity (Wood NS et al, 2003; Wilson-Costello D et al, 2007); birth weight (BW) (Vohr BR et al, 2004); sex, as risk factor for severity of hyaline membrane disease (HMD) (Greenough A et al, 2005; West JB, 2008; Gomella TL, 2009); Apgar score; birth

asphyxia; necessity of mechanical ventilation (Cools F et al, 2009; Greenough A et al, 2008; Göpel W et al, 2011); specific prematurity pathology (respiratory distress syndrome, intraventricular hemorrhage, periventricular leukomalacia) (Volpe JJ, 2008; de Vries LS et al 2009; Greisen G et al, 2001; Khashu M et al, 2009); Amiel –Tison neurologic exam at discharge (Amiel-Tison C, 2001); results of neurologic examinations during follow-up program; total numbers of evaluations; final results of evaluations, at two years corrected age.(Barrington KJ et al, 2006; Kirkegaard I et al, 2006; Petrini J et al, 2009).

The data were analyzed using the SPSS version 18.0. Correlations between variables were investigated using the Pearson product moment correlation coefficient (r), contingency coefficient, or by Spearman Rank correlation coefficient, as appropriate. The mean value differences were analyzed with Kruskal Wallis Test and the qualitative differences of variables were tested with Chi-2 test with nominal significance defined as $p<0.05$.

RESULTS AND DISCUSSION

Over the 6-year period of study, 45887 infants were admitted in our hospital. 13, 65% (n=6278) were premature and 29, 39% (n=1845) were introduced in the follow-up program and so were enrolled in this study. GA, varied between 23 weeks of gestation (wg) to 36 wg with mean GA of 30wg (Fig. 1).

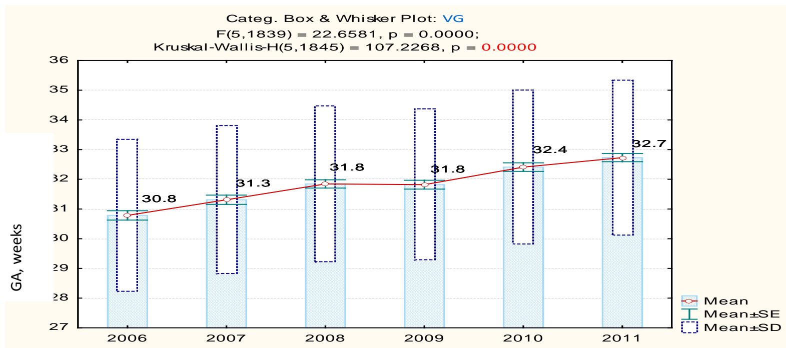


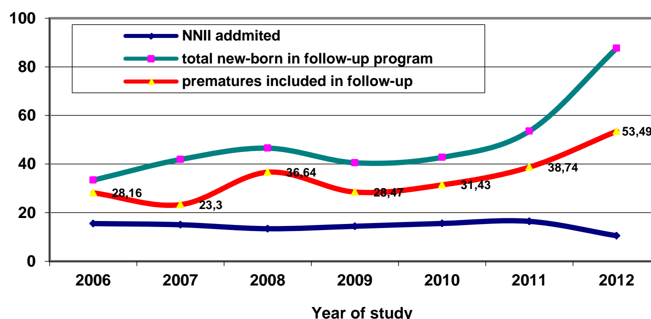
Fig. 1 Statistical analysis of gestational age

We noticed a constant predominance of male sex, over the studied period.

The mean birth weight was 1350 g (birth weight varies from 550g to 4550g). The mean Apgar score at one minute was 5 and 6 and at 5 minutes 6 and 7.

Complications of prematurity, developped by included patients and quantified as risk factors for outcome were : respiratory distress syndrome (70.51%), apneea of prematurity (40.76%), perinatal asphyxia(22.82%), intraventricular hemorrhage (17.01%), periventricular leukomalacia (5.42%), retinophaty of prematurity (25.47%).

Over the 6 year period of study, 6606 newborn were addmited in neonatal intensive care unit (NICU) and 47.26% (n=3122) were enrolled in the follow-up program. Among these, 33,03% (n=2182) were prematures with risk and from these, 1845 were enrolled in this study. We notice a constant increase in number of high risk prematures, included in follow-up, as shown in figure 2.



. **Fig. 2 Anual incidence of prematures included in follow-up program**

All infants were evaluated by the Amiel-Tison neurologic evaluation at discharge from hospital and 23.6% were found with low risk, 60.1% moderate risk and 16.3% severe risk, as presented in Table I.

Table I. Distribution of risk categories based on discharge evaluation

Discharge evaluation Risk category	n	%
Low risk	435	23.58%
Moderate risk	1109	60.11%
Severe risk	301	16.31%
	1845	

Infants included in low risk category had GA of 33 wg and over and birth weight 1800 g and over; medium risk had 32 wg or less and a medium birth weight 1600g and severe risk, 30 wg or less and birth weight 1260 g and less. Severe risk was inversely proportional with gerstational age, demonstrate by GA analysis and discharge evaluation , (F=69.65, p<<0.01, 95%CI) and with birth weight (F=50.94, p<<0.01, 95%CI).
Patients with Apgar score less than 5 at one minute and 6 at 5 minutes was in a larger percent categorized in severe risk group.
19.71% from infants with neonatal asphyxia were included at discharge in severe risk group and 50.59% in medium risk, aspect which emphises the corelation between asphyxia and degree of risk of newborn (χ^2=56.22, p<<0.01, r=0.65, p<<0.01, 95%CI).
Newborns ventilated by intermittent positive pressure ventilation (IPPV) associate severe risk in a percent of 33.3% versus 12.98% ventilated by continous positive pressure ventilation (CPAP).There is a significant corelation between type of mechanical ventilation and Amiel Tison exam at discharge (χ^2=249.03, p<<0.01, r=0.28, p<<0.01, 95%CI).
From infants with apneea of prematurity, 60.64% were included in medium risk group and 22.56% in severe risk.
All newborns with intraventricular hemorrhage (IVH) stage III, presented severe risk according to Amiel-Tison exam and moderate risk in 72% of cases. 73% of cases with periventricular leukomalacia (PVL) were categorized in severe risk (Fig. 3 and Fig. 4).

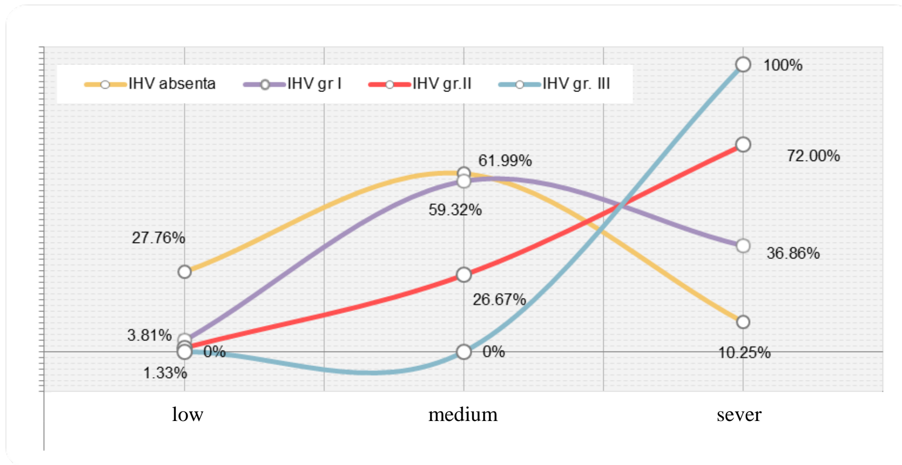


Fig. 3 Amiel Tison at discharge- risk category vs IVH

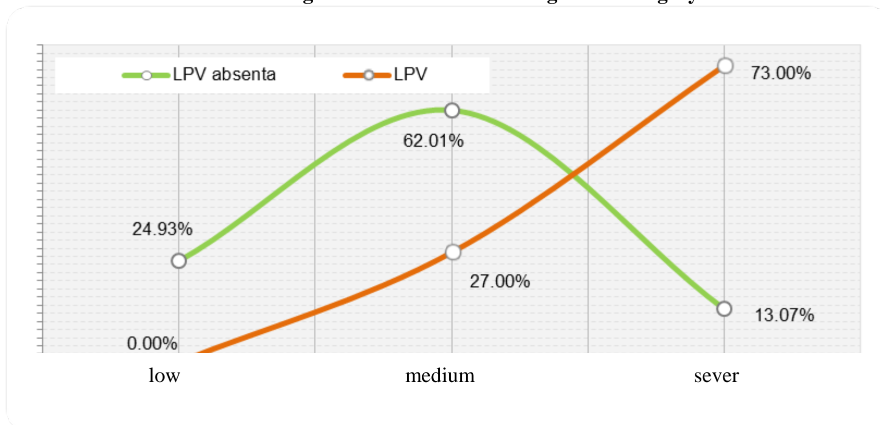


Fig. 4 Amiel Tison at discharge- risk category vs LPV

Mothers were informed about the significance and importance of the follow-up program and were asked to return for periodic evaluations according to a personalized schedule.
Statistic correlation from this study, demonstrate that infants who benefit of regular evaluations, reaches the highest percent with low risk, compare with those with only one evaluation.
According to the BSID II evaluation, at the end of the follow-up program, 11.03% were included in the severe risk group, 47.75% in the medium-risk group and 41.42% were considered to have a low risk of developing subsequent neurologic disabilities.
The results at two years corrected age were summarized into three domains: cognitive development, neuromotor development and both, cognitive and motor, according to main affected domain.
Motor deficit was predominant in 16.04% of cases, cognitive development was impaired in 12.68% of cases and mixt deficit was in 12.68% of cases. In 58.54% of cases there were no significant disabilities.
The results of evaluation at discharge were significant associated with final evaluation at the end of follow-up program (χ^2=1553.1, p<<0.01, r=0.65, p<<0.01, 95%CI).

This study reveal the presence of a significant correlation between Amiel-Tison examination at discharge and the results of dynamic neurologic evaluations, meaning that newborns with severe risk at discharge evaluates to medium risk through six month corrected age, meanwhile number of cases with severe risk, decrease in dynamic. The incidence of infants with low risk increase over the studied period and those with moderate risk slightly decrease, as shown below.

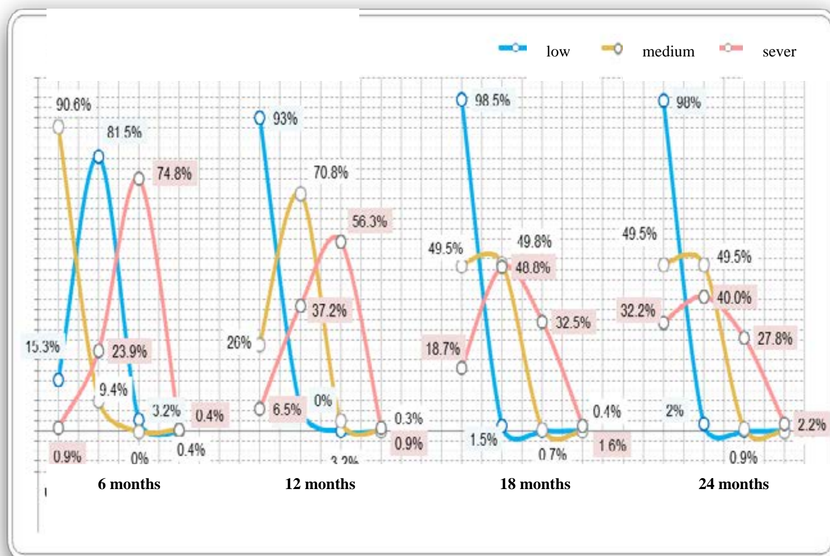


Fig. 5 Risk evaluation over follow-up period

From risk factors and complications of prematurity, we selected the following parameters in order to establish power of prediction: GA, Apgar score at one and five minutes, birth weight < 1500g, birth asphyxia, type of mechanical ventilation, apnea of prematurity, IVH, LPV, results of Amiel-Tison evaluation and number of total evaluations (Table II, Fig. 6).

Table II. Multiple correlations of predictive factors for final evaluation (BSID II)

Multiple correlations	Estimate values
r	0.45622
R^2	0.20813
F	0.20291
p	39.84229
Std.Err. of Estimate	0.00000

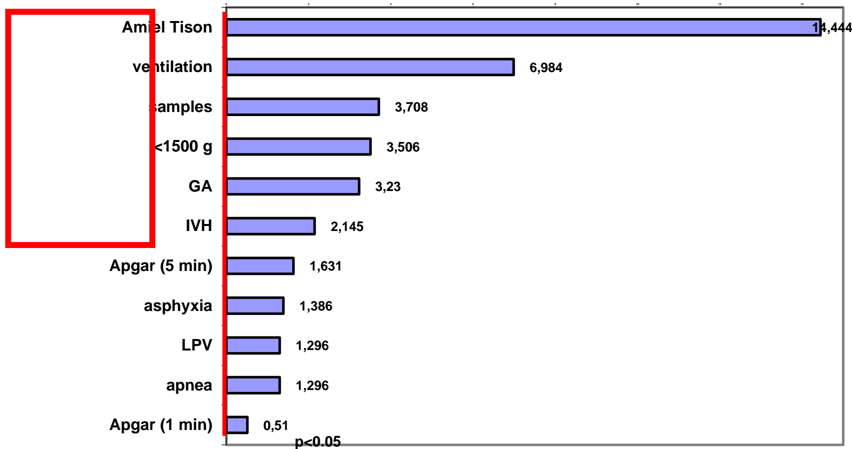


Fig. 6 Predictors in evaluation of BSID II

We found that birth weight less than 1500g, type of mechanical ventilation, intraventricular hemorrhage, results of Amiel-Tison evaluations and number of evaluations (adherence of patients to program) are predictive factors for results of final evaluation and for infant's outcome.
A reliable follow-up program depends on maintaining a high rate of participation which is strength conditioned by educational and socio-economic parental status.
As it reveals by many literature data (Aarnoudse-Moens CSH, et al, 2009; Johnson YR, 20111), we found a strong correlation between low gestational age and severe risk for neurologic sequels ($F=69.65$, $p<<0.01$, 95%CI)
Because high vulnerability (Volpe JJ, 2008; Volpe JJ, 2009) of premature brain, intraventricular hemorrhage is one of decisive complications of prematurity in increase the risk for adverse neurologic outcome ($p<0.05$).
Patients with LPV were initial categorized in high risk group because LPV is an ischemic white matter injury , frequently bilateral (Volpe JJ, 2008; Weindling M, 2010).
From both category of patients with IVH and LPV, those who benefit of early kinesiotherapy performed by a specialist, but also by instructed mothers, passes from severe to moderate risk.
The highest proportion of severe and moderate risk was reach at discharge and at three month corrected age mostly explained by the interest and orientation of mothers to "basic needs": feed and sleep. After infants' growth, parents seem to realize the importance of neurologic acquisitions and became more involved in stimulate motor and cognitive evolution of their infants.

CONCLUSIONS

Gestational age remains the leading factor in including premature infants in a specific group of risk ($F=69.65$, $p<<0.01$, 95%CI)
Birth weight under 1500g, type of mechanical ventilation, Amiel-Tison evaluation, intraventricular hemorrhage, number of evaluations (adherence to follow-up program) are predictive factors for the results of final evaluation.
Unfortunately, the cognitive and behavioral outcomes remain unpredictable, so all possible measures must be implemented so that premature infants can benefit from high-quality medical and family assistance.

REFERENCES

Aarnoudse-Moens CSH, Weisglas-Kuperus N, van Goudoever JB, MD, Oosterlaan J (2009). *Meta-analysis of neurobehavioral outcomes in very preterm and/or very low birth weight children*. Pediatrics; 124: 717-728

American Academy of Paediatrics and the American College of Obstetricians and Gynaecologists (2002). *Guidelines for Perinatal Care*. 5th Ed Elk Grove Village and Washington DC, AAP and ACOG; 199-201, 378-379.

American Academy of Paediatrics, Committee on Fetus and Newborn: Age Terminology during the Perinatal Period (2004). Policy Statement. Organizational Principles to Guide and Define the Child Health Care System and/or Improve the Health of All Children. Pediatrics; 114(5): 1362-1364.

Amiel-Tison C (2001). *Clinical assessment of the infant nervous system.* In: Levene MI, Chervenak FA, Whittle M, editors. Fetal and Neonatal Neurology and Neurosurgery. London: Churchill Livingstone; 99–120.

Barrington KJ, Saigal S (2006). *Long-term caring for neonates*. Paediatr Child Health; 11(5):265–6.

Bayley N (1993). *Scales of Infant Development*. 2nd ed. San Antonio, TX: Psychological Corporation, Harcourt Brace and Company.

Bayley N (2006). *Scales of Infant and Toddler Development*. 3rd Ed Technical Manual. Hartcourt Assessment Inc San Antonio Texas SUA.

Beck S, Wojdyla D, Say L, et al (2010). *The worldwide incidence of preterm birth: a systematic review of mortality and morbidity*. Bulletin of the World Health Organization; 88(1):31–8.

Berg AT (1991). *Menstrual cycle length and calculation of gestational age*. Am J Epidemiol, 1991; 133: 585-589.

Bhutta AT, Cleves MA, Casey PH, Craddock MM, Anand KJS (2002). *Cognitive and behavioural outcomes of school-age children who were born preterm: a meta-analysis*. J Am Med Assoc; 288: 728–37.

Cools F, Henderson-Smart DJ, Offringa M, Askie LM (2009). *Elective high frequency oscillatory ventilation versus conventional ventilation for acute pulmonary dysfunction in preterm infants*. Cochrane Database of Systematic Reviews 2009, Issue 3. Art. No.: CD000104. DOI: 10.1002/ 14651858.CD000104.pub3

de VRies LS, Counsell SJ, Levene MI (2009). *Cerebral ischemic lesions*, in Levene MI, Chervenak FA: Fetal and Neonatal Neurology and Neurosurgery. 4th Ed Churchill Livingstone Elsevier 2009; 431-437.

Fanaroff AA, Hack M, Walsh MC (2003). *The NICHD neonatal research network: Changes in practice and outcomes during the first 15 years*. Semin Perinatol; 27(4):281–7.

Gomella TL (2009). *Hyaline Membrane Disease (Respiratory Distress Syndrome) in Gomella TL Neonatology: Management, Procedures, On-call Problems, Disease, and Drugs.* Lange. 6th Ed Lange Clinical Science 2009; 477-481

Göpel W, Kribs A, Ziegler A, et al (2011). On *behalf of the German Neonatal Network. Avoidance of mechanical ventilation by surfactant treatment of spontaneously breathing preterm infants: an open-label, randomized, controlled trial.* Lancet; published online Sept 30. DOI: 10.1016/ S0140-6736(11)60986-0.

Greenough A, Dimitriou G, Prendergast M, Milner AD (2008). *Synchronized mechanical ventilation for respiratory support in newborn infants.* Cochrane Database of Systematic Reviews, Issue 1. Art. No.: CD000456. DOI: 10.1002/14651858.CD000456.pub3

Greenough A, Limb E, Marston L, Marlow N, Calvert S, Peacock J (2005). *Risk factors for respiratory morbidity in infancy following very premature birth*. Arch Dis Child; 90: F320–F323.

Greisen G, Vannucci RC (2001). *Is periventricular leucomalacia a result of hypoxic-ischaemic injury? Hypocapnia and the preterm brain.* Biol Neonate. 79(3-4):194-200

Hack M (2009). *Adult outcomes of preterm children*. J Dev. Behav Pediatr; 30:460–70

Hack M, Costello DW (2012). *Follow-up Outcomes of High Risk Infants* in Buonocore et al. (Eds). Neonatology.A practical Approach to Neonatal Diseases, © Spinger Verlag pg.122-130.

Hintz SR, Poole WK, Wright LL, et al. (2005). *Changes in mortality and morbidities among infants born at less than 25 weeks during the post-surfactant era*. Arch Dis Child Fetal Neonatal Ed; 90(2):F128–33

Howson CP, Kinney, M.V., Howson, C.P, McDougall, L., Lawn JE. (2012). Executive Summary for *Born Too Soon*: *The Global Action Rapport on Preterm Birth*. March of Dimes, PMNCH, Save the Children, World Health Organization.

Johnson YR (2011). *Long-term neurodevelopmental outcome of premature infants*. http://www.uptodate. com/home/index.html.

Khashu M, Narayanan M, Bhargava S, Osiovich H (2009). Perinatal *outcomes associated with preterm birth at 33 to 36 weeks' gestation: a population-based cohort study*. Pediatrics. 123(1):109

Kirkegaard I, Obel C, Hedegaard M, Henriksen TB (2006). *Gestational age and birth weight in relation to school performance of 10-year-old children: a follow-up study of children born after 32 completed weeks*. Pediatrics; 118:1600–1606.

Laroque B, Ancel P-Y, Marret S et al (2008). *Neurodevelopmental disabilities and special care of 5-year-old children born before 33 weeks of gestation (the EPIPAGE study): a longitudinal cohort study.* Lancet; 371:813−20

Mâtu E (2010). *Urmărirea nou-născutului cu risc pentru sechele neurologice si de dezvoltare.* Colectia ghiduri clinice pentru neonatologie. Ghidul 13/revizia 03.12.2010. Ghiduri clinice- Ministerul Sanatatii http://www.ms.ro

Petrini J, Dias T, McCormick M, Massolo M, Green N, Escobar GJ (2009). *Increased risk of adverse neurological development for late preterm infants*. J Pediatr, 154:169-176
Vohr BR, Wright LL, Dusick AM, et al (2004). *Center differences and outcomes of extremely low birth weight infants.* Pediatrics; 113 :781 –789
Volpe JJ (2008). *Intracranial hemorrhage: Germinal matrix-intraventricular hemorrhage of the premature infant.* In: Neurology of the Newborn. 5th ed. Philadelphia: WB Saunders;:517–584
Volpe JJ (2008). *Intrinsec vulnerability of cerebral white matter injury of premature newborn*. In: Neurology of the Newborn. 5th ed. Philadelphia: WB Saunders; 377-383.
Volpe JJ (2009). *Intraventricular hemorrhage in the premature infant—current concepts*. Part I. Ann Neurol 25:3–11
Weindling M (2010). *Clinical aspect of brain injury in the preterm infant.* The Newborn brain: Neuroscience and Clinical Aplication, 2edn., eds Hugo Lagercrantz, M.A Hanson, Cambridge University Press;301-303.
West JB (2008). *Respiratory physiology: the essentials,* 8th edn. Wolters Kluwer/Lippincott. Williams & Wilkins, Baltimore
Wilson-Costello D, Friedman H, Minich N, et al (2007). *Improved neurodevelopmental outcomes for extremely low birth weight infants in 2000–2002*. Pediatrics; 119(1):37–45.
Wood NS, Costello K, Gibson AT, Hennessy EM, Marlow N, Wilkinson AR, (2003) The EPI Cure study: *growth and associated problems in children born at 25 weeks of gestational age or less.* Arch Dis Child Fetal Neonatal Ed. 2003; 88:F492-500.

[1]Neonatal Intensive Care Unit, Clinical Hospital of Obstetrics and Gynaecology, ”Cuza-Voda”, Iasi, Romania
[2] University of Medicine and Pharmacy”Gr. T. Popa”, Iasi, Romania
[3] ”Alexandru Ioan Cuza” University, Iasi, Romania

*a.bivoleanu@yahoo.com

Acknowledgments/ The authors wish to thank the many families involved in the care of premature infants and would like to acknowledge their efforts to insure the best quality of life for their infants.

DETERMINATION OF THE POLYPHENOL OXIDASE ACTIVITY IN RELATIONSHIP TO TOTAL PHENOLIC AND ANTHOCYANIN CONTENT OF SOME ROMANIAN VINE VARIETIES (*VITIS VINIFERA* L.) FOR TABLE GRAPES

RĂZVAN V. FILIMON[1,*], ROXANA FILIMON[1], LILIANA ROTARU[1]

Keywords: polyphenol oxidase, phenolic compounds, anthocyanins, table grapes varieties, *Vitis vinifera* L.
Abstract. The undesirable change in the grape colour, flavour and texture is associated with the enzymes polyphenol oxidase. Therefore, it is important to control their effect, as well as to establish their characteristics associated to the fruits. Grapes (skin, pulp and seeds) of ten Romanian vine varieties for table grapes (Splendid, Cetăţuia, Milcov, Transilvania, Someşan, Napoca, Gelu, Coarnă neagră selecţionată, Purpuriu and Radames) which are grown in the Ampelographic colection of University of Agricultural Sciences and Veterinary Medicine Iaşi, Romania, were analyzed for determination of polyphenol oxidase activities, total phenolics (flavonoids and non-flavonoids) and total monomeric anthocyanin content. Total phenolic content was higher in seeds, ranging from 4.36 to 5.35 g gallic acid equivalent/100 g fresh weight, of which flavonoids were between 65 and 88%. The highest polyphenol oxidase activity was determined in the grape extract of Radames variety (7.11 U/g/min), while total anthocyanin content was the most important in Napoca variety grape skins (343.86 mg cyanidin-3-glucoside equivalent/100 g fresh weight). There were found strong negative correlations between polyphenol oxidase activity (PA) and anthocyanin content (R^2=0.8854) as well as between PA and total phenolic content of grape skins (R^2=0.8586) and pulp (R^2=0.8831), emphasizing the destructive effect of the enzyme on these chemical classes of compounds.

INTRODUCTION

Polyphenol oxidase (PPO) also known as o-difenoloxidaza, catechol oxidase or tyrosinase (EC 1.10.3.1.), plays an important role in respiration, catalyzing the aerobic oxidation of polyphenols and their derivatives, to produce the corresponding quinones (Rocha and De Morais, 2005; Artenie *et al*., 2008). The purified enzyme had both cresolase and catecholase activities. Catecholase activity had a pH optimum in a range 3.5–4.5 and was characterized by a relatively high stability to heat. Cresolase activity presents a lag period which is modulated by different factors (enzyme concentration, substrate concentration, temperature or pH). The presence of o-diphenols (phenol molecules containing two hydroxyl substituents) cease the lag period, these acting as co-substrates (Valero *et al*., 1988). Belonging to the class of oxidoreductases, PPO is responsible for undesirable change in the grape colour (enzymatic darkening), flavour and texture. In damaged berries an unsavoury flavour and loss of the skin colour can be developed, which will interfere in the quality of the final product (De Pieri Troiani *et al*., 2003). Although peroxidase and polyphenol oxidase are considered to be involved in the oxidative mechanism of the grape juice, the PPO is the main responsible for that oxidation (Yokotsuka *et al*., 1991). On the other hand, the action of the PPO can be considered beneficial because it is responsible for the higher resistance against attacks performed by pathogens (once quinones formed are highly toxic), thereby allowing them to reduce the action of invading microorganisms (Alvarenga *et al*., 2011). Considering all these aspects PPO has attracted much attention to the researchers becoming a complex scientific and technological issue. Regarding the dynamic of this enzyme activity should be mentioned that all phenol oxidases have the maximum activity at the beginning of grape maturation followed by a decrease, in parallel to the increase in the content of phenolic substances and the accumulation of anthocyanins (Beceanu *et al*., 2011).

Polyphenols are secondary plant metabolites (Teissedre and Chervin, 2011), generally involved in the defense against ultraviolet radiation or aggression by pathogens in plants (Pandey and Rizvi, 2009). In food, polyphenols may contribute to the bitterness, astringency, colour, flavour, odour and oxidative stability (Vermerris and Nicholson, 2006). Recent years epidemiological studies strongly suggested that long term consumption of diets rich in plant polyphenols offered some protection against development of cancers, cardiovascular diseases, diabetes, osteoporosis and neurodegenerative diseases (Graf *et al*., 2005). The phenolic compounds occurring in grapes include flavonoids, in particular flavan-3-ols (catechins and procyanidins), anthocyanins and flavonols as well as nonflavonoid compounds such as hydroxycinnamic and hydroxybenzoic acids (Ribéreau-Gayon *et al*., 2006a).

Belonging to the class of phenolic compounds (flavonoid subclass) (Bąkowska-Barczak, 2005), anthocyanins (gr. *anthos* – flower, *kyanos* – blue) are generally accepted as the largest and most important group of water-soluble pigments in nature (Horbowicz *et al*., 2008). The anthocyanins are responsible for the red-blue shades of grapes, being located mainly in the skin and, more unusually, in the pulp („teinturier” grape) (Davies, 2004). Their flavylium cation

structure includes two benzene rings bonded by an unsaturated cationic oxygenated heterocycle, derived from the 2-phenyl-benzopyrylium nucleus (Ribéreau-Gayon *et al*., 2006b; Daayf and Lattanzio, 2008).

Frequently, negative correlations were found between PPO activity and total phenolic and anthocyanin content (Orak, 2007), therefore it is important to control enzyme effect, as well as to establish their characteristics in relation to the grapes. The purpose of this paper was to analyse the polyphenol oxidase activities, total phenolics (flavonoids and non-flavonoids) and total anthocyanin content of ten Romanian *Vitis vinifera* L. varieties for table grapes, and to establish relationships between referred factors.

MATERIALS AND METHODS

The research has been carried out on the grapes (skin, pulp and seeds) of ten *Vitis vinifera* L. indigenous table grape varieties (Gelu, Milcov, Cetăţuia, Napoca, Someşan, Splendid, Transilvania, Coarnă neagră selecţionată, Purpuriu and Radames), which are grown in the Ampelographic Collection of the University of Agricultural Sciences and Veterinary Medicine Iasi, Romania, at grape maturity of consumption. Grapevines genotype were 25 years old, grafted on Kober 5 BB (Berlandieri × Riparia). Planting distances were 2.2 m (between rows)/1.2 m (between plants), half-high training system, bilateral cordon, with pruning in fructification rings providing an average load of 40–45 buds/vine. Soil maintenance was “black field” and technological operations were specific to industrial vineyard ecosystem.

Upon harvest, whole grapes were immediately frozen at –80 °C. Frozen berries were separated (skin, pulp and seeds) (5 g each) and ground separately in a mortar using sieved inert sand as a grinding aid, and extracted in the dark by stirring with 50 mL of 0.1% HCl (v/v) in methanol overnight at room temperature. The samples were filtered and the solid residue washed with an additional 50 mL of 0.1% HCl (v/v) in methanol. Filtrates were combined resulting in a final ratio of 1:20 (g/mL). The resulting solutions were kept at –20 °C until analysis (Gambuti *et al*., 2009).

The Folin–Ciocâlteau method (Singleton and Rossi, 1965) was conducted for the colorimetric estimation of total polyphenols, measuring the absorbance at 750 nm (UV-vis Shimadzu 1700 Pharmaspec Spectrophotometer). A standard curve using different concentrations of gallic acid solutions (R^2=0.991) was done to report the results as grams gallic acid equivalent (GAE) per 100 g fresh weight (f.w.).

Flavonoids were precipitated by formaldehyde at pH<0.8. Five milliliters of a 32% HCl in distilled water (50/50 v/v) and 5 mL of formaldehyde (8 mg/L, in distilled water) were added to 10 mL of each extract. The mixture was vortexed, then left 24 h at room temperature. Its absorbance was measured in the same way as for the total phenolics. The flavonoid content as percentage of dry matter is (X-Y)%, where X is the total phenolic content and Y the non-flavonoid phenolic content as calculated (Tibiri *et al*., 2010).

Total monomeric anthocyanin determination was carried out through the pH differential method (Lee *et al*., 2005). The coloured oxonium form exists at pH 1.0, and the colorless hemiketal form predominates at pH 4.5. The difference in the absorbance at 520 nm is proportional to the pigment concentration. Although the main anthocyanin in grapes is malvidin-3-glucoside, results were calculated as cyanidin-3-glucoside equivalents (CE), based on molecular weight (449.2 g/mol) and molar extinction coefficient, in order to be able to compare data with other species.

The polyphenol oxidase was assayed by spectrophotometry (420 nm), using catechol (Merck Romania) as a substrate according to Ermakov (1987), and reporting results as enzymatic units (U) per gram of f.w. and per minute (U/g/min). An enzymatic unit (U) represents the amount of enzyme that catalyzes the conversion of one micromole of catechol in one minute, at 25°C.

Relative humidity and total dry matter (OIV-MA-AS2-03A), total mineral content (OIV-MA-AS2-04), titratable acidity (OIV-MA-AS313-01) and evaluation by refractometry of the sugar concentration in grape (OIV-MA-AS2-02) were conducted according to the OIV (International Organisation of Vine and Wine) Compendium of international methods of analysis (2012).

Data have mentioned the standard deviation (± SD), and represent the average of three independent analyses. The method used to discriminate among the means was Fischer’s least significant difference procedure at 95% confidence level. P values lower than 0.05 ($p \leq 0.05$) were considered to be significant. Simple regression analysis was performed to look for relationships between data.

RESULTS AND DISCUSSIONS

Determination of moisture and total dry matter is essential in vegetal tissue analysis, high proportion of humidity causing a poor stability of samples, favouring microbiological and enzymatic activity, and hydrolysis reactions (Maltini *et al*., 2003; Beceanu *et al*., 2011). Grapes at full maturity have a moisture content of about 70-85% (Fregoni, 1998; Keller, 2010) and a

total mineral content of about 0.22-0.54% (Creasy and Creasy, 2009; Beceanu, 2010). Moisture, total dry matter, minerals, sugar and acidity of Romanian varieties grape berries at technological maturity were shown in table 1. Sugar content varied between 171.99 g/L and 235.85 g/L, with a mean of 188.58±19.29 g/L, amid of a titratable acidity of 6.25±0.93 g/L tartaric acid (t.a.) Thus, the calculated sugar/acid ratio of grapes (“maturation index”) ranged from 23.17 at Purpuriu variety to 42.50 at Radames variety, with a mean of 30.87, values that were within the range presented by Nicolaescu and Cazac (2012), and much higher than the minimum value (20:1) required by the OIV (2008) for table grapes at maturity.

Table 1. Main chemical characteristics and technological parameters of the grapes at harvest

Variety	Moisture (%)	SD (±)	Total dry matter (%)	SD (±)	Minerals (%)	SD (±)	Sugars (g/L)	SD (±)	Acidity (g/L t.a.)	SD (±)
Purpuriu	83.48*	1.02	16.52^{NS}	1.02	0.29^{NS}	0.09	183.05^{NS}	3.49	7.90***	0.10
Splendid	83.67*	0.87	16.33^{o}	0.87	0.37^{NS}	0.12	182.67^{NS}	3.03	5.87^{o}	0.42
Radames	79.19^{ooo}	2.01	20.81**	2.01	0.50^{NS}	0.08	235.85***	9.88	5.55^{ooo}	0.41
Cetăţuia	80.91^{NS}	1.45	19.09^{NS}	1.45	0.54^{NS}	0.14	180.41^{oo}	11.03	5.20^{ooo}	0.06
Coarnă neagră select.	83.91*	0.98	16.09^{o}	0.98	0.38^{NS}	0.11	201.30**	10.82	6.70**	0.46
Transilvania	82.66^{NS}	1.11	17.34^{NS}	1.11	0.39^{NS}	0.09	187.65^{NS}	9.28	6.50*	0.44
Someşan	84.84***	2.01	15.16^{oo}	2.01	0.39^{NS}	0.07	175.08^{NS}	5.83	5.93^{o}	0.12
Napoca	81.19^{NS}	1.16	18.81^{NS}	1.16	0.40^{NS}	0.11	172.42^{ooo}	3.40	6.50*	0.26
Gelu	80.94^{NS}	1.37	19.06^{NS}	1.37	0.43^{NS}	0.18	192.33^{NS}	11.44	4.94^{ooo}	0.08
Milcov	79.47^{oo}	2.11	20.53**	2.11	0.49^{NS}	0.06	171.99^{ooo}	14.90	7.37***	0.15
Mean	82.03	1.95	17.97	1.95	0.42	0.07	188.58	19.29	6.25	0.93
CV%	2.38	-	10.85	-	16.66	-	10.23	-	14.88	-

Note: Data expressed as mean values with standard deviation (n = 3). ^{NS}, *, **, *** - indicate non-significant and positive significant at $p \leq 0.05$, 0.01, 0.001, respectively; $^{o, oo, ooo}$ - negative significant at $p \leq 0.05$, 0.01, 0.001. CV% - coefficient of variation, ratio SD/mean (%).

According to Mazza and Miniati (1993) and Horbowicz *et al.* (2008), anthocyanin content (AC) of grapes varies greatly depending on many factors (e.g. genetic factor, light, temperature, technology etc.), ranging between 30 and 900 mg cyanidin-3-glucoside equivalent (CE)/100 g f.w. At Romanian *V. vinifera* L. varieties for table grapes studied, AC had a mean value of 260.31 mg CE/100 g f.w., with a high standard deviation (±) of 70.97 mg CE/100 g f.w. (table 2).

Table 2. Anthocyanin content (AC) and total phenolic content (TPC) of grapes (skins, pulp and seeds)

Variety	AC (mg CE/ 100 g f.w.)	SD (±)	Skin TPC (g GAE/ 100 g f.w.)	SD (±)	Pulp TPC (g GAE/ 100 g f.w.)	SD (±)	Seed TPC (g GAE/ 100 g f.w.)	SD (±)
Purpuriu	248.48^{ooo}	0.21	1.66^{NS}	0.09	0.33^{NS}	0.02	4.38^{NS}	0.45
Splendid	218.87^{ooo}	1.54	1.70^{NS}	0.11	0.33^{NS}	0.01	4.45^{NS}	0.12
Radames	103.70^{ooo}	1.00	1.56^{NS}	0.08	0.32^{NS}	0.05	4.06^{NS}	0.30
Cetăţuia	299.23***	2.29	1.77^{NS}	0.08	0.34^{NS}	0.01	4.58^{NS}	0.40
Coarnă neagră selecţionată	293.16***	2.16	1.81^{NS}	0.14	0.34^{NS}	0.02	5.07^{NS}	1.08
Transilvania	274.68***	1.02	1.75^{NS}	0.24	0.34^{NS}	0.02	5.15^{NS}	1.21
Someşan	318.92***	0.95	1.80^{NS}	0.07	0.34^{NS}	0.04	4.80^{NS}	0.68
Napoca	343.86***	1.74	1.80^{NS}	0.11	0.34^{NS}	0.08	4.88^{NS}	1.14
Gelu	198.33^{ooo}	1.33	1.67^{NS}	0.08	0.33^{NS}	0.02	4.49^{NS}	0.98
Milcov	303.84***	2.18	1.75^{NS}	0.06	0.34^{NS}	0.03	4.59^{NS}	1.27
Mean	260.31	70.97	1.73	0.08	0.34	0.01	4.65	0.33
CV%	27.27	-	4.60	-	2.11	-	7.16	-

Note: Data expressed as mean values with standard deviation (n = 3). ^{NS}, *, **, *** - indicate non-significant and positive significant at $p \leq 0.05$, 0.01, 0.001, respectively; $^{o, oo, ooo}$ - negative significant at $p \leq 0.05$, 0.01, 0.001. CV% - coefficient of variation, ratio SD/mean (%).

Among the three main parts of the grape berry, the most important total phenolic content was recorded in seeds (mean 4.65±0.33 g GAE/100 g f.w.), followed by skins (mean 1.73±0.08 g GAE/100 g f.w.) and pulp (mean 0.34±0.01 g GAE/100 g f.w.).

Statistical analysis indicate very significant differences to the mean for AC values ($p \leq 0.001$), while for TPC values of skins, pulp and seeds the influence of genetic factor (variety) was nonsignificant ($p>0.05$).

The phenylalanine metabolism products include the chemical groups of flavonoids and non-flavonoids. Major flavonoid groups in grapes are tannins (e.g. flavanol oligomers and polymers), flavonols and anthocyanins (Brossaud *et al*., 1999). The non-flavonoids form a smaller class (stilbenes, hydroxycinnamic and hydroxybenzoic acids) and are often associated with the flavonoids (Rentzsch *et al*., 2009). Within the berry, the non-flavonoids compounds are primarily found in the pulp (Creasy and Creasy, 2009), while flavonoids are found mainly in skin and seeds (Ribéreau-Gayon *et al*., 2006a; Cotea *et al*., 2009).

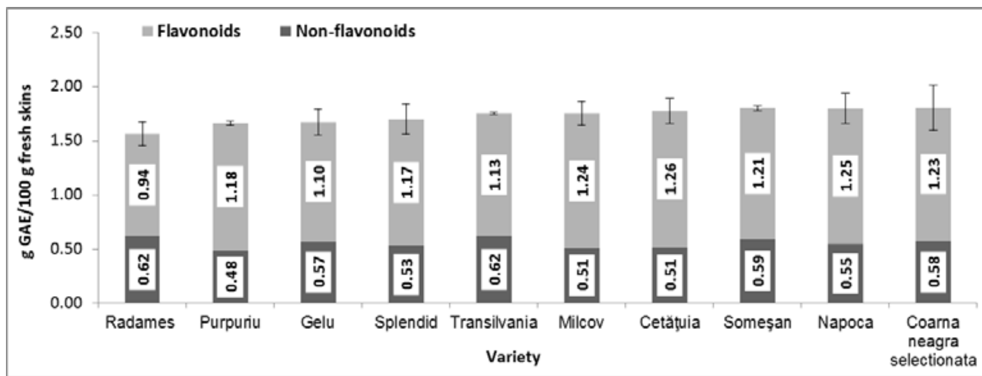


Fig. 1. Flavonoid and non-flavonoid content in grape skins of analysed varieties

In table grape samples of Romanian varieties, flavonoids were the most important class of phenolic compounds in skins (0.94–1.26 g GAE/100 g f.w.) (Fig. 1) and seeds (3.00–3.94 g GAE/100 g f.w.) (Fig. 2), while in pulp flavonoids were exceeded categorically by non-flavonoid phenolic compounds (Fig. 3).

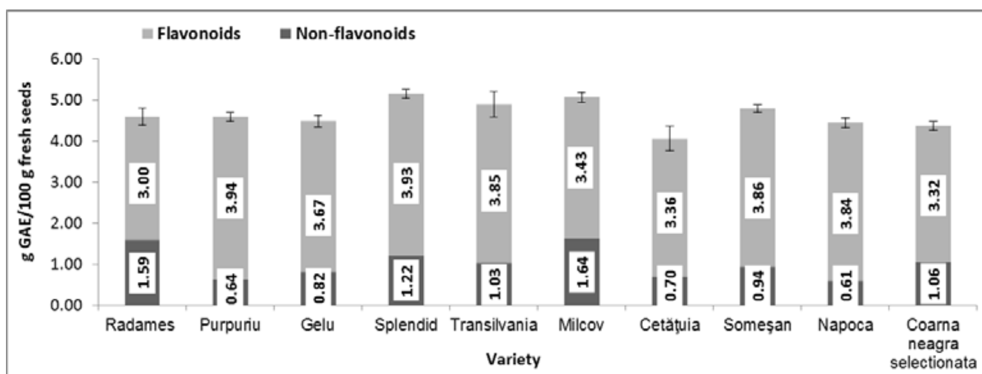


Fig. 2. Flavonoid and non-flavonoid content in grape seeds of analysed varieties

Percentage of flavonoids from total phenolic ranged in skins from 64.57% (Transilvania variety) to 71.05% (Cetăţuia variety), with a mean of 67.71±3.45%, in grape seeds from 65.28% (Milcov variety) to 86.35% (Splendid variety), with a mean of 78.13±7.08%, and in grape pulp from 9.35% (Gelu variety) to 27.27% (Purpuriu variety), with a mean value of 22.29±5.94%.

Ribéreau-Gayon *et al*. (2006a) mention that ripe grapes contain an orthophenol oxygen oxidoreductase, also known as cresolase or catechol oxidase, with an extremely variable activity depending on the grape variety and degree of ripeness. When physical disruption of cell occurs (due to mechanical damage or rot infections), phenolics are brought in contact with oxygen and polyphenol oxidases convert phenolics to quinones (Hernandez *et al*., 2009). This reaction is dominant if the grape pulp is rich in hydroxycinnamic acids (Keller, 2010).

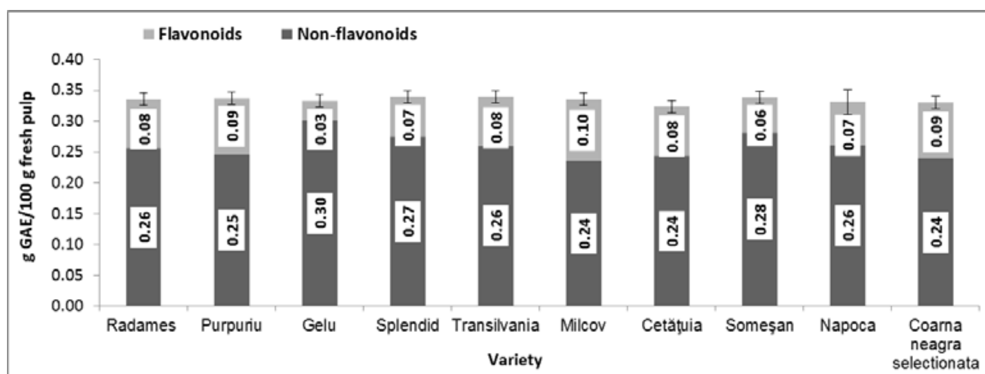


Fig. 3. Flavonoid and non-flavonoid content in grape pulp of analysed varieties

Polyphenol oxidase activity in *Vitis vinifera* L. Romanian varieties berries had a maximum of 7.11 U/g/min (Radames resistant variety), with a mean value of 5.30±0.99 U/g/min (Fig. 4).

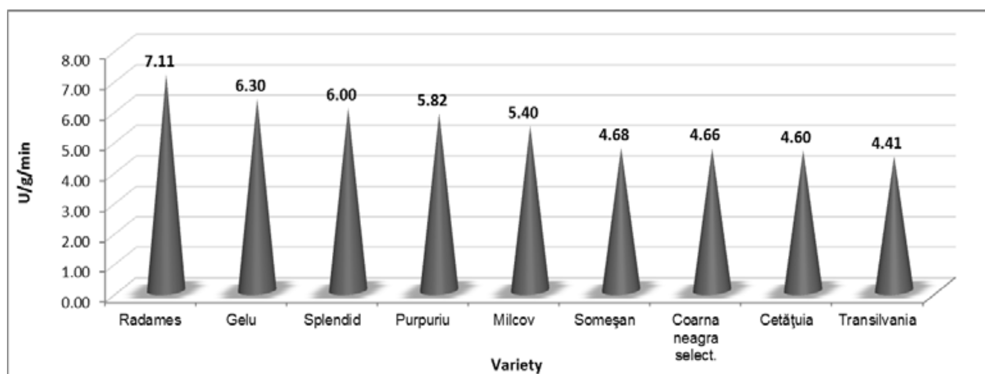


Fig. 4. Polyphenol oxidase activity of Romanian varieties grape berry

Should be noted the positive correlation between the anthocyanin and total phenolic content of grape skins (R^2=0.9291) (Fig. 5). This fact highlights that anthocyanins are part of the

phenolic compounds class, belonging to the flavonoid subclass, as suggested by the coefficient of determination (R^2=0.8471) of the anthocyanin – flavonoid relationship (Fig. 6).

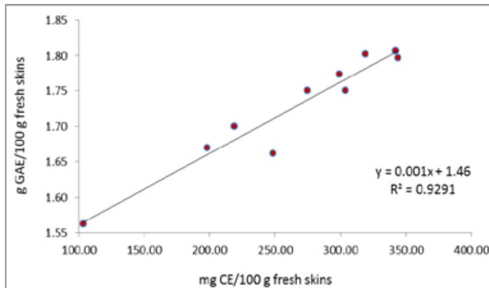


Fig. 5. Correlation of anthocyanin content and total phenolic content of grape skins

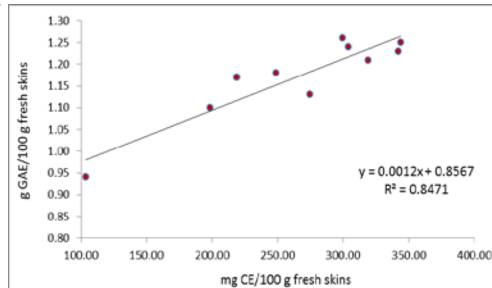


Fig. 6. Correlation of anthocyanin content and flavonoid content of grape skins

The main goal of this study was to identify correlations (positive or negative) between polyphenol oxidase activity and the concentrations of various classes of phenolic compounds in grape berry morphological parts. In this respect, in table 3 are presented the correlation coefficients of relationships identified between the experimental parameters.

Table 3. Correlation of the experimental parameters

Parameter	PPO	AC	Skin TPC	Skin NFC	Skin FC	Pulp TPC	Pulp NFC	Pulp FC	Seed TPC	Seed NFC	Seed FC
PPO	1										
AC	**-0.9180**	1									
Skin TPC	**-0.9266**	**0.9639**	1								
Skin NFC	0.0502	-0.2783	-0.1195	1							
Skin FC	**-0.7772**	**0.9204**	0.8711	-0.5917	1						
Pulp TPC	**-0.9397**	**0.8611**	**0.9020**	-0.0068	0.7358	1					
Pulp NFC	**0.8132**	-0.8174	-0.7567	0.3980	-0.8113	-0.7015	1				
Pulp FC	**-0.8833**	**0.8698**	0.8280	-0.3343	0.8377	0.8029	-0.9880	1			
Seed TPC	**-0.8713**	0.7948	**0.8401**	0.2337	0.5666	0.8770	-0.6483	0.7319	1		
Seed NFC	-0.3872	0.5598	0.4509	-0.0263	0.3791	0.3592	-0.4568	0.4598	0.5740	1	
Seed FC	-0.4561	0.1824	0.3521	0.2695	0.1526	0.4936	-0.1485	0.2310	0.3769	-0.5422	1

Note: AC – anthocyanin content; TPC – total phenolic content; NFC – non-flavonoid content; FC – flavonoid content.

Presence of polyphenol oxidase in a high concentration had a negative influence on the anthocyanin content of grapes (r = –0.9180; p<0.001), a greater enzyme activity corresponding to lower AC values. According to Kader *et al*. (1997), phenolases such as polyphenol oxidase are common anthocyanin degradation enzymes, but the destruction of anthocyanins is more efficient when other phenolic compounds (e.g. catechol, caftaric acid, chlorogenic acid) are present. Also, it is generally known that inactivation of enzymes improves anthocyanin stability in fruits (Garcia-Palazon *et al*., 2004).

Along with an increase in PPO activity, grape skins total phenolics followed the same trend as for anthocyanins (r = –0.9266; p<0.001), flavonoid fraction being more affected (r = –0.7772; p<0.05) than non-flavonoid one. A low level of total phenolic content was strongly correlated to a higher enzyme activity in pulp (r = –0.9397; p<0.001) and slightly lower in seeds (r = –0.8713; p<0.05).

It was observed that only in grape berry pulp non-flavonoid content was positively correlated to an intense PPO activity, while in skins and seeds were not set a relationship between these parameters. Thus, in the pulp an intense PPO activity was achieved on the background of a high content of phenolic acids (non-flavonoids). In parallel, it was noticed that varieties with a high content of phenolic compounds in the skin have also a high content of phenolic compounds in pulp and seeds.

CONCLUSIONS

Anthocyanin content of grape skins was highly correlated to the total phenolic content, and moreover with the flavonoid content of skins, confirming their appurtenance to this subclass of phenolic compounds. The flavonoid content was higher in grape skins and seeds, while non-flavonoids were predominant in grape pulp.

An intense polyphenol oxidase activity in grapes was negatively correlated to a lower total anthocyanin and total phenolic content of skins, pulp and seeds. Only in pulp non-flavonoid content was positively correlated to an intense polyphenol oxidase activity due to the fact that in pulp predominate phenolic acids that can be used by enzyme as substrate. Considering the possible destructive action of polyphenol oxidase on anthocyanins in grapes, further studies are necessary in order to evaluate the influence of an intense enzyme activity on the chromatic parameters of berry skins.

Experimental data obtained can be of interest to researchers in viticulture, vine breeding and pharmaceutical industry, and can serve as basis of comparison for future studies.

REFERENCES

Alvarenga, T. C., Da Silva Neto, H. F., Ogassavara, O., F., Arantes, F. C., Marques, M. O., Frigieri, M. C. (2011): *Polifenoloxidase: uma enzima intrigante.* Ciência & Tecnologia: FATEC-JB, Jaboticabal, 3 (1), 83-93.

Artenie, V., Ungureanu, E., Negură, A. (2008): *Metode de investigare a metabolismului glucidic şi lipidic.* PIM, Iaşi, Romania.

Bąkowska-Barczak, A., (2005): *Acylated anthocyanins as stable, natural food colorants - a review*. Pol. J. Food Nutr. Sci., 14/55 (2), 107-116.

Beceanu, D. (2010): *Tehnologia produselor horticole*. Vol. I. PIM, Iaşi, Romania.

Beceanu, D., Anghel, R. and Filimon, R. (2011): *Materii prime vegetale mai importante pentru industria alimentară*. PIM, Iaşi, Romania.

Brossaud, F., Cheynier, V., Asselin, C., Moutounet, M. (1999): *Flavonoid compositional differences of grapes among site test plantings of Cabernet franc*. Am. J. Enol. Vitic., 50 (3), 277–284.

Cotea, V. D., Zănoagă, C., Cotea, V. V. (2009): *Tratat de oenochimie*, Vol. I. Editura Academiei Române, Bucureşti, Romania.

Creasy, G.L. and Creasy, L.L. (2009): *Grapes*. CAB International, UK.

Daayf, F. and Lattanzio, V. (2008): *Recent advances in polyphenol research*, Vol. 1, John Wiley & Sons, UK.

Davies, K. (2004): *Plant pigments and their manipulation. Annual plant reviews*, Vol. 14, CRC Press, USA.

De Pieri Troiani, E., Tropiani, C. T., Clemente E. (2003): *Peroxidase (POD) and polyphenol oxidase (PPO) in grape (Vitis vinifera L.)*. Ciênc. agrotec., 27 (3), 635-642.

Ermakov, A. Y. (1987): *Methody biokhimicveskogo issledovaniya rastenii* (*Methods in Plant Biochemistry*), pp. 44 - 45. Leningrad, Agropromizdat.

Fregoni, M. (1998): *Viticoltura di qualità.* Stampe Grafiche Lama, Piacenza, p. 707.

Gambuti, A., Capuano, R., Lecce, L., Fragasso, M. G., Moio, L. (2009): *Extraction of phenolic compounds from 'Aglianico' and 'Uva di Troia' grape skins and seeds in model solutions: Influence of ethanol and maceration time.* Vitis, 48 (4), 193–200.

Garcia-Palazon, A., Suthanthangjai, W., Kajda, P., Zabetakis, I. (2004): *The effects of high hydrostatic pressure on b-glucosidase, peroxidase and polyphenol oxidase in red raspberry (Rubus idaeus) and strawberry (Fragaria × ananassa)*. Food Chem., 88, 7-10.

Graf, B. A., Milbury, P.E., Blumberg, J. B. (2005): *Flavonols, flavonones, flavanones and human health: Epidemological evidence*. J. Med. Food., 8, 281–290.

Hernandez, I., Alegre, L., Van Breusegem, F., Munne-Bosch, S. (2009): *How relevant are flavonoids as antioxidants in plants?* Trends in Plant Science, 14, 125–132.

Horbowicz, M., Kosson, R., Grzesiuk, A., Dębski, H. (2008): *Anthocyanins of fruits and vegetables - their occurrence, analysis and role in human nutrition.* Veg. Crops Research Bulletin, vol. 68, p. 5-22.

Kader, F., Rovel, B., Girardin, M., Metche, M. (1997): *Mechanism of browning in fresh highbush blueberry fruit (Vaccinium corymbosum L). Partial purification and characterization of blueberry polyphenol oxidase*. J. Sci. Food Agric., 73, 513-516.

Keller, M. (2010): *The science of grapevines: anatomy and physiology*. Academic Press, Elsevier Inc., USA.

Lee, J., Durst, R. W. and Wrolstad, R. E. (2005): *Determination of total monomeric anthocyanin pigment content of fruit juices, beverages, natural colorants, and wines by the pH differential method: collaborative study.* J. AOAC Intl., 88, 1269-1278.

Maltini, E., Torreggiani, D., Venir, E., Bertolo, G. (2003): *Water activity and the preservation of plant foods*. Food Chem., 82, 79–86.

Mazza, G. and Miniati, E. (1993): *Anthocyanins in fruits, vegetables, and grains*. CRC Press, USA.

Nicolaescu, G. and Cazac, F. (2012): *Producerea strugurilor de masă. Soiuri cu bobul roze şi negru (Production of table grapes. Pink and black bean varieties)*. Elan Poligraf, Chişinău, Moldova.

OIV (2008): *Standard on minimum maturity requirements for table grapes*. Resolution VITI 1/08. Organisation Internationale de la Vigne et du Vin, Paris.

OIV (2012): *Compendium of international methods of wine and must analysis*. Vol. II. Organisation Internationale de la Vigne et du Vin, Paris.

Orak, H. H. (2007): *Total antioxidant activities, phenolics, anthocyanins, polyphenol oxidase activities of selected red grape cultivars and their correlations*. Scientia Horticulturae, 111 (3), 235–241.

Pandey, K. B. and Rizvi, S. I. (2009): *Plant polyphenols as dietary antioxidants in human health and disease.* Oxid. Med. Cell. Longev., 2 (5), 270–278.

Rentzsch, M., Wilkens, A., Winterhalter, P. (2009): Non-flavonoid phenolic compounds. In M. V. Moreno-Arribas and M. C. Polo (Eds.) *Wine chemistry and biochemistry***.** Springer, New York, USA.

Ribéreau-Gayon, P., Dubourdieu, D., Doneche, B., Lonvaud, A. (2006a): *Handbook of enology. Vol.1. The microbiology of wine and vinifications*. 2nd Edition. John Wiley & Sons Ltd, UK.

Ribéreau-Gayon, P., Glories, Y., Maujean, A., Dubourdieu, D. (2006b): *Handbook of enology. Vol.2. The chemistry of wine stabilization and treatments*. 2nd Edition. John Wiley & Sons Ltd, UK.

Rocha, A. M. C .N. and De Morais, A. M. M. B. (2005): *Polyphenol oxidase activity of minimally processed 'Jonagored' apples* (*Malus domestica).* J. of Food Process. and Preserv., 29, 8–19.

Sarni-Manchado, P, Cheynier, V, Moutounet, M. (1997): *Reactions of polyphenol oxidase generated caftaric acid o-quinone with malvidin 3-O-glucoside*. Phytochemistry, 45, 1365-1369.

Singleton, V. L., Rossi, J. A. (1965): *Colorimetry of total phenolics with phosphomolybdic-phosphotungstic acid reagents*. Am. J. Enol. Vitic., 16, 144-158.

Teissedre, P.-L. and Chervin, C. (2011): Grape. In L.A.Terry (Ed.) *Health-promoting properties of fruit and vegetables*. CAB International, UK.

Tibiri, A., Sawadogo, W. R., Ouedraogo, N. (2010): *Evaluation of antioxidant activity, total phenolic and flavonoid contents of Entada africana Guill. et Perr. (Mimosaceae) organ extracts.* Res. J. of Medical Sci., 4(2), 81-87.

Valero, E., Varón, R., García-Carmona, F. (1988): *Characterization of polyphenol oxidase from Airen grapes*. J. of Food Sci., 53, 1482–1485.

Vermerris, W. and Nicholson, R. (2006): *Phenolic compound biochemistry.* Springer, Netherlands.

Yokotsuka, K., Shimizu, T., Shimizu, T. (1991): *Polyphenoloxidase from six mature grape varieties and their activities towards various phenols*. J. Biosci. Bioeng., 71, 156–162.

Authors' affiliation

[1]University of Agricultural Sciences and Veterinary Medicine Iaşi, 3, M. Sadoveanu Alley, 700490, Iaşi, Romania

*e-mail: razvan_f80@yahoo.com

Acknowledgements

This paper was published under the frame of European Social Fund, Human Resources Development Operational Programme 2007-2013, project no. POSDRU/159/1.5/S/132765.