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# CONTENT

# THE EFFECT INDUCED BY MILLIMETER WAVES WITH THE FREQUENCY 53.33 GHZ ON *SACCHAROMYCES CEREVISIAE* CNMN-Y-18 YEAST STRAIN

**AGAFIA USATÎI[1*], ELENA MOLODOI[1], NADEJDA EFREMOVA[1], LUDMILA FULGA[1]**

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**Keywords:** *Saccharomyces cerevisiae*, millimeter waves, mannoproteins, carbohydrates, cell biomass, protein, catalase activity.

**Abstract**: The effect of extremely high frequency electromagnetic waves on the biosynthetic activity of *Saccharomyces cerevisiae* CNMN-Y-18 yeast strain in dependence on the duration of irradiation was studied. The maximum amount of biomass, protein, carbohydrates, mannoproteins and catalase has been showed to accumulate when the yeast cells were irradiated with a frequency f = 53.33 GHz for 10 minutes. High degree of dependence between the content of cellular components (a correlation coefficient between $R^2 = 0.875$ and 0.926 ) it has been shown which demonstrates that biosynthetic processes were influenced by the same phenomenon - millimeter waves. A procedure for increasing of mannoprotein content in yeasts with the utilization of extremely high frequency waves has been proposed in this study.

## INTRODUCTION

The concept of millimeter wave electromagnetic oscillations includes extremely high frequency (30-300GHz). Recent research regarding the effect of millimeter waves focused on experimental investigation at cellular level on millimeter wave's therapy application (Cifra et al., 2011; De Vita et al., 2011). Studies have demonstrated the influence of millimeter waves on the cell multiplication and proliferation, enzymes activity and other biological processes (Markkanen Ari, 2009). In the field of millimeter wave investigation enters and theoretical modeling of possible mechanisms of interaction with biological sentities, including microorganisms. An important characteristic of millimeter waves is specific resonant character. Millimeter wave oscillations superimposed cell vibrations can initiate positive or negative responses. The biological effect is usually observed within the small millimeter wave frequency interval (Ruiz-Gomez et al., 2004). According to previously study of the influence of millimeter waves with frequencies 60,12 GHz, 53,33 GHz şi 42,19 GHz on biosynthetic activity of *Saccharomyces cerevisiae* CNMN-Y-18, millimeter wave frequency 53.33 GHz has had maximum stimulating effect among other (Molodoi et al., 2014).Therefore, the establishing of mechanisms of cell reaction to the influence of millimeter waves is one of the important problems in ensuring the effectiveness of their practical application.

The aim of this investigation is to evaluate the influence of millimeter wave frequency f = 53.33 GHz on biosynthetic processes in yeasts in dependence on the duration of irradiation.

## MATERIALS AND METHODS

*Object study* - *Saccharomyces cerevisiae* CNMN-Y-18 yeast strain, mannoproteins producer from National Collection of Nonpathogenic Microorganims of Institute of Microbiology and Biotechnology of Academy of Sciences of Moldova (Usatîi et al., 2013).

*Media and cultivation conditions*. Seed material obtained by growing yeast strain on the beer wort for 24 hours on shaker (200 rpm) was irradiated with millimeter wave frequency f = 53.33 GHz (corresponding to the wavelength $\lambda = 5.6$ mm) with the duration of treatment of 5, 10, 15, 20, 25 minutes. The irradiated inoculum in the amount of 5%, $2x10^6$ cells/ml concentration was transferred in nutritive medium YPD which consist of 1% yeast extract, 2% peptone, 2% glucose, water 1 L, pH 5.5 (Aguilar-Uscanga and Francois, 2003). Cell density was determined spectrophotometrically at 600 nm by UV-Vis spectroscopy. The submerged cultivation was carried out in Erlenmeyer flasks containing 0.2 L YPD, the concentration of oxygen - 81, 3..83,3 mg/l, cultivation period of 120 hours at temperature of 25˚ C.

As millimeter wave generator was used KWC-ND device, RS-232 (made in Russian Federation) with wavelength $\lambda =$ 4.9; 5,6; 7,1 mm,corresponding to the frequency *f*=60,12 GHz; 53,33 GHz; 42,19 GHz, kindly provided by the staff of the Institute of Electronic Engineering and Nanotechnologies ” D. Ghitu”. The device is certified and permitted for use in medical practice. The flask with 5 ml of inoculum was placed at the distance of 0,5 cm in relation to the generator.

*Methods of investigation*. Cell biomass was determined gravimetrically (Liu et al., 2009). Yeast total carbohydrates in the biomass were determined spectrophotometrically with PG T160 VIS Spectrophotometer at wavelength 620 mm and

anthrone reagent using D-glucose as standard (Dey and Harborn, *1993*). Determination of mannoproteins was done gravimetrically (Liu et al., 2011). Protein was determined according to Lowry method (Lowry et al., 1951). Catalase activity was determined by the method described by Efremova (Efremova et al., 2013).

Statistical processing of the results was performed using calculation of standard errors for average and relative values. Correlation between samples and statistical differences was assessed using t-Student criterion and materiality ($p < 0.05$)

## RESULTS AND DISCUSSIONS

In order to establish the time interval taken by the cell under the influence of extra high-frequency millimeter waves to activate its physiological processes, the content of total carbohydrate, mannoproteins, protein, catalase activity was determined.

The determination of cell biomass, mannans, carbohydrate content has demonstrated the positive reaction of yeast strain to the action of irradiation. Following the mathematic calculation, it was obtained biomass content of 5.68- 6.05 g/l, carbohydrate content 29.14 to 39.65% of dry biomass, mannoproteins varied from 11.7 to 13.93 % dry cell mass, which is by 21.0%, 50.58% and 30.06%, respectively, more than the control. Maximum biomass and carbohydrate content was accumulated at the duration of irradiation of 20 minutes and mannoproteins accumulation was established at 10 minutes of irradiation (Fig. 1).

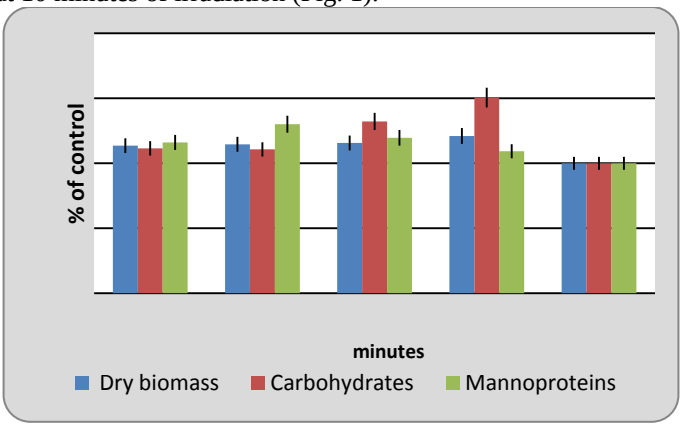


**Figure 1.** Effect of millimeter wave f = 53.33 GHz on the content of mannoproteins, carbohydrates and biomass *Saccharomyces cerevisiae* CNMN-Y-18 depending on the duration of irradiation

The calculation of linear correlation r (Pearson) for interpreting the biomass and mannoproteins content revealed a strong association. The coefficient of determination $R^2 = 0.926$ or 92.6% is determined by the variation of the other variable values (Figure 2a).

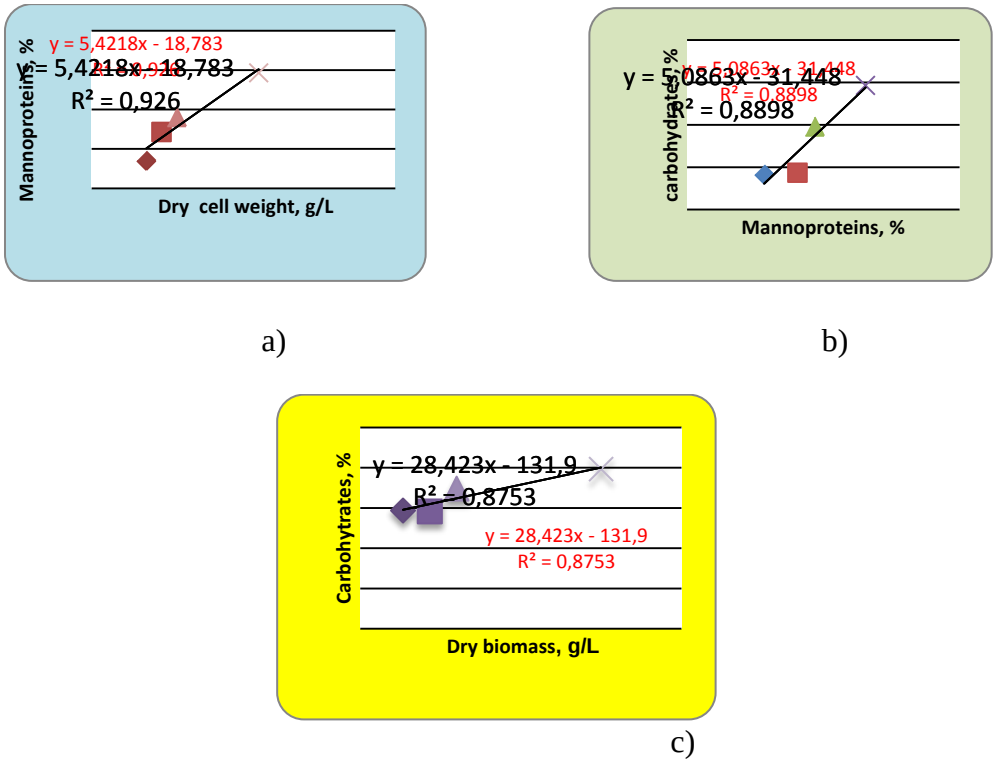


**Figure 2.** The interdependence between biomass, carbohydrates and mannoproteins content in *Saccharomyces cerevisiae* CNMN-Y-18, irradiated with millimeter wave f = 53.33 GHz.

The analysis of the correlation parameters between mannoproteins and carbohydrates content (fig.2b) established an ascending tendency values that imply variable an ascending tendency of other variables. The bond between them identified as $R^2 = 0.889$ or 88.9%, argues the hypothesis of the existence of real bonds in the base of those can be foretelled values on the base of the regression equation. The possible explanation would be that variables, the carbohydrate and mannoproteins content are influenced by the same phenomenon –millimeter waves treatment.

Calculation of the correlation coefficient for two variables - biomass and carbohydrate content $R^2 = 0.875$ or 87.5% demonstrated strong dependence between biomass and carbohydrate accumulation (Figure 2c).

The data referring to the protein content and catalase activity of *Saccharomyces cerevisiae* CNMN-Y-18 yeast strain are demonstrated in Figure 3. According to the obtained results, the highest values of protein content, as well as the catalase activity in the yeast strain has been determined by the duration of irradiation of 15 min, which was 39.26% and 2936 U/mg of protein, respectively, which was with 33.0% and 34.0% more than non-irradiated sample.

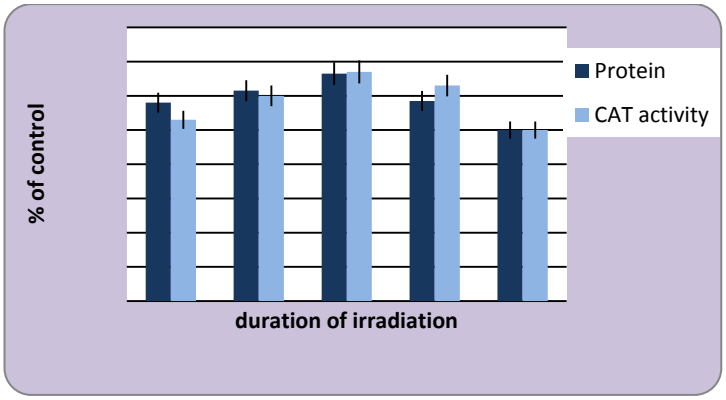


**Figure 3**. The effect of the duration of action of millimeter wave f = 53.33 GHz on protein content and catalase activity in *Saccharomyces cerevisiae* CNMN-Y-18 yeast strain.

The determination of the correlation between protein content and catalase activity in *Saccharomyces cerevisiae* strain CNMN-Y-18 confirms the existence of dependence between these parameters; correlation coefficient was 58.10% (Figure 4). Therefore, high levels of catalase activity are completely determined by the content of protein. Thus, extremely high frequency radiation has changed microbial metabolism and stimulated production of protein in yeast biomass. Oxidative stress condition induced by the irradiation has been reported to change enzymatic reaction by stimulating of catalase activity.

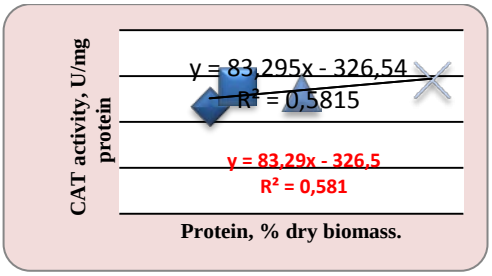


**Figure 4.** Interdependence between protein content and catalase activity in *Saccharomyces cerevisiae* CNMN-Y-18, irradiated with millimeter waves f = 53.33 GHz.

The analysis of selected indices of *Saccharomyces cerevisiae* CNMN-Y-18 yeast has demonstrated different levels of response to millimeter waves irradiation with different duration of treatment. Summarizing the experimental results it can be revealed that maximum amount of biomass and carbohydrates is accumulated for a irradiation time of 20 minutes. The treatment of yeast cell for 10 minutes provides high content of mannoproteins by 30% compared to the control. It was determined the optimum time for irradiation is 15 minutes whuich determines an increase of protein content and catalase activity in *Saccharomyces cerevisiae* CNMN-Y-18 yeast

strain. Analysis of the interdependence between biomass content, carbohydrates, mannoproteins, protein and catalase activity in irradiated yeast strains has established an ascending tendency of correlation.

According to research results, the new procedure of directed synthesis of mannoproteins at yeasts using extremely high frequency has been proposed. The elaborated procedure of activation of mannans biosynthesis includes the following stages: inoculums obtaining; the irradiation of inoculum with millimeter wave (f = 53.3 GHz) emitted on a continuous basis; subsequently seeding the fermentation medium with obtained sterile inoculum ($2x10^6$ cells ml $^{-1}$) at a concentration of 5%; submerged cultivation under continuous stirring (200 rpm) at 25˚ C for 120 hours. Yeast biomass is then further separated from the culture liquid, then mannoproteins subsequently extracted.

The scheme of the realization of procedure it is presented in figure 5 and includes the following stages:

*Stage I. Inoculum obtaining.*

The obtaining of 24 hours old inoculums by culturing *Saccharomyces cerevisiae* CNMN-Y-18 yeast in depth in the beer wort on the rotary shaker (200 rpm) at a temperature of 25˚ C.

*Stage II. Inoculum irradiation with extremely high frequency.*

Irradiation of inoculum with the millimeter wave f = 5,33 GHz for 10 minutes. The obtained sterile inoculum are used for further fermentation.

*Stage III. Submerged yeast cultivation.*

The cultivation of yeast strain on YPD nutritive medium at a temperature of $25^0$C for 96-120 hours. The separation of yeast biomass from the culture liquid by centrifugation 3000 rpm/min for 20 min.

*Stage IY. Extraction of mannoproteins.*

Spent brewer’ s yeasts (20 g dry weight) were first sieved (mesh diameter 125 mm), then the yeast suspension was centrifugated at 4500 g for 10 min, followed by re-suspension of the deposition in sterile distilled water. The procedure was repeated 5–6 times until the supernatant was clear, then the yeast was purified. After purifying, 1 L of 2% NaOH (w/v) was added to the cell wall sediment. This was placed in a boiling water bath and agitated at 150 rpm/min for 2 h. The preparation was centrifuged and the supernatant was collected. The residue was washed with little deionized water and combined with supernatant extracts. After that, the pH was adjusted to 6.5 with 10% acetic acid, and the supernatant was concentrated to one fifth of the original volume by the evaporation, ethanol was added to supernatant with the aim to precipitate mannoproteins. The precipitated mannoprotein was dissolved in water and centrifuged; the supernatant was precipitated again by the addition of ethanol and recentrifuged. The obtained white sediment was washed twice with ethanol and once with ether, then dried at 70 ˚C

The proposed method provides an increase in mannoproteins content with 26 to 30% compared to the control.

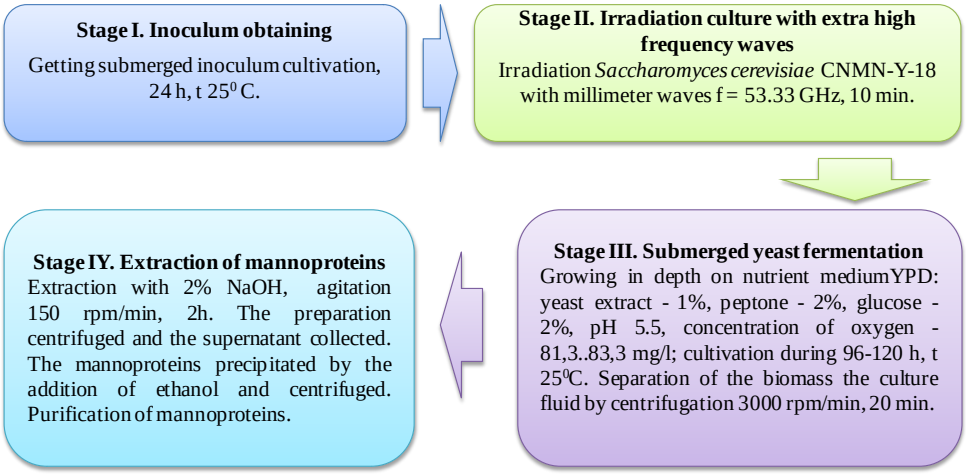


**Figure 5**. Scheme of performing the process of increasing in mannoproteins content in *Saccharomyces cerevisiae* CNMN-Y-18 by using extremely high frequency.

## CONCLUSIONS

*Saccharomyces cerevisiae* CNMN-Y-18 yeast strain has demonstrated various response to the extremely high frequency waves (f = 53.33 GHz) irradiation. Positive influence on the accumulation of biomass and carbohydrates exhibited irradiation time of 20 minutes, the protein content and catalase activity - after 15 minutes of irradiation and biosynthesis of mannoproteins was activated after 10 minutes of irradiation. High dependence has been established between biomass, carbohydrates, mannoproteins content at selected yeast strain (coefficient of correlation $R^2$ = 0.875 ... 0.926), which demonstrates that these cellular components are influenced by extremely high frequency. The new procedure of increasing of the biosynthesis of mannoproteins in *Saccharomyces cerevisiae* CNMN-Y-18 yeast strain, based on the use of millimeter waves as the stimulating factor with duration of irradiation for 10 minutes allows its successful employment in the industrial production of mannoproteins.

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# PAIN ANIMAL MODELS IN ALZHEIMER`S DISEASE

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**Keywords:** Alzheimer, pain, animal models.

**Abstract.** Animal models offer valuable tools for evaluating new therapeutic strategies for treatment of human diseases, as well for studying the pathological mechanisms involved in the disease processes. However, they reproduce, in general, just certain aspects of human diseases.
In this way, there are numerous aspects of nociception studies, describing these tests as a simple matter of first accounting for the nature of the stimulus (electrical, thermal, mechanical, or chemical) and then describing the behavioural parameters that are measured, but assessments vary with the scale used, while also the scales can be very subjective.
Thus, animals will withdraw an injured body part from a stimulus, where different levels of stimulation affect the latency or force of withdrawal. This withdrawal response is considered a measure of pain, which correlates highly with more integrative nocifensive behaviours (e.g. some behavioural signs are usually associated with pain), such as licking of the injured body part and guarding behaviour.
Moreover, the vocalizations are important indicators of pain in several species. In this way, animals in pain may lick, bite, scratch, shake, or rub the site of injury, as described in reference works such as the Guidelines for the Care and use of Mammals in Neuroscience and Behavioural Research.
In addition, it is important to mention that pain studies are affected by a wide range of modulatory factors, including sex, genotype and social communication, all of which must be taken into account when using an animal model.

## INTRODUCTION

Alzheimer`s disease (AD) represents 60-80% of all dementia according to Alzheimer`s Association, making it the most common dementia.

Pain is a subjective, multidimensional experience that can have marked impact on both physiological and psychological state of an individual (Moriarty O et al 2011**).**

It is known that AD affects both cognitive and affective functions, and on the other hand the global pain experience results from a complex integration of sensory, cognitive and affective processes (Melzack and Casey, 1968; Rainero I.et al, 2000), therefore it is not a surprise that several studies showed alterations of both acute and chronic pain in AD and demented patients (Farrell et al, 1996). In contrast with the polymorphic nature of the pain that is described as a sensation in humans, pain in animals can be estimated only by examining their reactions (Le Bars D et al, 2001). Due to lack of complete understanding of the aetiology of AD, all the available models have limitations, which have to be carefully considered when using them (Benedikz E et al., 2009).The difficulty of identifying pain reactions is essentially the same as the one faced by the pediatrician, the geriatrician, or the psychiatrist dealing with patients incapable of expressing themselves verbally. In those cases as well, the symptomatology is not unequivocal—it has to be taken in context and placed in an inventory, because its meaning will differ depending on the degree of maturation (or degradation) of the nervous system (Le Bars D et al, 2001).

Considering the importance of studying pain perception, there had been imagined several pain animal models. This article`s purpose is to present different types and certain detail aspects of pain animal models that are currently used.

## SEVERAL GENERAL ASPECTS OF ALZHEIMER DISEASE

Characterization of Alzheimer Disease

Alzheimer`s disease (AD) is characterized by progressive cognitive decline, where memory of recent facts, spatial orientation, attention and executive functions are one of the first affected, followed by speech and behavioural problems, which affect every day life (Almkvist O,1996; Benedikz E et al, 2009).

As it is widely known, for AD at histological level is representative the presence of neurodegenerative plaques and neurofibrillary tangles. The hyperphosphorylated tau protein is the main constituent of neurodegenerative plaques and neurofibrillary tangles. Senile plaques present in Alzheimer dementia is composed of β amyloid, the amyloid precursor protein (APP), dystrophic neuronal extensions, activate microglia and reactive astrocytes (Behl 1997). Additionally, the formation of Aβ peptide occurs by proteolitic cleavage of its precursor APP (Padurariu M et al, 2013).

There is relatively consistent evidence in the literature which showed that free radicals may be involved in the etiopathogenesis of Alzheimer dementia (Ferreiro et al, 2012; Padurariu at al, 2012; Balderas et al, 2008; Greilberger et al, 2008).

Free radicals, oxidative stress, antioxidative system

Free radicals are toxic biochemical compounds due to their instability that result from oxidative stress, a process of imbalance between pro-oxidants and antioxidants. The instability of free radicals is granted by their single electron structure, therefore they easily attach to various molecules to reach stable energy states.

The reaction of pairing the single electron in free radicals is called oxidative reaction. The oxidative reactions leading to compound stability are coupled with reduction reactions as so-called redox reactions. Most free radical injuries concern lipidic structures, in particular the polyunsaturated fatty acids, which are produced from lipidic peroxidation reactions.

Brain is particularly vulnerable to oxidative stress, due to its high concentration of polyunsaturated fatty acids, increased oxygen demand, relatively low levels of antioxidants (Evans 1993).

The antioxidants are the elements of the antioxidant system, which is the arsenal of protection against oxidative stress. Under normal conditionts it is very effective. This system consists of antioxidant enzymes such as superoxide dismutase (SOD), glutathione peroxidise (GPX), catalase or aldehyde dehydrogenase and non-enzymatic antioxidants factors such as: uric acid, lipoic acid, ascorbic acid, glutathione, beta-carotene, bilirubin, melatonin, selenium, NADP, mannitol, benzoate, reduced Co Q10, tocopherol.

Glutathione is the one that is remarked due to its propriety of reducing lipid peroxidation processes through direct blocking of reactive oxygen species activity (Padurariu M et al, 2013). There is no natural model of AD, so most of the research is performed using models simulating the disease phenotypes by active manipulation of the animals (rat models of cholinergic-dysfunction, Aβ-based models of AD) or more recently using transgenic animal models (Benedikz E et al, 2009).

Pain phenomenon and its pathways

Pain is termed nociceptive (*nocer* – to injure or to hurt in Latin), and nociceptive means sensitive to noxious stimuli. Noxious stimuli are stimuli that elicit tissue damage and activate nociceptors. Nociceptors are sensory receptors that detect signals from damaged tissue or the threat of damage and indirectly also respond to chemicals released from the damaged tissue.

Nociceptors are free (bare) nerve endings found in the skin, muscle, joints, bone and viscera. Recently, it was found that nerve endings contain transient receptor potential (TRP) channels that sense and detect damage. The TRP channels are similar to voltage-gated potassium channels or nucleotide-gated channels, having 6 transmembrane domains with a pore between domains 5 and 6. They transduce a variety of noxious stimuli into receptor potentials, which in turn initiate action potential in the pain nerve fibers. This action potential is transmitted to the spinal cord and makes a synaptic connection in lamina I and/or II. The cell bodies of nociceptors are mainly in the dorsal root and trigeminal ganglia. No nociceptors are found inside the CNS.

Nociceptors respond when a stimulus causes tissue damage, such as that resulting from cut strong mechanical pressure, extreme heat, etc. The damage of tissue results in a release of a variety of substances from lysed cells as well as from new substances synthesized at the site of the injury. Some of these substances activate the TRP channels which in turn initiate action potentials.

The cell bodies of the primary afferent pain neurons from the body, face, and head are located in the dorsal root ganglia (DRG) and in the trigeminal ganglia respectively. Some of these cell bodies give rise to myelinated axons (A delta fibers), and others give rise to unmyelinated axons (C fibers).

The free nerve endings arise from both A delta fibers and the unmyelinated C fibers, which are scattered together. The synaptic terminals of the axons of the dorsal root ganglion, which carry noxious information arriving to Rexed layers I and II , release neurochemical agents such as substance P (SP), glutamate, aspartate, vasoactive intestinal peptide (VIP), cholecystokinin (CCK), somatostatin, calcitonin gene-related peptide (CGRP), galanin, and other agents.

These agents activate the nocineurons. It was shown that when SP and CGRP are applied locally within the spinal cord dorsal horn, glutamate is released. The release of glutamate excites the nocineurons. Furthermore, SP receptors (neurokinin receptors) and NMDA receptors (glutamate) interact which result that the NMDA receptors will become more sensitive to glutamate, which results in central sensitization. The functions of these peptides are largely unknown but they presumably mediate slow, modulatory synaptic actions in the dorsal horn neurons. The neuropeptides are always co-localized with other "classical" neurotransmitters [http://neuroscience.uth.tmc.edu/s2/chapter06.html]-cited 12 October 2014.

There are two main pathways that carry nociceptive signals to higher centres in the brain. The spinothalamic tract: secondary afferent neurones decussate within a few segments of the level of entry into the spinal cord and ascend in the contralateral spinothalamic tract to nuclei within the thalamus. Third order neurones then ascend to terminate in the

somatosensory cortex. There are also projections to the periaqueductal grey matter (PAG). The spinothalamic tract transmits signals that are important for pain localisation.

The spinoreticular tract: fibres also decussate and ascend the contralateral cord to reach the brainstem reticular formation, before projecting to the thalamus and hypothalamus. There are many further projections to the cortex.

This pathway is involved in the emotional aspects of pain. The somatosensory cortex is important for the localisation of pain. However, imaging techniques such as functional magnetic resonance imaging (fMRI) have demonstrated that a large brain network is activated during the acute pain experience. This is often called the ‘pain matrix’.

The commonest areas activated include the primary and secondary somatosensory (S1 and S2), insular, anterior cingulated cortex and prefrontal cortex, and the thalamus, demonstrating that these areas are all important in pain perception (Fisher-Morris Mary et al, 1997). The way pain is quantified is through pain scales. They are adapted to age and pathology. Not surprisingly, several studies have shown alterations of both acute and chronic pain in AD and demented patients (Farrell et al., 1996).

Pain in Alzheimer Disease context

In an observation report of two patients with AD who had experienced trauma of different kinds, neither of the patients exhibited normal pain behaviour or gave verbal reports of pain commensurate with the tissue damage they had incurred. Although, various types of physical trauma have been observed occurring in patients- burns, fractures, invasive tumours, herpes zoster – all capable of creating different types of pain and involving a variety of different types of structures (nerves, soft tissue, bone, superficial skin, deep tissues and so on) none of them showed signs of normal pain perception [https://www.ucl.ac.uk/anaesthesia/StudentsandTrainees/PainPathwaysIntroduction]-cited 12 October 2014.

Different pain animal models – reactions and contribuitions

Studying intact animals allows the multidimensional nature of pain to be examined. A number of animal models have been developed, reflecting observations that pain phenotypes are mediated by distinct mechanisms. Animal models of pain are designed to mimic distinct clinical diseases to better evaluate underlying mechanisms and potential treatments (Gregory Nicholas S et al, 2013).

Animal models of nociception (pain) date back to the late 19th century and have been crucial in our understanding of pain processes (von Frey M, 1896; Gregory Nicholas S et al, 2013). Since then, there have been a large number of animal models of disease developed to better understand pain from a variety of disease states, both acute and chronic, and these have proven useful in further advancing disease-specific questions and processes (Bennett G.J et al, 1988; Berberich P et al, 1988; He Y et al, 2012; Schaible H.G et al,1985; Woolf C.J.,1983; Gregory Nicholas S et al, 2013). It has become increasingly clear that pain is a heterogeneous phenomenon that differs widely based on the affected tissue (skin, muscle, joint, viscera, etc)(Gregory N S et al,2013;Hoeger B.M.K et al,2007; Ness T.J et al,1990; Sluka K.A,2002) and the mechanism of injury (thermal, mechanical, inflammatory, neuropathic, etc)(Gregory N S et al,2013; Milligan E.D et al,2009; Schmidt B.L.et al,2013; De Santana J.M.et al, 2008).

Descriptions of the “signs” of pain have been published on several occasions in a veterinary or an animal-welfare context (Gibson and Paterson, 1985; Morton and Griffith, 1985; Flecknell, 1986; Sanford et al, 1986; American Veterinary Medical Association, 1987; Sanford, 1992, 1994; Baumans et al, 1994). Above all else, it must be emphasized that these signs have no unequivocal value and that each species expresses pain in a manner related to its own behavioral repertoire (Le Bars D et al,2001).

Although animals cannot communicate verbally, they exhibit motor behaviours and physiologic responses similar to those of humans in response to pain. Those behaviours may include simple withdrawal reflexes; complex, unlearned behaviours such as vocalization and escape; and learned behaviours such as pressing a bar to avoid further exposure to noxious stimulation. However, there are species-specific behaviours that animals may express in response to pain (Bolles, 1970; Guidelines for the Care and Use of Mammals in Neuroscience and Behavioural Research.National Research Council (US) Committee on Guidelines for the Use of Animals in Neuroscience and Behavioral Research.Washington (DC): National Academies Press (US); 2003).

The most used models of animals are rodents, rats being the first mammalian species domesticated for scientific research over 180 year ago(Gibbs RA et al,2004; Benedikz Eirikur et al,2009). Since then, it has been one of the extensively studied model organism, particularly in cardiovascular, cancer, toxicology, behavioural, neurodegeneration and aging research (Gill TJ 3rd et al, 1989;Benedikz E et al,2009).

The rat`s contribution to human research cannot be overestimated (Benedikz Eirikur et al,2009; Gibbs RA et al,2004) and it has been the organism of choice for most physiological and behavioural research for decades (Benedikz E et al,2009).

Zimmermann (1986) re-interpreted the IASP definition of pain so that it could be applied to animals: “an aversive sensory experience caused by actual or potential injury that elicits progressive motor and vegetative reactions, results in learned avoidance behaviour, and may modify species specific behaviour, including social behaviour”.

Among other models of animals use in the study of pain there are rabbits, cats, dogs, non human primates.

Here are the pain expressions of these different animal models according to Guidelines for the Care and Use of Mammals in Neuroscience and Behavioural Research: rodents show decreased activity; excessive licking and scratching; self-mutilation; may be unusually aggressive; abnormal locomotion (stumbling, falling); writhing; does not make nest; hiding, rapid, shallow respiration; decreased food/water consumption; tremors; dogs show excessive licking; increased aggression; increased vocalizations, inclusive of whimpering, howling, and growling; excessive licking and scratching; self-mutilation, decreased food/water consumption; increased respiration rate/panting; cats react by hiding; increased vocalizations, inclusive of growling and hissing; excessive licking; increased aggression, also decreased food/water consumption; rabbits sign of pain are head pressing; teeth grinding; may become more aggressive; increased vocalizations; excessive licking and scratching; reluctant to locomote, rapid, shallow respiration; decreased food/water consumption, non-human primate show signs of increased aggression or depression; self-mutilation; often a dramatic change in routine behaviour (e.g., locomotion is decreased); rubbing or picking at painful location, decreased food/water consumption(Guidelines for the Care and Use of Mammals in Neuroscience and Behavioural Research.National Research Council (US) Committee on Guidelines for the Use of Animals in Neuroscience and Behavioral Research.Washington (DC): National Academies Press (US); 2003).

According to the Guide cited above: "fundamental to the relief of pain in animals is the ability to recognize its clinical signs in specific species" (p. 64). Pain can be assessed by evaluating behavioural measures such as eating, socializing, and withdrawal reflexes, and physiologic measures such as heart rate and respiration rate.

However, species, and even strains and individuals of the same species, may vary widely in their perception of and response to pain (NRC, 1992; Wixon, 1999). Even for an individual animal, pain sensitivity varies among different tissues and organs (Baumans et al, 1994), and pain sensitivities can be altered by pathologic processes or experimental procedures (Carstens and Moberg, 2000).

For example, during the initial phase of lipopolysaccharide-induced fever, rats exhibit hyperalgesia, whereas they exhibit hypoalgesia during the later stages of the illness (Carstens and Moberg, 2000). The existence of these differences underscores point out that pain and distress exist as a continuum of experience.

In addition, some animals may hide signs of pain; for example, it has been suggested that rats may mask pain during the dark-cycle hours to avoid displaying abnormal activity and increasing their risk of predation (Roughan and Flecknell, 2000).It is important to note that it is usually incorrect to infer that an animal's pain tolerance level is signaled by the onset of avoidance or escape behaviour, as some avoidance-escape behaviour is an appropriate adaptive response. It is only when the animal's behaviour is dominated by avoidance-escape attempts that the behaviour becomes maladaptive, signaling unacceptable levels of pain (NRC, 1992).

Quantifying pain in animal models

Pain assessment will vary with the pain scale or scoring system used. Scoring systems involve assigning a numeric score to constellations of behavioural, physical, and physiologic observations, and this process can be subjective. There are no generally accepted objective criteria for assessing the degree of pain that an animal is experiencing, and different species or strains can vary in their response to pain. Physiologic measures include heart rate, blood pressure, and respiration rate, but obtaining most of the measures requires some degree of intervention, which may not be feasible or desirable (Baumans et al, 1994).

Experimental studies on conscious animals are often designated "behavioural studies". Sometimes, this may seem to be stretching the meaning of the word "behavioural", but what it means is simply and implicitly that all responses—including simple withdrawal reflexes—are part of an animal's behavioural repertoire. Describing these tests should be a simple matter of first accounting for the nature of the stimulus (electrical, thermal, mechanical, or chemical) and then describing the behavioural parameters that are measured.

Pain tests

In humans and in animals, experimental studies of the mechanisms underlying acute pain necessitate the use of appropriate stimuli to provoke the sensation. To be adequate, these stimuli have to be quantifiable, reproducible, and noninvasive (Beecher, 1957; Lineberry, 1981; Le Bars D et al, 2001).

Nociceptive tests use electrical, thermal, mechanical, or chemical stimuli (Le Bars D et al, 2001). Some of them rely on the latency of appearance of avoidance behaviour, usually a withdrawal reflex of the paw or the tail. In this case the stimulus may be considered as fixed.

The concerned tests that use thermal stimulation include the tail flick test, the hot- or cold-plate tests, and the radiant heat paw-withdrawal test .The application of electrical stimuli has the advantages of being quantifiable, reproducible, and noninvasive and of producing synchronized afferent signals (Barrot M, 2012).

Thermal pain tests

The tail-flick test is a test of acute nociception in which a high-intensity thermal stimulus is directed to the tail of a mouse or a rat. The time from onset of stimulation to a rapid flick/withdrawal of the tail from heat source is recorded. It is held that the tail-flick test of pain depends on the spinal reflex because a similar response is observed in spinal transacted rats(King TE et al,1997).

The Hot Plate test is a common sensorimotor task that measures thermal nociception in rodent models of CNS disorders. This test measures the nociceptive responses of mice when they are placed on a warmed metal plate either at a standard, constant temperature or at slowly increasing temperature, starting from non-noxious levels to a standard, constant temperature. Subjects are tested for their baseline latency; then in test conditions, subjects are treated with an analgesic agent and tested for their sensitivity to pain. The latency to a nociceptive response is recorded, defined as the time elapsed until the subject licks or flicks its hind paw (http://sbfnl.stanford.edu/cs/bm/sm/bmst_hot.html)- cited 12 October 2014.

A cold plate apparatus was designed to test the responses of unrestrained rats to low temperature stimulation of the plantar aspect of the paw (Jasmin L et al, 1998). The primary method for studying responses to ambient temperature changes was the “dynamic cold plate” (Yalcin et al, 2009 and Descoeur et al, 2011), in which animals are put on a room temperature Peltier device which is then rapidly cooled (1 °C/min) until it reaches 1 °C. Behavioural responses including licking, rearing, and jumping are measured at different temperature ranges and used to estimate cold responsiveness (Brenner DS et al,2014)

The plantar test (Hargreaves et al. 1988) is used to differentiate pre- and post-treatment hind paw responses to heat. Before starting the test, each mouse is placed into clear acrylic boxes on a Plexiglas floor for 20-30 minutes for acclimatization. The radiant heat source is placed under the hind paw, and the paw withdrawal latency (PWL) is recorded as the time from the start of the radiant heat stimulus to paw withdrawal or licking. The mean PWL were determined from the average of 3 separate trials taken at 5 min intervals to prevent thermal sensitization (http://www.mitopain.com/pain/)- cited 12 October 2014.

Mechanical pain tests

Another factor that nociceptive tests follow is the stimulus threshold necessary to elicit an avoidance behaviour. The stimulus is either variable, with increasing value, or the test may use successive incremental stimuli at a fixed value. These tests concern mechanical stimulation and include the von Frey filaments, the Randall–Selitto analgesimeter, and recent tests based on strain gauges held by forceps or fingers (Barrot M, 2012).

Mechanical allodynia is measured by use of a series of calibrated von Frey filaments (Stoelting, Bioseb), which range in bending force from 0.008 to 300 g (typical force range used in rat testing is 0.4–15 g). The animal to be tested is placed on a wire-mesh floor with an inverted plastic shoebox-type rodent cage placed over it. In order to access the plantar surface of the animal's foot, the rodent cage and wire mesh flooring can be suspended on a stainless steel surgical instrument tray with the tray removed.

The von Frey filaments are applied starting in ascending order at right angles to the midplantar surface of the hind paw through the mesh floor. Each filament is applied to the foot until it bends. Once the filament bends, continued advancement produces more bending but not more force (Piel Margaret Jet al, 2013).

An electronic von Frey unit—a dynamic plantar aesthesiometer (Bioseb) is available that allows measurement of the sensitivity threshold in one test with high repetitiveness. It consists of a moveable touch-simulator unit, a framed metal mesh, a 2-compartment animal enclosure, and a microprocessor controlled electronic unit. The animal moves freely within the enclosure positioned on the metal mesh. Once the animal has acclimatized to the apparatus and ceased exploratory behaviour, the operator places the touch simulator below the animal's paw. The unit then automatically raises the filament at a preset force until a signal is received that the animal has either moved its paw or the greatest preset force has been met. Latency to paw withdrawal and force exerted are recorded (Piel MJ et al, 2013).

Randall Selitto test is based on the use of mechanical nociceptive stimuli applied to the paw or tail. The test consist of the application of an increasing mechanical force, in which the tip of the device is applied onto the medial portion of the plantar or the dorsal surfaces of both fore and hind paws until a withdrawal response results. Randall Sellito test, the tail flick test and the hot plate test are all sensitive to the training phenomenon – decrease in the pain response with repeated exposure of animals to experimental conditions (Santos NE et al, 2012).

Observation and scoring pain tests

Lastly, some nociceptive tests can rely on the observation and scoring of specific behaviours (Barrot M, 2012).The Mouse Grimace Scale (MGS) was developed based on the premise that animals are capable of demonstrating facial expressions suggestive of pain or discomfort. Such scoring systems are being developed for all domesticated species and provide a unique, validated adjunct to behaviour-based and reflex response systems for assessing pain in mice that is applicable to clinical and laboratory research settings. The MGS is particularly useful in the laboratory setting, where researchers and animal care staff may have limited knowledge of behavioural signs of pain in animals (Wiese Ashley J**,** submitted for publication).

For rating pain in rats, the MGS was adapted, because it was noticed differences between the two rodent species. Therefore, it appeared the rat grimace scale containing 4 units: *Orbital Tightening-r*ats in pain display a narrowing of the orbital area, manifesting either as (partial or complete) eye closure or eye "squeezing";*Nose/Cheek Flattening- r*ats in pain display successively less bulging of the nose and cheek (see above), with eventual absence of the crease between the cheek and whisker pads;. *Ear Changes-t*he ears of rats in pain tend to fold, curl and angle forwards or outwards, resulting in a pointed shape. The space between the ears may appear wider; w*hisker change-t*he whiskers of

rats in pain move forward (away from the face) from the baseline position, and tend to bunch, giving the appearance of whiskers standing on end (Sotocinal SG al,2011).

Irritant, algogenic, chemical agent based test

There are tests based on the use of long duration stimuli ("tonic pain") that involve using an irritant, algogenic chemical agent as the nociceptive stimulus. They differ from the vast majority of other tests in that they abandon the principle of determining the nociceptive threshold and involve a quantitative approach to the behaviour observed after the application of a stimulus with a potency that is going to vary with time. They can be thought of as a kind of model for tonic pain. However, they are not models for chronic pain because their duration is only in the order of some tens of minutes (Le Bars D et al, 2001).

The intradermic formalin test is one of these tests, which is predominantly used with rats and mice, involves moderate, continuous pain generated by injured tissue. In this way it differs from most traditional tests of nociception which rely upon brief stimuli of threshold intensity (Tjølsen A et al, 1992).

The formalin test in mice is a valid and reliable model of nociception and is sensitive for various classes of analgesic drugs. The noxious stimulus is an injection of dilute formalin (1% in saline) under the skin of the dorsal surface of the right hindpaw. The response is the amount of time the animals spend licking the injected paw (Hunskaar S et al, 1987). The response to formalin shows an early and a late phase (Tjølsen A et al, 1992). From the two distinct periods of high licking activity identified, there is an early phase lasting the first 5 min and a late phase lasting from 20 to 30 min after the injection of formalin (Hunskaar S et al, 1987).

The early phase seems to be caused predominantly by C-fibre activation due to the peripheral stimulus, while the late phase appears to be dependent on the combination of an inflammatory reaction in the peripheral tissue and functional changes in the dorsal horn of the spinal cord. These functional changes seem to be initiated by the C-fibre barrage during the early phase( Tjølsen A et al,1992).The intensities of these behaviours are dependent on the concentration of formalin that is administered (Rosland JH et al, 1990; Aloisi AM et al., 1995; Clavelou P et al, 1995).

## CONCLUSIONS

Although there are many means of studying pain, the phenomenon hasn`t been researched enough in Alzheimer`s demented persons. It should be a future purpose considering statistical indicators of people suffering from dementia is estimated at 35 million across the world.

The demographic changes and the high number of aging population will face us with the problem of finding an appropriate treatment and care, therefore we highlight the need of further research in this field.

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# CURRENT STATUS OF RESEARCH ON TRANSGENIC ANIMAL MODELS FOR ALZHEIMER'S DISEASE

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**Keywords:** Alzheimer's disease, genetics, transgenic mice, animal models.

**Abstract.** Each and every pathological visible effect is mostly due to molecular or biochemical reactions on cellular and intracellular level. This was the reason why in the last decade many incurable or poorly understood diseases were at least partially described. This is also the Alzheimer's disease case, extremely aggressive and highly unpredictable. Many studies showed various conclusions used in further research to develop treatments and biochemical therapies in order to slow the degenerative effects or to eradicate the means and motives of this disease. Generally, in order to cure a certain disease, one must surely know the mechanisms of actions involved in its pathology and the start point, also very important. In Alzheimer's disease, there has been studied an entire cohort of hypothesis and by using animal models the researchers reached possible cause variants. Because of the unknown idiopathic Alzheimer's disease etiology, animal models were based on genetic mutations associated with familial Alzheimer, supposing that following events are identical. These genetic models are highly valuable to elucidate the molecular mechanisms which determine the disease's progression, although no animal model can replicate the entire disease symptomatology. For this reason, every animal model type can be used to study one aspect of the disease. For example, transgenic mice overexpressing APP gene exhibit a similar pathology as humans. Age dependence and beta-amyloid accumulation speed was determined also by using transgenic mice. Beta-amyloid plaques discovered in transgenic mice brains were extremely similar to humans ones. Using the same manner of research, it was found a link between beta-amyloid plaques and alfa-synuclein. Plaques coloring method was also developed by various coloring techniques used on histological transgenic mice brain tissue smears. Alzheimer's disease pathology was well studied using transgenic animal models in order to characterize the molecular deficiencies observed in humans. Because of the incapacity of study on human subjects due to ethical reasons and specific human characteristics, several animal models have been developed, which facilitated the study Alzheimer's disease features one at a time.

## INTRODUCTION

Alzheimer's disease (AD), nowadays the main cause of dementia, affects over 12 million people worldwide; only 4.5 million of these are in America and there has been predicted to almost triple by 2050 (Herbert *et al.*, 2003; Citron, 2004). Transgenic animal models of AD provide an excellent tool for investigating pathogenic mechanisms and further treatments. Here, we review several animal models used in Alzheimer's disease research.

To begin, we will discuss the pathology of Alzheimer's disease that transgenic models are designed to follow. The first and most common symptom of AD is cognitive function loss (short memory loss). Patients also undergo general cognitive decline including temporal and geographic disorientation, impairment of judgment and problem solving, and deterioration of language abilities (Faber-Langendoen *et al.*, 1988). Behavioral and personality changes also occur until the end stages when troublesome behaviors disappear along with most personality traits (Rubin *et al.*, 1987; Swearer *et al.*, 1988). In the severe stages of the disease, motor complications often develop (Morris *et al.*, 1989; Romanelli *et al.*, 1990) which along with the dementia, leave people completely bedridden and dependent on caregivers.

Recently it has become obvious that AD starts decades prior to its clinical manifestation (Jack *et al.*, 2010; Sperling *et al.*, 2011; Bateman et al., 2012). Exploring the neuropathology of AD in human "pre-clinical" stages is not an easy task. This is the purpose of transgenic animal models – they can shed at least some light on the disease progression factors or on yet undescribed biomarkers or therapeutic targets. This article aims to review transgenic AD animal models currently used, effective or less effective and other relevant studies on the efficiency of transgenesis in AD research.

## NEUROPATHOLOGY AND GENETICS

In neuronal tissue, Alzheimer's disease causes an active neuron loss and a synapse number decrease, more often in cortex and sub cortical regions (Wenk *et al.*, 2003). This loss worsens in time leading to mass atrophy of the impaired regions to almost entire inactivation of parietal and temporal lobes, frontal cortex and cingulate gyrus (Braak and Del Tredici, 2012). This degenerative process also occurs in the nuclei of the locus coeruleus. Nuclear resonance studies show that

brain tissue of the Alzheimer patients tends to shrivel like a dried walnut as the patients' behavior worsens (Desikan *et al.*, 2009*;* Moan, 2009).

From a cyto-histopathological point of view, amyloid plaques (βAP) and neurofibrillary tangles (NFT) are highly visible during microscopy examination on Alzheimer's patients' brain tissue smears and sections using specific pigmentation or electronic microscopy. βAP are dense, mainly insoluble β-amyloid (βA) aggregations found particularly in extracellular compartment. NFT are tau protein aggregations formed by aberrant forms' hyperphosphorylation which leads to thread-like aggregates causing disorders in the microtubular system. These are common to most elders, but AD patients exhibit a more increased frequency of aggregate formation, mostly in temporal region. Also, is has been shown that Lewy bodies are quite common to Alzheimer disease but it seems that this is a secondary Alzheimer's effect (Bouras *et al.*, 1994; Kotzbauer *et al*., 2001).

Biochemically speaking, Alzheimer's disease remains to be caused by a characteristic protein missfolding process mainly causing temporal lobe βA accumulation and secondarily in other brain regions (Hashimotot *et al.*, 2003). The small proteins that form the plaques are mostly formed from 39–43 amino acids residues and are the final product of the amyloid precursor protein (APP) pathway. APP is a transmembrane protein which in association with membrane lipoproteins connects the extracellular and intracelullar environment in brain tissue (Priller *et al*., 2006). It seems that APP is involved in neuron maturation, survival and repair. The fact that explains its contribution to Alzheimer's development is yet to be discovered, but it is supposed that the poor APP split during proteolysis leads to smaller fragments than normal (Turner *et al.*, 2005). This may be caused by genetic faults of the APP or of any of the enzymes implicated in the pathway. βA forms extracellular aggregates that break synapse flow. Apart from this, there are other 42 residues fragments highly soluble in the cytosol which activate the casein kinase 2 involved synaptic function inhibition (Ohnishi and Takano, 2004; Hooper, 2005).

At the same time, a fault in the tau protein structure can occur, and cause a high adhesivity leading to tau aggregation with other cellular components. Neurons cytoskeleton is mostly formed by sub cellular structures called microtubules involved in neuron nutrient supplying from neuron soma through axon. Tau protein stabilizes cytoskeleton components structures and it is modulated by a phosphorylation-dephosphorylation mechanism. In Alzheimer, this protein undergoes a succesive phosphorilation serie which bring it in a highly active state protein which gain a supplemental binding activity and therefore supplemental adhesivity. This way NFT are formed and they lead to neuron instability caused by nutrient transport system disturbance. This can occur due to translation faults, posttranslational maturation errors or modulation process slip. The most possible picture is considered to be specific phosphorilase activity disturbance (Hernandez and Avila, 2007).

However, the exact pathogenic mechanism is mostly unknown from a molecular point of view and the way this mechanism is influenced by the βA is yet unclear (Van Broeck *et al*., 2007; Huang and Mucke, 2012). Some studies show that βA accumulation triggers neuronal degeneration, but others show that this accumulation is just another secondary effect (Yankner *et al*., 1990; Chen and Yan, 2006). On the other hand, it has been shown that the βA is an important component of the mitochondrial triggered apoptosis mechanism alongside ionic calcium homeostasis and glucose neuronal usage regulation (Greig *et al*., 2004).

Various research molecular approaches led to conclusions that can offer a starting point for further therapy development which can slow degenerative processes or stop disease trigger. Generally, in order to cure a disease, it is important to know for sure the pathogenic model and the triggering point. Regarding AD, there were many hypothesis of action that led to many possible cause variants. In present, partly because of the current affinity to molecular dimension of life, the biochemistry, genetics and molecular pathogenesis of diseases are mainly studied.

Current research approaches trigger the finding of the real cause. Since 2012, the safety and efficiency of over 400 therapy products has been tested in worldwide clinical trials, but under a fourth of them reached the third phase (Waldemar *et al*., 2007). This was due to the many individual variables as physiological differences, studies say, but the truth is that the predicted effects of the tested products were thought using assumptions not facts. Whether these compounds were not target specific, miss absorbed or metabolized, whether they triggered unpredicted immune reactions, this was possible because of the not fully known pathogenic mechanism. In the end, all clinical trials were suspended because of this and all scientific efforts were directed to disease pathway study.

## CURRENT RESEARCH ON ANIMAL MODELS

Current research directions regarding neurodegenerative diseases are mostly targeting diseases' molecular aspects. If until now researchers were desperately trying to find an efficient treatment through pharmaceutical means, now all attention is focused on finding the real cause of the diseases. Because pharmaceuticals only ameliorate the effects of the diseases and mainly of the lack of the real cause, most of the pharmaceuticals' researches were seized.

Many compounds were tested aiming for different pathological features such as βA levels reduction (apomorphine (Lashuel *et al.*, 2002), immunotherapy and APP-based vaccines (Hawkes and McLaurin, 2007; Dodel *et al*., 2010)). The most recent study uses cDNA coated nanoparticles which encodes a monoclonal antibody proven extremely dangerous to

APOEε4 allele carriers, but not as dangerous as another vaccine, ACC-001 (Woodhouse *et al.*, 2007), which can trigger meningoencephalitis development to more than 5% of the patients. The only partially functioning vaccine is bapineuzumab, structurally identical with the natural antibody induced in the βA presence (US NIH, 2008a). There have been tested many other ways of stopping AD development using TNFα receptor fusion proteins (US NIH, 2008b), tau protein phophorilase inhibitors or anti-inflammatory agents but all studies were abandoned (Tobinick *et al.*, 2006) and all attention was focused on animal model research and testing. The most important and developed research branch is the genetic manipulation and transgenic animal models research.

Because the idiopathic Alzheimer's etiology is mostly unknown, animal models research is based on mutation associated with familial Alzheimer's, thinking that the events following triggering mechanism are very similar. These animal models are extremely valuable in determining the molecular mechanism through which the disease evolve, but it has to be said that no animal model can resume all of the disease's features at the same time. Thus every animal model is designed to allow the analysis of only one feature of the disease per model.

Over time, researchers used many animal models based on many mammalian and non-mammalian animals. It is true that the invertebrates could not accurately reproduce AD pathology, but they were a low cost, easy breading alternative to explore cascade mechanisms triggered in AD. As these mechanisms were at least partly discovered, researchers developed more complex transgenic organisms such as mice and rats. Recently, it has been shown that the most eloquent animal model to study is the rat, not the mouse (Do Carmo and Cuello, 2013).

## NONRODENT TRANSGENIC MODELS

The most commonly used invertebrate model organisms in the study of brain neurodegeneration are *Drosophila melanogaster* and *Ceanorhabditis elaegans*, an insect and a worm. This is proving that it is not necessarily to study complex animals in order to find responses to complex questions. In fact there is more suitable to study as simpler animal models as we can to find the bases of the mechanisms we are trying to explain. As the evolution and adaptation of all organisms permits us to compare the simplest organisms with the more complex ones, researchers became convinced that it is more suitable to start from more simple animal models.

Therefore a team of researchers found that a homolog of APP is expressed in *Drosophila* and named it amyloid precursor protein-like protein (APPL) (Diagle and Li, 1993). Later they found that the round worm also expresses an APP homolog: the amyloid precursor-like protein 1 (APL-1) (Gunawardena and Goldstein, 2001). Both of these are similar to human APP except the amyloid precursor region. Furthermore, this study showed that expressing human APP in fruit flies and round worms induced neuronal apoptosis dependent upon the presence of the C-terminal and amyloid precursor regions. Studies of APP in *Coenorhabditis elaegans* show that expression of βA in body wall muscles induces progressive paralysis (Link, 1995; Fay *et al.*, 1998).

More than these, both fruit flies and round worms express presenilin homologs, and invertebrate studies have contributed to identification of secretase complex components (Chung and Struhl, 2001, Goutte *et al.*, 2002, Francis *et al*., 2002).

Tau protein has also been studied in both species. Overexpression of wild-type and FTD mutant tau in *Drosophila* results in adult-onset neurodegeneration but without neurofibrillary tangle formation (Wittmann *et al.*, 2001). Overexpression of tau with a tau phophorylation enzyme homolog induces neurofibrillary pathology, though with a different conformation than that seen in human. Coexpression of both tau and APPL in *Drosophila* leads to neuronal dysfunction and disrupted axonal transport. In *C. elaegans*, tau overexpression leads to aggressive pathological changes and behavioral abnormalities (Kraemer *et al.*, 2003).

There is another invertebrate used to study Alzheimer's disease, the sea lamprey, *Petromyzon marinus*. The studies on this fish were important because its central nervous system is characterized by six giant neurons. Microinjections in these were meant to induce chronic tau overexpression and led to rapid degeneration starting with distal dendrites (Hall *et al.*, 2001). Furthermore, with these tests, it has been identified a low molecular weight, lipid-soluble compound that retards the progression of tau-induced degeneration (Hall *et al.*, 2002).

## MOUSE TRANSGENIC MODELS

One of the first transgenic models of AD overexpressed amyloid precursor protein in order to reproduce amyloid pathology. This was based on the amyloid hypothesis that predicts that damaged amyloid synthesis pathway leads to Alzheimer-like pathology. Thus, amyloid precursor protein can be processed in two ways - fibrillogenic that leads to plaque accumulation and nonfibrillogenic, with cytosolic location. Familial mutations of APP are associated with accumulation of βA in senile plaques. In order to develop amyloid pathogenesis to mice, many researchers tried to genetically manipulate mice to overexpress human APP under specific promoters.

The most convincing mouse AD model is the PDAPP mouse (Games *et al.*, 1995) that overexpress human APP carrying V717P mutation under the control of PDGFβ promoter 10-fold higher than wild-type normal expressing endogenous APP

mice. It has been shown that they develop Alzheimer-like neuropathologies starting with hippocampus and then extending to cortical and limbic areas with regional specificity as seen in AD pathology (Masliah *et al.*, 1996; Irizarry *et al.*, 1997). Behavioral tests certified that amyloid pathology is age-dependent as it has been shown in human. More than that, it has been shown that the amyloid pathology in PDAPP mice is similar to that observed in AD even in ultrastructural details. Although entorhinal cortex, CA1, or cingulate cortex neuron loss was not observed (Irizarry *et al.*, 1997), the pattern of neuron loss was observed mimicking human AD patterns (Urbanc *et al.*, 2002).

Tg2576 mice line was another mice AD model that overexpressed APP double Swedish mutation (K670N and M671L) cDNA controlled by hamster prion protein promoter (Hsiao *et al.*, 1996). The APP production was lower than PDAPP mice, but the effects were very similar to those seen in AD. During behavioral tests it has been observed that some functional disruptions may underlie some of the observed memory deficits.

APP23 mice, APP cDNA with Swedish mutation under control of a murine promoter carriers, develop both amyloid plaques and cerebral amyloid angiopathy starting earlier that the other transgenic mice. Also, they develop memory deficits as assessed by behavioral tests (Lalonde *et al.*, 2002; Kelly *et al.*, 2003; Van Dam *et al.*, 2003).

There have been other attempts to genetically manipulate mice for AD study but they do not develop plaques until 18 months (line APP Swe C3-3) (Borchelt *et al.*, 1996; Borchelt et al., 1997), or trigger premature death (TgCRND8 mouse model) (Christi et al., 2001; Dudal *et al.*, 2004).

Besides APP mutations, there has been shown that presenilin genes mutations can trigger familial AD, probably by altering the processing of APP in favor of βA production to generate animal models. Overexpression of either M146L or M146V mutations under the PDGFβ promoter causes a selective increase in $\beta A_{42}$ production (Duff *et al.*, 1996; St George-Hyslop, 2000).

To model the NFT pathology, researchers have been developed tau transgenic mice. The tau protein located in axons, appears in AD hyperphosphorylated in cell bodies and dendrites. Mutations of MAPT gene cause FTDP-17 syndrome (frontotemporal dementia and parkinsonism linked to chromosome 17) by reducing tau's ability to bind to microtubules or splice the tenth exon increasing aberrant isoforms (containing 4 microtubule-binding domains) (Hutton *et al.*, 1998).

One of the first transgenic tau animal models was transgenic mice expressing wild-type 4 repeat human tau. Hyperphosphorylation of tau and aberrant localization, but not NFT, were observed in these mice (Gotz *et al.*, 1995). Other studies showed that regardless the gene promoter used, the transgenic mice developed hyperphosphorylation of tau and pathologies along association neurons' axons in central and spinal cord, but never NFT (Spittaels *et al.*, 1999; Gotz *et al.*, 2000; Probst *et al.*, 2000). These showed that overexpression of human wild-type tau is not sufficient to induce NFT. Rare NFT were observed in transgenic mice overexpressing wild-type human 3 repeat tau alongside hyperphosphorylation of tau in the hippocampus, amygdala, and entorhinal cortex, though very late in life.

After MAPT mutations discovery, many groups begun to develop animal models using them. Thus NFT formation was observed in P301L mutation mice carriers (JNPL3 line) in spinal cord, brainstem, cerebellum, diencephalon, and basal telencephalon (Lewis *et al.*, 2000; Arendash *et al.*, 2004)[67,68]. New P301L tau transgenic mice that can demonstrate progressive neurofibrillary pathology and neuronal loss in AD has recently been developed (Gotz *et al.*, 2001). In P301S transgenic mice were observed high expression of tau in spinal cord, brainstem, hippocampus, and neocortex, death of half of the motor neurons in spinal cord and hyperphosphorylation of tau, but no NFT (Santa Cruz *et al.*, 2003).

There have been developed many transgenic mice based on interactions between APP, tau protein, βA, presenilins and other risk factor genes that could explain why overexpression or mutations are not always enough for AD triggering. For example, the most common association demonstred via transgenic mice was the association between apolipoprotein E ε4 allele presence with increasing risk of AD seen in amyloid pathology context. Recent research show that ApoE may affect tau phosphorylation and accumulation processes. Overexpression of ApoEε4 and ApoEε3 induces aggregation of phosphorylated tau in regions vulnerable in AD of the brain of the transgenic mice (Brecht *et al.*, 2004). Changing expression promoter, they show that ApoEε4 may be neuron-specific (Brecht *et al.*, 2004). Other studies used conformational change knock down or up constructs showing that tau processing enzymes mught also be involved in AD pathology and NFT formation (Ahlijanian *et al.*, 2000; Lucas *et al.*, 2001)

## TRANSGENIC MICE DRAWBACKS

Using transgenic mice, many groups discovered the AD pathology features. Beginning with βA role, inflammatory responses, APP and presenilins functions, and ending with posttranslational processing of tau protein, all of which have been studied using different transgenic mice models. However, the hallmark of AD – massive or selective neuron death – has not been exhibited by any mice model published so far, with one exception (Santa Cruz *et al.*, 2003). It seems that massive neuron loss is a human specific feature of AD due to longer life or a more vulnerable brain tissue. More than that, the βA and NFT distribution in mice was never exactly reproduce to that in human. And, to be more accurate, recombinant DNA used in transgenic mice was under an exogenous promoter, in other words, there weren't any regulating native sequences in the transgenic construct (Aronov *et al.*, 1999). Also, in order to mimic the exact human

conditions, there must be done a transgenic construct that can express all of the six tau isoforms. This can be done by constructing an entire tau human gene expressing model but it has been observed that this is correlated with a three or four-fold increase in tau mice brain concentration and absence of murine tau expressing, and in absence of a tau mutation, these mice did not develop any aberrant phenotype (Duff *et al.*, 2000).
There has been made a correlation between cognitive performance and tau and βA pathology in behavioral studies and the possibility of βA plaques removal in immunological studies, but the precise mechanism and relationship between removal and memory function is not fully understood.
Despite all the limitations mentioned, transgenic mice have been extremely valuable in AD research (Hartley *et al*., 1999). However, the production of transgenic mice takes time and resources and it has been proved an inefficient task. This is true for single transgenic mice, but even more pronounced in two or three transgenic lines. However, the transgenic strategies are never abandoned but improved. Given that there are many species in the rodent family, it seems that there is another valuable rodent available for research – the rat.

## THE RAT – THE NEW AD MODEL

Along time, mice have always been preferred in transgenesis studies in spite of the rats mainly because of the size and needs. More than that, rat's zygotes are more demanding regarding the transgene injection perform (Charreau *et al.*, 2004) and success. The gene replacement or loss of function mutation strategies (knock in/knock out) are hampered by the difficult to obtain rat embryonic cells. These few drawbacks are insignificant compared to the numerous advantages.
Firstly, between rat and mouse, the rat is physiologically, genetically and morphologically closer to humans than mice (Lin, 1995; Jacob and Kwitek, 2002; Gibbs *et al*., 2004). Because it is larger, the intraventricular administration of different drugs and cerebrospinal fluid sampling is easier (Tesson *et al.*, 2005).
Biochemically speaking, rat genome contains informations for the expression of six tau isoforms, as human genome does too (Hanes *et al*., 2009), although there is a slightly difference between major isoforms ratio. More than that, both human and rat share almost 73% of the ApoE protein amino acid sequence (McLean *et al*., 1983; Rajavashisth *et al.*, 1985), and the rat ApoE features are similar to ApoE3 in human (Tran *et al.*, 2013).
Behavioral speaking, it is well known that the rats are behaviorally well characterized. It has been shown that they have finer and more accurate motor coordination than mice and their behavioral display is more observable than in mice. They express social behavior and age-related aggression (Whishaw *et al.*, 2001). Since rat's life environment is a complex one, combining both terrestrial and aquatic elements, behavioral test on land or water is more facile (Whishaw *et al*., 2001). These differences are possible because of the post-natal brain development occurred both in rats and humans, but not in mice.
Thus rat models should permit a more complex characterization of behavior, cognition and pathology levels. More than that, they are more valuable tool in drug and therapies testing. Based on all these advantages, rats are increasingly used to mimic pathological hallmarks of Alzheimer's disease (Taravini *et al*., 2011; Kitamura *et al*., 2011; Nuber *et al.*, 2013). What is the most important is that there have been developed certain transgenic rats models that are a more accurate representation of the human pathology model.

## CONCLUSIONS

Alzheimer's disease pathology was well studied using transgenic animal models in order to characterize the molecular deficiencies observed in humans. Because of the incapacity of study on human subjects due to ethical reasons and specific human characteristics, several animal models have been developed, which facilitated the study Alzheimer's disease features one at a time. Over time, researchers used invertebrates such as fruit fly and common round worm and vertebrates such as the sea lamprey or mammals to mimic pathologies of interest, but it seems that a more valuable tool have been discovered for genetic manipulation and study – the rat. It has been shown that the rat posses certain features that make him closer to human than the previously used animal models.

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# CURRENT KNOWLEDGE OF PAIN INVOLVEMENT IN ALZHEIMER`S DISEASE

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**Abstract:** Alzheimer`s disease is one of the most encountered dementia, since according to Alzheimer`s Association it represents approximately 60-80% of all types of dementia. It is a progressive neurodegenerative disorder which affects memory, cognitive processes, communication abilities and produces important mood changes.
A complex psycho-physiological process, pain, is a unique for every individual, being described by IASP (International Association for the Study of Pain) as “an unpleasant sensory and emotional experience associated with actual or potential tissue damage, or described in terms of such damage”(1979).
There have been few studies made on pain involvement in patients with Alzheimer`s disease, making it a field that raises interest more and more.
In this way, patients with Alzheimer`s disease report less clinical pain than their cognitive intact peers. Moreover, patients suffering from Alzheimer`s disease are administered fewer analgesics, as compared with unaffected cognitive subjects with similar level of painful disease or injury. Also, according to the newer hypothesis, perception and pain processing are affected in Alzheimer`s disease and are not diminished as some older studies stated, raising questions about the ways that is dealt with pain in this highly dependent and vulnerable patient group.
However, it is still unclear whether the observed difference in pain report and management occurs as a result of impaired communication and memory of pain, and/or whether the perception and experience of pain is altered as a result of the progressive degeneration of cortical and subcortical regions involved in the transmission and processing of nociceptive information.

## INTRODUCTION

Alzheimer`s disease (AD) is characterized by a progressive cognitive decline, where memory of recent facts, spatial orientation, attention and executive functions are ones of the first affected. This is followed by speech and behavioural problems, which affect everyday life (Almkvist O,1996; Benedikz E et all 2009). Pain is a subjective phenomenon, described by IASP as an unpleasant sensory and emotional experience associated with actual or potential tissue damage, or described in terms of such damage.

Alzheimer`s disease affects both cognitive and affective functions, and the global pain experience is known to result from a complex integration of sensory, cognitive and affective processes (*Melzack and Casey*, 1968). Assessment and management of pain in dementia is problematic for at least three reasons: first, there are difficulties in pain assessment in patients progressively less able to communicate; second, neurodegenerative changes affect pain processing at different levels; third, different subtypes of dementia show specific changes along the pain pathways(Scherder E et al,2009; Scherder E et al 2005; Carlino E et al, 2010).

Although it is a known fact that age is the highest risk factor for both dementia and pain, this issue has only recently started to be studied, despite its physiological and clinical relevance in the aging population(Scherder E et al,2009; Scherder E et al 2005; Carlino E et al, 2010) One of the major conclusion during the last 2 decades of studies focusing on the experience, assessment , and treatment of pain in older persons with dementia, is that pain is still undertreated in this population due to the complexity of reliable pain assessment, which is a prerequisite for effective pain treatment(Scherder E et al,2009; Nanda de Knegt et al, 2011).

The purpose of this article is to follow pain perception in AD, and its numerous aspects, through a wide range of studies and to highlight the necessity of understanding pain phenomenon in AD, but most important, to recognize it, so that methods of treatment can be performed.

## GENERAL ASPECTS OF ALZHEIMER`S DISEASE

Alzheimer disease-factors that arise or contribute to its occurrence

Alzheimer disease, one of the most common dementia, is characterized by pathological changes in the brain. They consist of abundant extracellular amyloid plaques (deposits of β-amyloid) and intracellular neurofibrillary tangles (NFTs), accompanied by synaptic and neuronal loss, and brain inflammation (Ball MJ,1977; Braak H et al,1991; Scheff SW et al,2007; Schultzberg M et al,2007; Benedikz E et al,2009).

Synaptic loss is one of the strongest reasons of cognitive impairment in patients with AD. Several lines of investigation support the notion that the synaptic pathology and defective neurogenesis in AD are related to progressive accumulation of Aβ oligomers rather than fibrils(Crews L et al,2010). Aβ is associated with the formation of reactive oxygen (ROS) and nitrogen (RNS) species, and induces calcium-dependent excitotoxicity, impairment of cellular respiration, and alteration of synaptic functions associated with learning and memory (Querfurth H.W et al,2010; Butterfield D.A et al,2014). The Aβ peptide can consist of 39-43 amino acid residues, but the two major forms are Aβ40 accounting for approximately 90% of all the Aβ released from cells and the longer Aβ42 accounting for only approximately 10%.

Aβ42 is more hydrophobic and more prone to aggregation than Aβ40 (Verdile G et al,2004), and is the predominant form found in the amyloid plaques of AD (Lippa CF et al,1998; Benedikz E et al,2009).

Increasing evidence suggests a role for caspase activation and apoptosis in AD neuropathogenesis (Holtzman DM et al,1997; Lunkes A et al,1998; Namura S et al,1998; Kim TW et al,1997; Loetscher H et al,1997; Barnes NY et al,1998; Kovacs DM et al,1999; Su JH et al,1994; Su JH et al,1997; Tesco G et al,2007; Haijun Shao et al,2014; .Mattson MP et al,2002; Raina AK et al,2003), even though the contribution of apoptosis to neuronal loss in AD remains debatable (LeBlanc AC et al,2005;Cribbs DH et al,2004; Haijun Shao et al,2014).

A recent study suggest that caspase activation even without apoptosis can contribute to AD neuropathogenesis (Haijun Shao et al,2014; Yao J et al,2009).

Mitochondrial dysfunction may also contribute to AD neuropathogenesis (Haijun Shao et al,2014;Querfurth HW et al,2010; Zhu X et al,2013; Louneva N et al,2008). Furthermore, a recent study has shown that AD patients may have an age-dependent decrease of gama-aminobutyric acid (GABA) currents in the AD brain, and this reduction was associated with decreased mRNA and protein levels of GABA receptor subunits(Limon A et al,2012).

A large body of evidence implicates oxidative damage in AD pathogenesis (Beal M.F. et al,2005; Solfrizzi V. et al,2006; Padurariu M et al, 2009). It is believed that oxidative damage to critical molecules occurs early in the pathogenesis of AD and precedes pronounced neuropathological alterations (Baldeiras I et al,2008; Lovell MA et al,2007; Halliwell B,2007; Padurariu M et al, 2009).

What is oxidative stress?

Oxidative stress is defined as the biomolecular damage caused by the attack of reactive species upon constituents of living organisms (Halliwell B et al,2004).

The oxidative stress refers to a serious imbalance between reactive species production and antioxidant defences (Halliwell B,2007; Sies H et al,1991). As described by Sies, oxidative stress is a disturbance in the pro-oxidant-antioxidant balance in favour of the former, leading to potential damage (Halliwell B,2007; Sies H et al,1991).Chemically speaking pro-oxidants are oxidant agents and antioxidants are reduction agents.

Chemical reactions of oxidation and reduction happen on a common basis in the organism and are named redox reactions. During redox reactions a process of giving and accepting electrons is taking place. In physiological conditions there is a balance between oxidation and reduction. When this balance is broken, oxidative stress arises. That is the moment when free radicals appear, producing oxidative damage.

What are Free radicals?

They are an atom or a molecule that have in their structure an unpaired electron. This fact causes their instability, and makes them possess a high energy. Their quest is to find a matching structure that will bring back the missing electron, the equilibrium state being characterized by paired electrons and therefore less energy. In nature there is a large variety of free radicals, making their classification difficult. Depending on their structure there are several types of free radicals, and most studied are: superoxide anion, hydroxyl radical, hydrogen peroxide, nitric oxide, peroxyl and reactive aldehyde. The difference between free radicals, which depends on their structural and biochemical features, is crucial because it confers the compound its oxidative power, i.e. its toxicity.

Depending on their oxidation power, there are two types of free radicals: free radical with lesser reactivity and more aggressive free radicals, with larger reactivity.

Sources of free radicals

The sources of reactive oxygen species are many and varied and have not yet been fully identified. The free radicals are the result of diverse physiological and pathological processes not only endogenous but also exogenous, such as aging, excessive caloric intake, infections, inflammatory states, environmental toxins, certain drugs, emotional and psychological stress, tobacco smoke, ionizing radiation, alcohol or unbalanced nutrition (Ranjana et al., 2012). One of the main endogenous source of free radicals is the respiratory chain that takes place in the mitochondria, but they can also be synthesized by activated microglia. Microglial activation is a type of immune response to some brain lesions, a process that involves the generation of cytotoxic compounds such as superoxides that maintain a vicious cycle of neuronal damage (Nakajima et al., 2001; Padurariu M et al,2013).

Effects of oxidative stress

Cells show a wide range of responses upon exposure to reactive species, ranging from increased proliferation, prevention of cell division, senescence, necrosis, apoptosis, or cell death mechanisms with features of both (Halliwell B et al,2004; Tang SY et al,2004). The effects are to some extent cell-type-specific, being influenced by such parameters as the presence of certain cell-surface receptors and signal transduction mechanisms, as well as antioxidant defence levels (Halliwell B et al,2004; Halliwell B,2003; Burdon RH et al,1990; Burdon RH et al,1995).
Most free radical injuries concern lipidic structures, in particular the polysaturated fatty acids, which are produced from the lipid peroxidation reactions (Padurariu M et al, 2013).It should be noted that there are also other structures vulnerable to oxidative attack, of which DNA and proteins are definitely worth mentioning (Padurariu M et al, 2013). DNA oxidative changes may include alterations varying from nitrogenous base losses to DNA repairing system damage. The highly toxic hydroxyl radical can easily access the cell nucleus causing the degradation of various nitrogenous bases such as guanine, adenine and pyrimidine, with the formation of toxic compounds such as hydrodeoxyguanosine, hydroxiadenine, peroxide thymine or glycol thiamine (Padurariu M et al, 2013 ).
The oxidative stress theory explains neuronal death as caused by free radicals that attach and change composition of neuronal fat molecules, altering membrane fluidity and permeability and disturbing some of the membrane functions, such as transport and barrier-like functions. The consequences of these disturbances are directed mainly towards the traffic of $Ca^{2+}$ ions that cross the membrane structure, with the alteration of the signal transduction processes (Rowan et al., 2004; Padurariu M et al, 2013).
It is now understandable that free radicals are involved in many diseases, including cancer and atherosclerosis, chronic inflammation and diabetes (Halliwell & Gutteridge 2007; Evans 1993). The role of oxidative stress in neuropsychiatric disorders is well known, including schizophrenia, Parkinson`s Disease, Alzheimer dementia, anxiety or bipolar affective disorder (Uttara et al., 2009; Padurariu et al.,2010, Ciobica et al, 2010,2011,2012, Stefanescu et al., 2012).
Brain is particularly vulnerable to oxidative stress as a result of the relatively low levels of antioxidants, high levels of polysaturated fatty acids and increased need of oxygen (Padurariu M et al, 2010; Burdon RH et al,1990).

Antioxidative system

Against the oxidative attack the organism has its own developed mechanism to fight it. It consists of an arsenal of processes that offer protection against oxidative stress, called the antioxidative system. The elements that compose the antioxidative system are: antioxidant enzymes and non-enzymatic factors. Between the antioxidant enzymes most mentioned are superoxide dismutase (SOD), glutathione peroxidise (GPX), catalase or aldehyde dehydrogenase. These enzymes catalyze the reaction of reduction of free radicals, conducting to diminishing their power and also the oxidative cytotoxicity.

Non-enzymatic factors may be considered homeostatic role molecules that act as “scavengers” towards the pro-oxidant compounds. Their origin has been described as endo- or exogenous and they include: uric acid, glutathione, lipoic acid, bilirubin, melatonin, ascorbic acid, beta-carotene, bilirubin, selenium, NADPH mannitol, benzoate, reduced CoQ10 or tocopherol. Glutathione appears to be most important, reducing lipid peroxidation processes by directly blocking the activity of reactive species. Also, it maintains vitamins E and C in reduced forms, conferring them antioxidant proprieties (Singh et al., 2004; Padurariu M et al, 2013).

Biological indicators of oxidative stress are determined in various biological fluids (such as blood, cerebrospinal fluid ,urine, saliva, tissue level) since measurement of free radicals isn`t possible, we follow the resulting compounds of the oxidative process. The DNA oxidation markers 8-oxo-2`-deoxyguanosine and 8-oxoguanine and lipid peroxidation markers represented by 4-hydroxynonenal, malondialdehyde or F2 isoprostanes. Furthermore, increases in the specific activities of antioxidant enzymes, such as SOD2 in the hippocampus, especially in CA1 region and the amygdale (Massaad et al. 2011) have been observed (Padurariu M et al, 2013).

The causal relationship between oxidative stress and the changes identified in dementia has not yet been fully elucidated. It is not known which is the primary etiological factor, whether oxidative stress is a consequence if the degeneration processes of dementia or the oxidative compounds produce the characteristic lesions in dementia (Padurariu M et al, 2013).

## PAIN- GENERAL ASPECTS

What is pain?

Pain is a subjective multidimensional experience that can have a marked impact on both physiological and psychological state of an individual. The IASP(International Association for the Study of Pain) definition of pain considers it more than a purely sensory modality but as a perception that needs cognitive processing for pain to be consciously experienced (Burdon RH et al ,1995).

Pain mechanism

At somatic and visceral level there are receptors, named noxic receptors or nociceptors composed of free arborescent endings of afferent fibers. Nociceptors can be divided into two general types. A-fiber nociceptors have lightly myelinated axons, conduct action potentials rapidly, and have medium to large-diameter cell bodies. A-fibers mediate the fast, pricking quality of pain. C-fibers have unmyelinated axons, conduct action potentials slowly, and have small-diameter cell bodies. C-fibers mediate the slower, burning quality of pain. C-fibers comprise around 70% of all nociceptors.

Two classes of C-fibers have been identified. One class contains a variety of neuropeptides, including substance P and calcitonin gene-related peptide, and expresses trkA receptors, the high-affinity receptor for nerve growth factor (Stucky C et al,2001).These neurons project to the outermost region of the spinal dorsal horn (lamina I and outer lamina II) and terminate largely on spinal neurons that project to higher-order pain centers in the brain(Stucky C et al,2001). The other class contains few neuropeptides but expresses a surface carbohydrate group that selectively binds to a plant lectin called isolectin B4 (IB4). This subpopulation of neurons is supported by glial-derived neurotrophic factor during early postnatal development (Stucky C et al,2001). The IB4-binding neurons project to a different region of the spinal dorsal horn (inner lamina II) that contains primarily local spinal interneurons (Stucky C et al,2001).

In addition to the Aδ and C fibres that carry noxious sensory information, there are primary afferent Aβ fibres that carry non-noxious stimuli. Each of these fibre types possesses different characteristics that allow the transmission of particular types of sensory information. Aδ and C fibres synapse with secondary afferent neurones in the dorsal horn of the spinal cord. The dorsal horn can be divided histologically into ten layers called Rexed laminae. Aδ and C fibres transmit information to nociceptive -specific neurones in Rexed lamina I and II, in addition to projections to other laminae. Primary afferent terminals release a number of excitatory neurotransmitters including glutamate and substance P. Complex interactions occur in the dorsal horn between afferent neurones, interneurones and descending modulatory pathways. These interactions determine activity of the secondary afferent neurones. Glycine and gamma-aminobutyric acid (GABA) are important neurotransmitters acting as inhibitory interneurones. There are two main pathways that carry nociceptive signals to higher centres in the brain.

The spinothalamic tract: secondary afferent neurones decussate within a few segments of the level of entry into the spinal cord and ascend in the contralateral spinothalamic tract to nuclei within the thalamus. Third order neurones then ascend to terminate in the somatosensory cortex. There are also projections to the periaqueductal grey matter (PAG) (Danielle Reddi et al, 2014).The spinothalamic tract transmits signals that are important for pain localisation.

The spinoreticular tract: fibres also decussate and ascend the contralateral cord to reach the brainstem reticular formation, before projecting to the thalamus and hypothalamus. There are many further projections to the cortex. This pathway is involved in the emotional aspects of pain.

The somatosensory cortex is important for the localisation of pain. However, imaging techniques such as functional magnetic resonance imaging (fMRI) have demonstrated that a large brain network is activated during the acute pain experience. This is often called the 'pain matrix'. The commonest areas activated include the primary and secondary somatosensory(S1 and S2), insular, anterior cingulated cortex and prefrontal cortex, and the thalamus, demonstrating that these areas are all important in pain perception (Danielle Reddi et al, 2014).

## PAIN IMPLICATIONS IN ALZHEIMER DISEASE

Not surprisingly, several studies have shown alterations of both acute and chronic pain in AD and demented patients (Farrell et al., 1996). In an observation report of two patients with AD who had experienced trauma of different

kinds, neither of the patients exhibited normal pain behaviour or gave verbal reports of pain commensurate with the tissue damage they had incurred.

Although, various types of physical trauma have been observed occurring in patients- burns, fractures, invasive tumours, herpes zoster – all capable of creating different types of pain and involving a variety of different types of structures (nerves, soft tissue, bone, superficial skin, deep tissues and so on) none of them showed signs of normal pain perception (Fisher-Morris M. et al, 1997).

It has been observed that the incidence of headache following lumber puncture in demented patients is only 2% (Blennow et al., 1993) compared to 40% of non-demented patients (Knutz et al., 1992). Similarly, Cornu (1975) found a modification of the body image which resulted in a poor localization of noxious stimuli. In a recent study (Benedetti et al., 1999), it was found that tolerance to electric shock pain and ischaemic arm pain was increased in AD patients, whereas pain thresholds were unchanged compared with controls.

These data indicate that sensory-discriminative component of pain is preserved in demented patients whereas pain tolerance, which is associated with the affective-emotional counterpart of pain experience (Prince, 1988), undergoes significant changes. Porter et al. (1993,1996) found altered heart rate responses prior to, during and following venipuncture in elderly patients with poor cognitive abilities and in demented patients, suggesting altered emotional responses (Rainero I et al, 2000).

A study conducted by Rainero I et al, 2000, during which a series of clinical tests were ran on AD patients aimed at recording heart rate, systolic blood pressure and pain perception after electrical stimulation. The results of the investigation confirmed the notion that pain processing is altered in dementia, also stated in other studies (Cornu, 1975; Jonsson et al., 1997; Blennow et al, 1993; Farrell et al., 1996). The findings of Rainero I et al, 2000, indicate that the autonomic responses to noxious stimuli depend on stimulus intensity and not on the pain experience *per se*. When pain stimulation is mild autonomic responses are blunted and pain perception is normal, whereas when pain stimulation is strong autonomic responses are almost normal (e.g. blood pressure) and pain experience is blunted. This suggests that threshold for autonomic activation is increased in AD patients (Rainero I et al, 2000).

Cole et al, 2006, 2011 used pressure pain stimuli and found an increased threshold for just noticeable pain(Jensen-Dahm C et al,2013). Others have also suggested that the perception of acute pain is preserved, while the experience of chronic pain may be altered (Pickering et al., 2006; Cole LJ et al, 2006).

These contradictory findings might be attributable to methodological differences in regard to pain induction, pain assessment, and severity of AD. Another explanation is that it is unclear whether the methods are appropriate in patients with AD. Patients with AD have impairment of short-term memory and may have difficulties understanding simple instructions (Jensen-Dahm C et al, 2014).

In 2014, a study to evaluate some aspects of reliability (i.e. Coefficient of variation) to know if the prior used methods in different studies were appropriate, was conducted. The team examined the test-retest reliability and agreement of different pain sensitivity models using quantitative sensory testing, i.e. , assessments of thermal and mechanical thresholds, and assessments of tolerance to cold and pressure stimuli, in patient with AD. The results of the study concluded that patients with mild to moderate AD were able to reliably cooperate with standardized thermal and mechanical pain sensitivity tests, compared to age- and gender-matched control group. The pain thresholds did not differ between AD patients and control subjects, but a significantly lowered mechanical pain tolerance was observed in AD patients (Jensen-Dahm C et al, 2014).

Patients with AD might express their pain and discomfort behaviourally, as agitation, aggression, pacing, wandering, screaming, yelling, and sleep disturbances, although these behaviours are often not recognized as symptoms of pain but instead as behavioural and psychological symptoms of dementia (Coehen-Mansfiel J et al, 2012) so that individuals with dementia are more likely to receive psychotropic medication and not pain medication, to treat these manifestations of pain (Kamble P et al, 2009).

A randomized controlled trial reported that the use of a stepwise pain protocol based on the treatment recommendations of American Geriatrics Society significantly reduced behavioural disturbances and pain in individuals with moderate to severe dementia (Husebo BS et al, 2011), indicating the adequate treatment of pain reduces behavioural disturbances, but currently available drugs for management of pain such as acetaminophen, nonsteroidal anti-inflammatory drugs, opioids, antidepressants and antiepileptic drugs often not effective or cause serious adverse reactions in older persons.

Recent studies have demonstrated the potential of cannabinoids, including delta-9-tetrahydrocannabinol (THC) and cannabidiol (CBD), to manage the symptoms of pain and dementia (Lynch ME et al, 2011). Two canabinoid receptors, CB1 and CB2, mediate the psychoactive, behavioural and analgesic effects of cannabinoids. CB1 receptors are highly expressed in the cortex, basal ganglia, cerebellum and hippocampus whereas CB2 receptors are mainly expressed in the immune system (Baker D et al, 2003). Cannabinoids also interact with other receptors and neurotransmitters in the brain, such as acetylcholine, dopamine, gamma-aminobutyric acid, serotonin, glutamate, norepinephrine, prostaglandins and opioid peptides (Baker D et al, 2003). This wide range of interactions reflects the potential pharmacological effects of cannabinoids in the management of behaviour, mood, and pain(Ahmed A et al, 2014).

Accurate assessment of pain is crucial for adequate pain management. In turn, this requires a reliable and valid observational tool for assessing pain in nonverbal individuals with dementia in clinical and nonclinical settings (Ahmed A et al, 2014).

Many brain areas affected in AD are relevant for pain transmission, for example, the amygdala, the hypothalamus, the thalamic intralaminar nuclei, the prefrontal regions (Mann DMA et al, 1988). Enhanced fMRI pain-related activity in sensory and affective brain areas has been observed in mild AD (Cole LJ et al, 2006) and both facial responses to pain (Kunz M et al, 2007; Lints-Martindale AC et al, 2007) and nociceptive motor reflexes (Kunz M et al,2009) have been found to be preserved or even increased in heterogeneous group of cognitively impaired patients (Carlino E et al, 2010).

Patients on a psychogeriatric ward where pain prevalence and intensity were found to be lower than on a somatic ward, received less pain medication than patients on the somatic ward, even when these pain parameters were matched (Achterberg WP et al,2007; Scherder E et al, 2009).

In one study, patients with dementia (subtype not otherwise specified, NOS) recovering from hip fracture surgery received only 1/3 the amount of morphine sulphate equivalents administered to non-demented adults and 76% of patients with dementia had no standing order for post-operative analgesia (Scherder E et al, 2009; Morrison RS et al, 2000).

In another study, only 33% of AD patients received appropriate analgesic medication compared to 64% of non-demented adults (Scherder E et al, 2009; Scherder EJ et al, 1997).

## CONCLUSION

As most of the studies show, it is a fact that pain perception is altered in AD. Some argue that the patient doesn`t realise the amount of pain he is experiencing or he is experiencing less pain of a certain kind. Others believe that pain manifestations are different from the ones that non-demented persons experience. Altogether, the available studies, suggest undertreatment of pain in patients with Alzheimer disease, which is not a surprise considering the complexity of this cognitive disorder and the intricate pathways of the pain phenomenon.

Therefore, we emphasise on the need of further studies in this complex matter of pain perception in Alzheimer dementia, so that patients with AD suffering from pain can be treated adequately.

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# THE CONSEQUENCES OF THE CONGENITAL INFECTION WITH *TOXOPLASMA GONDII* ON THE OFFSPRING

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**Abstract.** Statistically, 90% of the pregnant women who are also infected with *Toxoplasma gondii* do not show any symptoms. During the parasitemia that is consecutive to the primary site of infection, the parasite disseminates to the placenta through blood, so the infection can be transmitted to the fetus through the placenta. Most of the times, the routine examination of the newborns, does not show the usual signs of congenital toxoplasmosis. The study aims to determine the frequency of cases of congenital toxoplasmosis, to assess the health of babies and children from mothers with acute infection, and also to illustrate the consequences of the infection with *Toxoplasma gondii* on the offspring and to correlate them with the gestational age. This prospective study was conducted on a total sample of 152 pregnant women who were hospitalized and investigated in “Elena Doamna” Iasi Clinic of Obstetrics and Gynaecology. In the period of time between 2001 and 2014 we identified a number of 108 children (69%) showing a damage of the central nervous system (CNS) and 44 children (28%) with generalized manifestation of the disease. The mortality rate recorded in these children was 12%, without any significant differences between those with CNS damage and those with generalized disease ($p<0.05$). About 85% of the survivors developed mental retardation, 75% convulsions, spasticity, paralysis and 50% impaired vision. More than 80% of these children have an IQ < 70, most of them showing convulsions and severely impaired vision. The cutaneous manifestations of congenital toxoplasmosis include: rash, petechiae, bruising or massive hemorrhage secondary to thrombocytopenia. Also present in other congenital infections, the consequences on the offspring will be all the more severe as the transmission of the parasite toxoplasma infection from the pregnant woman to the foetus occurs earlier in pregnancy.

## INTRODUCTION

Toxoplasmosis, a parasitic disease that often shows no symptoms in the mother, has important repercussions on the baby and is more frequent than it is statistically estimated. The data published in literature show a level of sero-prevalence of 30-40%, higher in occupational exposure categorized as risk of infection with this parasite (Abbasi, 2003; Ministerul Sănătății, România, 2011).
Because of its extent and distribution, its frequency in the general population and also because of the severity of manifestations in adults and especially in the newborns, because of the difficulties in getting a diagnosis of certainty, toxoplasmosis is a matter of real importance in the work of health units.

**Table 1.** Serological diagnosis of congenital infection with *T. gondii* in the newborn (Remington, 1990)

| Infant age | Examination performed | Remarks |
|---|---|---|
| First day of life | Serological testing to determine specific IgM, with cord blood testing and peripheral blood confirmatory study.<br><br>Comparison of the immunological maternal and cord levels. | IgM-ISAGA and DS-IgM- ELISA testing is positive in 70% of the cases with congenital toxoplasmosis.<br>RIF-IgM testing is positive in just 25% of the cases with congenital infection.<br><br>The difference is significant if titer in the cord blood is four times higher than the one in maternal blood. |
| 15 days | IgM testing in the child’s blood. | The positive result in the cord blood that turns into a negative result for the peripheral blood harvested afterwards suggests the possibility of contamination with maternal blood.<br>The negative result in the cord blood that turns into a positive result for the peripheral blood harvested afterwards suggests the transmission of the infection shortly before birth. |
| 1,2,4,6,8 | Comparison of immune levels in | An increase or the maintaining of a steady level proves the |

| Infant age | Examination performed | Remarks |
|---|---|---|
| months | recent samples in relation to the previous samples. | synthesis of specific antibodies by the child's body.<br>A decrease in the antibodies level suggests the absence of their synthesis due to the absence of infection or the reduced antigenic stimulus, proving the efficiency of the treatment (the immune level monitoring is useful in assessing the effectiveness of the treatment). |

The transplacental transmission rate of *T. gondii* increases in relation with the age of the pregnancy at the moment when the primo-infection occurred (Rădulescu, 2000; Silveira, 2003).
The speciality literature cites an average of 14% - 17% cases of congenital transmission from the total number of cases with acute infection in pregnancy for the first trimester of pregnancy. The percentage increases in the second trimester to 25%, in the third trimester it goes over 59%, and in the last three weeks of pregnancy the transmission of the parasite occurs in over 90% of the cases with acute infection. (Couvreur, 1983; Lupea, 2000).
In congenital toxoplasmosis, the parasite shows an obvious tropism for the central nervous system and for the ocular system, regardless of other systemic sites of infection, more or less obvious. This preferential localization does not seem to result from an organo-tropism, but from a weaker strength of the brain and ocular tissue. The importance of lesions at these levels is amplified by the reduced capacity for regeneration of these tissues compared with the remarkable regenerative capacity of the other tissues in the body.
Rarely, *T. gondii* can be located in the lungs, causing an interstitial pneumonia; in the myocardium, causing necrosis and inflammation; the parasite can persist as a parasitic cyst. At pericardium level it causes acute, then chronic pericarditis; in the kidney, it induces glomerulo-nephritis by antigen-antibody immune complexes. When the parasite is located in the liver or spleen, the organism reacts by hepatosplenomegaly. Other locations mentions, rarely met, are: cortico-suprarenal, pancreatic, in the digestive tract, thyroid, thymus, ovaries or testes, in teguments, skeletal muscles and bone marrow (Popoviciu, 1993; Rădulescu 2000, Stănilă, 1996; Stamatin, 2003).
There can also be some endocrinological abnormalities by affecting the hypothalamus, pituitary gland or peripheral glands. Studies mention and describe myxedema, persistent hypernatremia with Vasopressin-Sensitive Diabetes Insipidus, with no polyuria or polydipsia, precocious puberty, and hypopituitarism (Lloyd, 2013).

## MATERIAL AND METHODS

The study was performed on 152 children, coming from mothers hospitalized and investigated in "Elena Doamna"Iasi Clinic of Obstetrics and Gynecology, with a definite diagnosis of neurological impairment (n=108) and systemic impairment (n=44), following an intra-uterine infection with *T.gondii*.

**Table 2. Signs and symptoms that appear after the diagnosis in the children with acute congenital toxoplasmosis**

| Signs and symptoms | Frequency of events (%) for the subjects with | |
|---|---|---|
| | Neurological disease | Systemic disease |
| Newborns | 108 subjects | 44 subjects |
| Chorioretinitis | 102 (94%) | 29 (66%) |
| Pathologic LCR | 59 (55%) | 37(84%) |
| Anaemia | 55 (51%) | 34 (77%) |
| Convulsions | 54 (50%) | 8 (18%) |
| Cerebral calcifications | 54 (50%) | 2 (4% ) |
| Icterus | 31 (29%) | 35 (80%) |
| Fever | 27 (25%) | 34 (77%) |
| Splenomegaly | 23 (21%) | 40 (90%) |
| Lymphadenopathy | 18 (17%) | 30 (68%) |
| Hepatomegaly | 18 (17%) | 34 (77%) |
| Microcephaly | 14 (13%) | 0 |
| Vomiting | 17 (16%) | 2 (48%) |
| Diarrhea | 7 ( 6% ) | 11 (25%) |
| Cataract | 5 ( 5 %) | 0 |
| Glaucoma | 2 ( 2 %) | 0 |

| Signs and symptoms | Frequency of events (%) for the subjects with | |
|---|---|---|
| | Neurological disease | Systemic disease |
| Newborns | 108 subjects | 44 subjects |
| Microphtalmia | 2 ( 2 % ) | 0 |
| Eosinophilia | 6 ( 4 %) | 8 (18 %) |
| Pathologic bleeding | 3 ( 3 %) | 8 (18 %) |
| Hypothermia | 2 ( 2 %) | 9 (20 %) |
| Optic nerve atrophy | 2 ( 2 %) | 0 |
| Cutaneous rash | 1 (1 % ) | 11 (25%) |
| Pneumonia | 0 | 18 (41%) |

The macroscopic examination reveals a pale and edematous placenta, with an increased volume. Microscopic examination shows free or cystic parasites that are located in the villous, especially in the stroma, trophoblast, in the endothelial cells of the chorionic vessels and Wharton’s jelly, and also in the decidua. Villous lesions are mononuclear inflammatory infiltrate type, histiocytic and with giant cells. Chorionic villi edematous degeneration is relatively frequent.

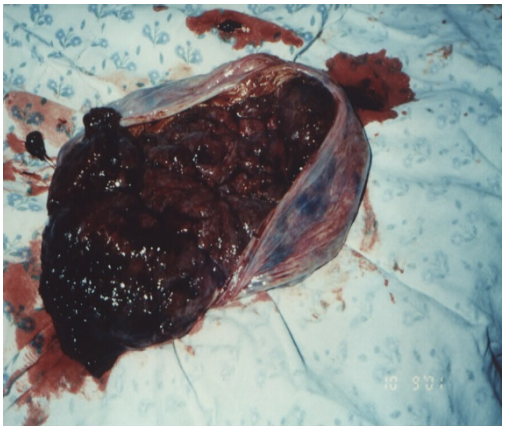

**Fig. 1. Placenta. Macroscopic aspect post-infection with *Toxoplasma gondii* (“Elena Doamna” Iaşi Hospital collection)**

Ocular fundus examination may also reveal a papillitis that is secondary to the adjacent inflammation or a papilledema that is consecutive with the hydrocephalus. Extended lesions can cause the central atrophy of the retina. The parasites with an intracellular location can be found in all choroid and retina layers. The choroid appears thickened, with hyperemia, infiltrated. When the choroid is affected, it frequently causes damage to Bruch’s membrane and proliferation of connective tissue in the sub retinal space. As a result, the retina and the choroid will be fixed to one another by a scar.

## RESULTS AND DISCUSSIONS

Congenital infection was suspected, most often following a serological screening of pregnant women with acute infection with *T. gondii*. 21 cases were discovered (13.8%) as having severe congenital toxoplasmosis involving the central nervous system, the visual system and also showing general systemic lesions. 56.7% of them manifested a mild form of the disease, with a normal general clinical examination except retinal scarring and intracranial calcifications.

If the foetus gets infected with *T. gondii* when the pregnancy age is 10–24 weeks, the consequences will be extremely serious. They will be: congenital malformations, severe impairment of the central nervous system, systemic damage, and lead in most cases to spontaneous abortion, intrauterine death, prematurity and dysmaturity, severe neonatal infections

with plurivisceral touch, hemorrhagic syndrome, icterus, hepatosplenomegaly, encephalopathies with lethal ending in 5–15% of the cases, or to progressive sequelae of a fetal disease with hydro- or microcephaly, intracranial calcifications and chorioretinitis.

**Systemic damage**. From the total number of children with a disease that was clinically manifested at birth, 25-50% are born before term, getting a low Apgar score. This group includes those children with intrauterine growth retardation and instability in temperature control. Other systemic manifestations include: lymphadenopathy, hepatosplenomegaly, myocarditis, nephritic syndrome, vomiting, diarrhea and eating disorders. There can be some transparent metaphyseal bands and irregularities of the provisional calcification line of the epiphyseal plate without periosteal reaction in the ribs, vertebrae and femur. (Ambulatory Child Health, 2000).

The **central nervous system** impairment is manifested by diffuse or focal meningoencephalitis, accompanied by tissue necrosis, microglial nodules, perivascular mononuclear inflammatory infiltrates.

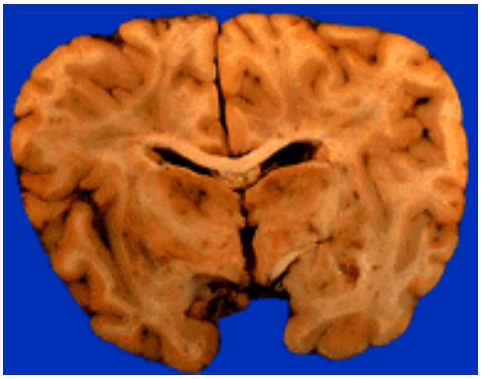

**Fig. 2**. **Toxoplasmic encephalitis.** Multiple outbreaks of hemorrhagic necrosis and cerebral edema (www.gtmer.ch)

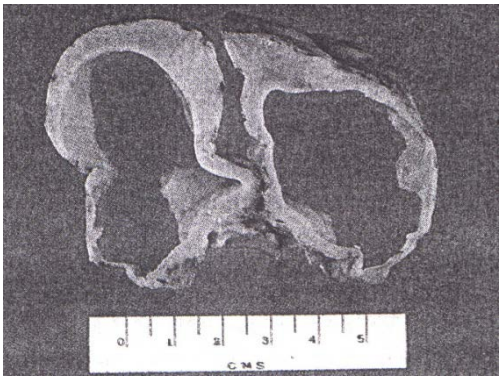


**Fig. 3**. C**ongenital toxoplasmosis**. Ventricular dilatation postencephalitic fasciitis (Umberto de Girolami, 1999)

**The neurological** m**anifestations** of congenital toxoplasmosis vary from massive acute encephalopathy to subtle neurological syndromes. Toxoplasmosis must be considered the cause of an undiagnosed neurological disease in children under one year old, especially if retinal lesions are present.
Hydrocephaly can be the only manifestation of congenital toxoplasmosis and it can exist either compensated, or requiring the execution of a shunt during the perinatal period or later.

**The eyes** are affected mainly by chorioretinitis. It is estimated that both in the U.S.A. and in Europe, *T. gondii* is the most common etiologic agent of chorioretinitis, causing about 35% of the cases (Brezin, 2003; Pinon, 2003).
Most children with congenital toxoplasmosis will develop subsequent episodes of chorioretinitis, if not treated. (Leblebicioglu, 2002).

Toxoplasmic chorioretinitis is, most frequently, the consequence of a congenital infection and very rarely of an acquired one (1%); the maximum frequency is attained between 20-40 years old and very rarely over 50 years old.

In **toxoplasmic chorioretinitis** the lesion is characterized by the appearance of macular plaque of chorioretinitis, usually for both eyes. The atrophic oval plaque has net pigmented slightly protruding margins. The centre is white, and the sclera is visible because of the chorioretinal necrosis. The parasite is present in the lesion and the more numerous it is, the more severe the lesion. Occasionally it can be observed without an associated inflammatory reaction, in apparently normal areas of the retina, at the periphery of inflammatory foci, alone or in groups, free inside the cell or as a cyst and rarely in the choroid. It was described as being located in the tissues of the optic nerve and optic nerve head. More rarely met in the vitreous body, the hemorrhages can be accompanied by signs connected to the appearance of strabismus, nystagmus, the paralysis of the VIth pair of cranial muscles and optic atrophy.

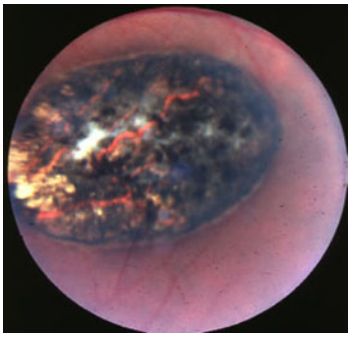

**Fig. 4. Chorioretinitis in congenital toxoplasmosis (www.opt.indiana.edu/.../Text3beta.html)**

**Liver damage/impairment**, manifested through icterus, or **lung impairment** manifested through interstitial pneumonia, can be present, and so can the secondary **edemas** of myocarditis or the nephritic syndrome. Icterus and conjugated hyperbilirubinemia can persist for months (Lloyd, 2013).
The severity of the consequences in the congenital infection decreases gradually if it occurred further from the $26^{th}$ week of pregnancy. Thus, if the transplacental infection occurs in the last trimester of pregnancy, the newborn can show a subclinical form of infection at birth, which can be overlooked. In the absence of an adequate treatment that is applied immediately, the infection will develop mild forms of the disease in 85% of cases, immediately afterwards or in childhood. The sequelae can be: *ocular* (isolated microphtalmia, strabismus, episodes of chorioretinitis with different degrees of vision impairment, up to blindness), *neurological* (hypotonia, transitory drowsiness, delays in the physical and mental development, *hepatic* (icterus at an early age of a few weeks).

## CONCLUSIONS

The prevalent signs and symptoms for the children with congenital toxoplasmosis were: staturo-ponderal hypotrophy, convulsive syndrome, microcephaly, psychomotor retardation of different degrees, ophthalmologic changes (mainly chorioretinitis).

In the infections that occur shortly before conception and in the first 10 weeks of pregnancy, the congenital transmission rate of *T. gondii* to the offspring is very low, below 1%. If the infection occurred, this will result in over 90% of the cases in a pregnancy stopped in evolution, death and miscarriage.

There is therefore an inversely proportional relationship between the incidence of fetal infection and the severity of the destruction caused by it, in relation to the gestational age.

It must be mentioned that once the placental infection occurs, the placenta will stay infected throughout pregnancy, even after overcoming the phase of toxoplasmic parasitemia.

About 2/3 of the cases with congenital toxoplasmosis do not show the known symptoms, as there are oligosymptomatic forms of the disease, and often the diagnosis of toxoplasmosis is generally established in the chronic phases of the disease, unaccompanied by high or increasing titers of antibodies. That is why, a large scale serologic screening programme should be considered in order to prevent and decrease the severe consequences met in congenital toxoplasmosis.

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# THE ANALYSIS OF CYTOCHROME B SEQUENCES FOR *BISON BONASUS* (ARTIODACTYLA: BOVIDAE) INDIVIDUALS FROM VÂNĂTORI-NEAMȚ AND NEAGRA-BUCȘANI NATIONAL PARKS

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**Abstract:** Mitochondrial DNA (mt-DNA) has proven to be a useful tool in molecular phylogenetic studies, because evolutionary relationships can be inferred in depth, between recently divergent groups, populations, species, and even individuals. In this experiment we investigated the genetic variability of *Bison bonasus* individuals from Vânători Neamț and Neagra Bucșani National Parks, by analyzing *cyt b* nucleotide sequences. The study allowed us to identify the haplotypes, the nucleotide frequencies, mt-DNA divergence and phylogenetic relationships within studied populations. In this study we observed 3 haplotypes at Vânători Neamț population and 2 haplotypes at Neagra Bucșani population.

## INTRODUCTION

DNA-sequence data from the mitochondrial genome are frequently used to estimate phylogenetic relationships among animal taxa. The advantage to using DNA-sequence data is that many of the processes governing the evolution and inheritance of DNA are already understood (Brown et al. 1979). DNA data, however, do not guarantee the correct phylogenetic tree because of problems associated with shared ancestral polymorphisms and multiple substitutions at single nucleotide sites (Simon et al. 1994). Another characteristic of mt-DNA, generally accepted as a real advantage for populational genetic studies, is the lack of recombination (Wilkinson and Chapman 1991, Loftus et al. 1994, Ishiba et al. 1995). The majority of the animals have sexual reproduction. After the recombination process and the independent segregation of chromosomes during the first meiosis, genes from both parents are transmitted to the daughter cells, but because organelle genes (such as mt-DNA) are uniparentaly transmitted, these don’t recombine (Birky, 2001).

The biggest mammal species in Europe, the European bison (*Bison bonasus* L.), went through a severe bottleneck at the beginning of the 20th century. Reintroduction of wild populations started in the 1920s and it was based on a few animals from private and public collections. After World War I, the survival of this species was secured only in a few European zoological gardens (Sztolcman 1924). In total, only 54 individuals with proved pedigrees remained (29 males and 25 females), descending from 12 animals (Slatis 1960; Raczynski 1978; Pucek 1991).

After an intensive breeding period in the zoological gardens, the first animals were released in the Bialowieza National Park, in 1952, which is the core of the Bialowieza Forest, stretching across eastern Poland and western Belarus. With approximately 540 animals, the Bialowieza bison population is globally the largest (Kita et al. 2003; European Bison Pedigree Book - EBPB 2001). In 2000, 2864 individuals were registered worldwide (EBPB 2001; Hilton-Taylor 2000).

Tokarska et al. in 2011 suggest that European bison has been the subject of extensive genetic studies. The low level of genetic variability has been widely confirmed by genetic analyses using a variety of methods. However, there is no evidence that this will inevitably lead to extinction. In 2006 Anderung et al. developed a study based on ancient mt-DNA of just four European bison from the 13th to 14th centuries and revealed two haplotypes. Another analysis conducted by Wojcik et al. in 2009 revealed three haplotypes for 200 individuals of a 1429 base pair of control region. An earlier study based on the analysis of the variability of mt-DNA sequence in the European bison by Burzynska et al. (1999) revealed eight distinct haplotypes of a 1026 base pair fragment of control region.

The Vânători Neamț Natural Park and Neagra Bucșani Reservation are two protected areas established in 1999, as a site of the Nature 2000 ecological network, with both communitarian and avi-faunistic protection importance and one of the objectives is the release of the bison in its natural milieu. The bison is the symbol of the Carpathians and the most impressive herbivorous animal in Europe (800-1000 kg/ex. the male and 500-700 kg/ex. the female).

The aim of this study was to investigate the genetic variability of two Romanian bison populations Vânători Neamț and Neagra Bucșani, in order to provide new information on the genetic diversity of this species. Mitochondrial *cyt b* analysis was used for 23 individuals. We identify the total number of haplotypes, the nucleotide diversity, and phylogenetic relationships within studied populations.

## MATERIAL AND METHODS

**Biological material**:

The studied individuals of *Bison bonasus* are originated from two different Romanian protected areas: Vânători Neamț National Park and Neagra Bucșani Reservation. Sixteen European bisons, with random age, were sampled during 2010 to 2014. From both areas the same number of individuals was analyzed: eight from Vânători Neamț National Park (seven adults and one death calf) and another eight adults from Neagra Bucșani Reservation.

The analyzed samples consist of blood or muscle tissue. The blood samples have been collected through puncture of the jugular vein, after the animals were tranquilized for translocations, 2 ml Vacutainer-type tubes being employed. One muscle tissue sample has been collect from a death calf, fresh blood sample being unavailable. The muscle tissue was sampled by cutting with a bistoury a small fragment from the leg. The blood samples were stored in Queen's Lysis buffer (Seutin et al., 1991) and muscle tissue in 98% ethanol.

For analyzing the phylogenetic relationships and quantify genetic diversity within the studied populations, DNA had to be isolated, amplified and sequenced.

**DNA extraction and polymerase chain reaction**:

Total genomic DNA was isolated from biological material by proteinase K treatment followed by phenol chloroform extraction (Gorgan, 2007, 2008). DNA was eluted in TE buffer (pH=8.0) and kept at -20° C. Polymerase chain reaction (PCR) was used to amplify the entire *cyt b* gene.

Two specific primers were used (Watanobe et al., 1999), and their sequences were: $mitL_1$ 5'-ATCGTTGTCATTCAACTACA-3', $mitH_2$ 5'-CTCCTTCTCTGGTTTACAAG-3'. The cycling conditions consisted of an initial denaturation step at 94°C for 4 min followed by 40 cycles of 94°C for 30 sec, 50°C for 45 sec, 72°C for 1 min and a final extension step at 72°C for 10 minutes.

**The sequencing process** was based on the dye-terminator Sanger method (Sanger et al. 1977) using the CEQ 8000 Genetic Analysis System (Beckman Coulter, Switzerland) and process according to manufacturer protocol.

**Sequences analysis:** For this study we used 16 *cyt b* sequences obtained from our individuals and another 7 sequences were downloaded from GenBank (accession numbers are listed in Table 1) and included in this analysis. The phylogenetic relationships were determined by the Neighbour-Joining method (Saitou 1987) and the evolutionary history was inferred using the Maximum Composite Likelihood method. The complete *cyt b* sequences were aligned using Clustal W (Thompson et al., 1994) in the MEGA 5.0 (Tamura et al., 2011) phylogenetic package.

Table 1 Accession numbers from GenBank

| GenBank accession | Species | Location | Reference | Length (bp) |
|---|---|---|---|---|
| AY079126 | *Bison bonasus* | Artis Zoo, Amsterdam | [1] | 1140 bp |
| HM045017 | *Bison bonasus* | Wrocław Zoo, Poland | [2] | 1140 bp |
| NC014044 | *Bison bonasus* | Wrocław Zoo, Poland | [2] | 1140 bp |
| JN632602 | *Bison bonasus* | Paris, France | [3] | 1140 bp |
| AY689186 | *Bison bonasus* | Paris, France | [4] | 1140 bp |
| Y15005 | *Bison bonasus* | Frankfurt zoo, Germany | [5] | 1140 bp |
| HQ223450 | *Bison bonasus* | - | [6] | 1140 bp |

[1]Verkaar,E. L. et. al., (2004);[2]Zeyland J et. al., (2004);[3]Hassanin A. et. al., (2012);[4]Hassanin A.et. al., (2004);[5]Zimmermann S. et. al, (1998);[6]Derr, J. N.-(2010), Direct submission to NCBI.

## RESULTS AND DISCUSSION

**Haplotype data**

After the sequence analysis, we identified the existence of 6 different haplotypes. Haplotype frequencies were determined using DnaSP 4.50.3 software (Rozas et al., 2003).

For Vănători-Neamț population 3 haplotypes were observed. Haplotype 1 (H1) is the most common, and it was observed at four male individuals (*Bb01m, Bb02v Bb04m,Bb05m, Bb03m*) and at two females (*Bb04f* and *Bb06f*). Haplotype 2 (*Bb03f*) and haplotype 3 (*Bb02f*) were found only in the Vânători Neamț population.

Because we not found other cyt b sequences on GenBank for comparison, we can affirm that so far both of them are local haplotypes. Haplotype diversity (Hd) for this population is 0.46. The average number of differences (K) is 1.75.

For Neagra Bucşani population we identified only 2 haplotypes. Haplotype 1(H1) has a high frequency, and it was detected at seven individuals: four males (*Bb02m, Bb03m, Bb06m,* and *Bb07m*) and 3 females (*Bb06f, Bb07f* and *Bb08f*). A single individual (*Bb01f*) presents haplotype 4 (H4) so we can conclude that this is a local haplotype.

Haplotype diversity in this population is 0.25, a much lower value compared to haplotype diversity from Vânători Neamț, where the same parameter is 0.46. This means that at Neagra Bucşani, the population presents a lower genetic diversity, with only two haplotypes in comparison to Vânători Neamț population, where three haplotype were identified.

For the sequences from GenBank, 3 haplotypes were found (H1, H5 and H6). Similar to the other two populations, haplotype 1(H1) is the most common one, being found at 5 individuals (AY079126, HM045017, NC014044, JN632602, AY689186). The fifth haplotype is Y15005, and it was identified at an animal in Frankfurt zoological garden (Zimmermann et al. 1998). The sixth haplotypes is HQ223450.

In Romanian National Parks, haplotypes H5 and H6 were not found. A possible cause may be the existence of two separate genetic lines of *Bison bonasus* species (Lowland and Lowland-Caucasius). In Romanian natural reservations, all the bisons originate from the Lowland Caucasius genetic line.

**Nucleotide diversity**

To calculate the nucleotide diversity (Pi) for both populations we used DnaSP 4.50.3 software (Rozas et al., 2003).We can observe (Figure 1) that the alignment of *cyt b* sequences for population 1 (P1) contains a great nucleotide variability. Pi increases at the beginning of the sequence, and after nucleotide position 800, it remains constant. The number of polymorphic sites for P1 is 7 and the average number of nucleotide differences is k=1.750. We calculated nucleotide diversity for P1 and obtained Pi= 0.00149. The total number of nucleotides differences for population 1 (P1= Vânători Neamț population) is 7.

For the second population, Pi varies at the beginning of alignment, until nucleotide position 100. After this, the nucleotide diversity is invariable but it increases again near the end of the sequences. The number of polymorphic sites for P2 is 2, the average number of nucleotide differences is k=0.500 and the nucleotide diversity is Pi=0.00043. The total number of mutation for P2 is 2. (P2 = Neagra Bucşani population).

**Total data**: Regarding the nucleotide diversity we can say that the total number of polymorphic sites for both populations is 9. The nucleotide diversity Pi(t) is 0.00096. The average number of nucleotide differences between populations is k=1.125. The average number of nucleotide substitution per site between populations is Dxy= 0.0009.

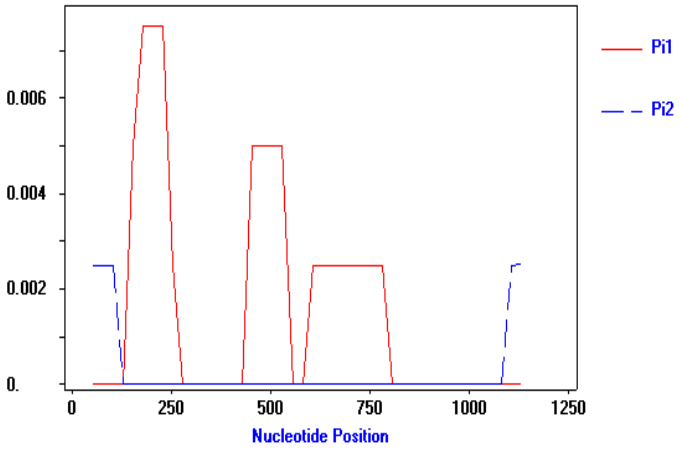


Figure 1. Nucleotide diversity for P1 and P2 (Pi1= nucleotide diversity for P1; Pi2=nucleotide diversity for P2

**Network Haplotypes estimations**

Using Network software, v.4.621 (Polzin et al., 2003) we could create the haplotype network (Figure 2). Five haplotypes were identified in our study; however, this surprisingly high variability was not confirmed in further studies. Only Burzyńska et al. 1999 observed eight haplotypes after developed a study on 14 specimens. They followed genetic variation on mitochondrial DNA D-loop sequences.

Historical studies on ancient mtDNA of just four European bison from the 13th to 14th centuries by Anderung et al. (2006) revealed two haplotypes. Extensive analyses of the 1429 bp sequence of the control region (D-loop) performed on nearly 200 individuals showed that there are just three haplotypes Wojcik et al. 2009.

Based on our study haplotype 1 has a high level of frequency for the analyzed individuals. It was identified for eighteen European bisons, thirteen of them belonging to the two Romanian populations (Vânători Neamț and Neagra-Bucșani). Also, haplotype 1 occupies a central position with the highest number of connections. It is likely to be the ancestor for the other 5 haplotypes during the expansion. H3, H4 and H6 are the closest to the H1, differing from it by the presence of a single nucleotide. H2 and H5 are two important haplotypes with a great number of transversions and transitions. For example between H1 and H2, we identified five substitutions, and between H1 and H5 seven substitutions and three indels were observed.

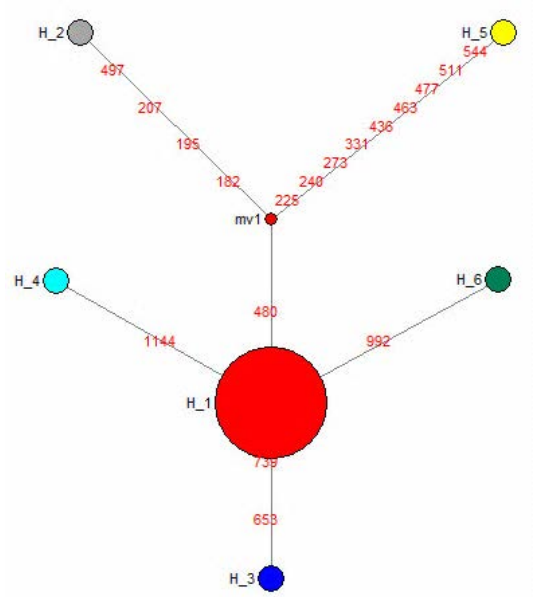


Figure 2. Maximum Likelihood Network Haplotypes estimations

**Maximum Likelihood Estimate of Substitution Matrix**

Each entry represents the probability of substitution (r) from one base (row) to another base (column). Substitution pattern and rates were estimated under the Tamura-Nei (1993). Rates of different transitional substitutions are shown in **bold** and those of transversional substitutions are shown in *italics* (Table 3).

Table 3. Maximum Likelihood Estimate of Substitution Matrix

| From\To | A | T | C | G |
|---|---|---|---|---|
| **A** | - | *2.40* | *0.99* | **31.42** |
| **T** | *1.98* | - | **7.26** | *2.17* |
| **C** | *1.98* | **17.57** | - | *2.17* |
| **G** | **28.63** | *2.40* | *0.99* | - |

Relative values of instantaneous r should be considered when evaluating them. For simplicity, sum of r values is made equal to 100.

The nucleotide frequencies are A = 26.24%, T/U = 31.83%, C = 13.14%, and G = 28.79%. For estimating ML values, a user-specified toplogy was used. The maximum Log likelihood for this computation was -2027.098. The analysis involved 24 nucleotide sequences. All positions containing gaps and missing data were eliminated.

There were a total of 1172 positions in the final dataset. Evolutionary analyses were conducted in MEGA5 (Tamura-Nei., 2011).

## CONCLUSIONS

The study of genetic variability in two *Bison bonasus* populations from Romania inferred from the cytochrome b gene revealed the following:

For the alignment of the 23 sequences of *cyt b*, six haplotypes were identified. There were identified 3 haplotypes in population of Vânători-Neamț, and 2 haplotypes in Neagra Bucşani population. We consider that the presence of one more haplotype in the Vânători Neamț population is probably due to the introduction of new individuals (received from Germany, Switzerland and France) in the last years.

For the sequences downloaded from GenBank, 3 haplotypes were observed.

Haplotype diversity (Hd) is higher for Vânători-Neamț population (0.46), comparative with Neagra Bucşani population, which is 0.25.

The most common haplotype is H1, observed at 18 individuals, so we can assume that it is probably the oldest haplotype. The identified haplotypes seems to radiate from H1 suggesting a conservation of this genetic form through the time and a starting point for other haplotypes. The convergence of H2 and H5 into a lost ancestral haplotype mv1, support our previous assumption that actual individuals of *Bison bonasus* evolved from a H1 like genetic group. Our point of view is also confirmed by historical records of European bison repopulation strategy which started from two genetic lines: one specific to Poland and another from Romania (Olech et al. 2008).

In the two studied populations we found 5 haplotypes, the fact which shows an important genetic variability taking into account the small number of individuals analyzed.

Nucleotide diversity is higher in Vânători-Neamț population (0.00149), comparative with Neagra-Bucşani populations (0.00043).

The nucleotide diversity between *cyt b* sequences shows a high similarity, but the presence of major differences and the presence of the substitutions and indels confirm that this species presents a high genetic variability.

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**RECENZIE**

## Grigorie Stefanescu - *Gregor Mendel*. Editura ”Risoprint”, Cluj-Napoca, 2014, 306p.

Prin Declarația din aprilie 1964, România acelor vremuri lua cumva distanță față de ”marele ei prieten” de la Răsărit - URSS, fapt salutat cu entuziasm de întregul popor român, dar mai ales de către tânăra generație. Până atunci, chiar și unele domenii ale științei avuseseră de suferit, au fost bântuite de dictatură. Într-o astfel de situație s-a aflat Genetica, disciplină devenită după 1953 cea mai dinamică și mai fecundă ramură a biologiei în lume, ale cărei descoperiri de marcă au fost onorate cu numeroase premii Nobel. Între 1948 și 1964, genetica adevărată, mendelo-morganistă, fusese înlocuită în fosta URSS, și implicit la noi, cu o aberație, cu așa-zisa genetică miciurinistă, promovată de către un impostor în știință (T. D. Lîsenko). M-am aflat chiar în prima generație de studenți biologi la Universitatea ”Al. I. Cuza” din Iași care, în 1966, audiam un curs (de un semestru) de genetică autentică, susținut de profesorul Corneliu Zolyneak. Pe atunci, în bibliotecile de profil nu existau manuale sau cărți de acest gen, pentru că ele erau interzise, așa încât am trăit o mare satisfacție, o adevărată revelație aflând de la profesorul meu cât de interesantă și incitantă este genetica - știința eredității și variabilității lumii vii.

O carte recent apărută, intitulată ”*Gregor Mendel*”- semnată de prof. dr. Grigorie Ștefănescu de la Universitatea de Nord din Baia Mare, mi-a amintit de momentul mai sus relatat și de impactul imens pe care l-ar fi avut în acele vremuri genetica și legile eredității stabilite de Mendel asupra elevilor, studenților și profesorilor. O carte ca aceasta ar fi fost pur și simplu devorată de cititorii avizați. Eu cred că și acum ea este binevenită și va fi bine primită pentru că, deși au trecut aproape 5 decenii de la repunerea în drepturi a geneticii în România, nimeni de la noi nu a încercat să-i dedice o lucrare de amploare primului și celui mai genial dintre geneticieni - Johann Gregor Mendel. Lucrarea nu conține doar o prezentare critică a operei marelui savant, ci și multe aspecte din viața acestuia, școlile urmate, greutățile cu care s-a confruntat de-a lungul vieții, neîmplinirile, cunoștințele acumulate până la el în domeniul hibridării la plante (de către predecesori), contextul vremii în care a lucrat, scrisorile adresate de Mendel lui K. W. Nägeli (un alt hibridolog al epocii, care nu l-a înțeles) etc, aspecte ce vor stârni și mai mult interesul cititorilor.

By The Declaration of April 1964, Romania of those times somehow took distance from "her great friend" from the East - USSR, an act welcomed with enthusiasm by the entire Romanian people, but mostly by the younger generation. Until then, even some fields of science were affected and haunted by the dictatorship. In such a situation was Genetics, a discipline that has became after 1953, the most dynamic and fecund branch of Biology in the world, whose remarkable discoveries have been honored with numerous Nobel Prizes. Between 1948 and 1964, the true Genetics, Mendelist-Morganist theory, was replaced in the ex- USSR, and implicitly in our country, with an aberration, the so-called Michurinist Biology (Genetics), promoted by an impostor in science, T. D. Lisenko. In 1966, as biologist student at the University "Al. I. Cuza" of Iasi, I was attending an authentic Course of Genetics (during one semester), presented by Professor Corneliu Zolyneak. At that time, there were no manuals or books of this kind in specialized libraries, because they were forbidden, so I lived a great satisfaction, a true revelation by finding out from my professor how interesting and exciting is Genetics - the science of heredity and variability of the living world.

A recently published book entitled "*Gregor Mendel*" - signed by PhD Prof. Grigorie Ștefănescu from North University of Baia Mare, reminded me about the time above-mentioned, and the huge impact that Genetics and the Laws of Heredity set by Mendel would have had on students and teachers, in those times. A book like this would have been simply devoured by interested readers. I think that is welcomed and now, and it will be well received, because although nearly 5 decades have passed since the reinstatement of Genetics in Romania, nobody from our country tried to dedicate the first and the most genial among geneticists - Johann Gregor Mendel, a major work. This book includes not just a critical presentation of the work belonging to this great scientist, but many aspects of his life, the attended schools, difficulties encountered throughout his life, unfulfillments, knowledge gained till him about hybridization of plants (by the predecessors), the times when he worked, the letters adressed by Mendel to Carl Nägeli (another hybridologist of that time, who did

În plus, autorul a avut ideea salutară de a insera în cartea sa lucrarea de căpătâi a lui Mendel ”*Versuche über Pflanzen Hybriden*” (”*Experienţe asupra hibrizilor la plante*”) şi vor putea ei înşişi s-o analizeze şi interpreteze, lucrare ale cărei concluzii fuseseră prezentate în 1865 în cadrul Societăţii de istorie naturală din Brno şi publicată un an mai târziu în revista aceleiaşi societăţi.

Lectura cărţii ne va procura prilejul de afla informaţii interesante şi inedite despre locul natal şi familia lui Mendel, despre studiile efectuate de acesta înainte de a intra în mănăstire, despre dificultăţile materiale prin care a trecut pentru a se instrui, despre faptul că a fost un elev strălucitor şi că, pentru a-şi putea continua studiile, pentru a experimenta şi verifica unele din ideile sale, dar şi din convingeri religioase, a urmat cariera ecleziastică, fiind acceptat ca novice în Mănăstirea augustină ”Sf. Toma” din Alt Brün (Brno, Cehia). De reţinut că intrarea în mănăstire nu era o simplă formalitate, ci presupunea cunoştinţe solide din partea candidaţilor, Johann Mendel concurând în 1843 pe 4 locuri cu alţi 12 aspiranţi. Ca monah al mănăstirii, Mendel va primi prenumele de fratele Gregorius. Starețul mănăstirii, Franz-Cyrill Napp, era un prelat luminat, care promova ştiinţa şi încuraja cercetarea ştiinţifică în mănăstire (în special în agricultură). Mănăstirea ”Sf. Toma” dispunea de o bibliotecă remarcabilă, de colecţii de minerale şi plante, în 1830 părintele Napp a înfiinţat o grădină experimentală în curtea mănăstirii, iar mai apoi (în 1844) o seră. Mendel îşi va desăvârşi pregătirea teologică (1844-1848) la Institutul Teologic din Brno, dar în paralel (din 1846) a frecventat şi cursuri de agricultură, pomicultură şi viticultură la Institutul de flilozofie din acelaşi oraş. Între 1851 şi 1853 Mendel urmează o serie de cursuri la Universitatea din Viena, pentru a căpăta dreptul de a preda fizica şi istoria naturală (la gimnaziul din Znaim), unde a avut o serie de profesori renumiţi ai vremii în domeniul fizicii, matematicii, anatomiei şi fiziologiei plantelor, zoologiei, chimiei etc. Într-o lucrare dedicată genezei operei marelui savant (publicată în 1984 în ”La Recherche”), J. L. Serre consideră că o înrâurire specială asupra lui Mendel au avut probabil profesorii Franz Unger – adept al metodei reducţioniste şi analitice în cercetare (spre deosebire de doctrina la modă, promovată de Lorenz şi susţinută de Goethe şi Hegel, care era anti-reducţionistă, ne-transformistă), şi Christian Doppler - în privinţa manierei în care şi-a conceput experienţele de hibridare şi a explicării segregării

not understand him) etc, aspects that will arouse the interest of the readers and more. In addition, the author had the salutary idea to insert in his book Mendel's fundamental work "*Versuche über Pflanzen Hybriden*" ("*Experiments on hybrids in plants*") and they will be able to analyze it and interpret it themselves, the work whose conclusions were presented in 1865 within the Natural History Society in Brno and published a year later, in the Journal of the same Society.

The reading of this book will procure us the opportunity to find out interesting and original information about Mendel's birthplace and family, his studies before entering the monastery, the material difficulties which he passed for his education, about the fact that he was a brilliant student, and that to continue his studies, to experience and check out some of his ideas, but from religious beliefs, he has followed the ecclesiastic career, being accepted as novice in the Augustine Monastery "St. Thomas" in Alt Brün (Brno, Czech Republic).To note that the entrance to the monastery was not a simple formality, supposing solid knowledge from the candidates, Johann Mendel being in competition with 12 other aspirants (for 4 places), in 1843. As monk of the monastery, Mendel will receive his first name Brother Gregorius. The abbot of the monastery, Franz-Cyrill Napp, was an enlightened prelate who promoted science and encouraged the scientific research in the monastery (especially in agriculture). The Monastery "St. Thomas" had a remarkable library, collections of minerals and plants, and in 1830, father Napp founded an experimental garden in the courtyard of the monastery, and later (1844) a greenhouse. Mendel will perfect his theological training in 1848 at the Theological Institute in Brno, but in parallel he attended agriculture, fruit production and viticulture courses at the Institute of Philosophy in the same town. Between 1851 and 1853, Mendel follows a series of lectures at the University of Vienna, in order to have the right to teach physics and natural history (at the Gymnasium in Znaim), where he had a number of famous teachers of that time, in physics, mathematics, anatomy and plant physiology, zoology, and chemistry etc. In a paper dedicated to the genesis of the great scientist's work (published in 1984 in "La Recherche"), J. L. Serre considered that a special influence on Mendel had probably the teachers Franz Unger - follower of the reductionist and analytical method in research (opposed to the

caracterelor în generația a doua (fenomen ce ar semăna cu trecerea luminii albe printr-o prismă, care asigură separarea ei pe componente). În epocă domnea ideea *eredității prin amestec* la hibrizi. Or, tocmai un prelat (ce ironie a sorții!), înarmat cu altă metodă de lucru și foarte exact în cuantificarea rezultatelor obținute în hibridări, vine și arată că trăsăturile contrastante ale unui caracter se reunesc în hibrid, dar se separă, segregă la descendenți. Același J. L. Serre consideră că este posibil ca anii petrecuți la Viena să-l fi înarmat pe Mendel și cu alte informații utile, cum ar fi cele furnizate de citologii Hoffmeister și Amici, care arătau că polenul și ovulul au rol strict egal în fecundare la plantele cu flori. În plus, cum am amintit deja, Mendel era la curent cu toate lucrările de hibridologie publicate până la el de către botaniști, agronomi, hibridologi și în special cu rezultatele obținute de J. G. Kölreuter și de K. F. Gärtner, pe care îi citează adeseori în lucrarea sa, care i-au servit în conceperea experiențelor sale de hibridare și l-au orientat pe Mendel inclusiv asupra materialului biologic principal pe care a experimentat – mazărea.

Pentru profesorul Ștefănescu imboldul de a scrie această carte a fost de a-l repune în drepturi pe Mendel, de a-l prezenta întocmai, așa cum a gândit, cum a lucrat etc, de a-i reda opera așa cum a fost ea, plecând de la constatarea că în diverse manuale și cărți de genetică publicate în România (dar nu numai) de-a lungul anilor, se găsesc o serie de inadvertențe privind opera acestuia, că nu există consens nici măcar în privința numărului legilor descoperite de el, a formulării acestor legi etc, fapt ce denotă că diverșii autori nu l-au citit pe Mendel ci s-au ”inspirat” din comentariile altora. În acest context, autorul cărții precizează că în urma experiențelor de hibridare efectuate în special pe mazăre, dar și pe fasole și alte specii, Mendel a stabilit trei legi, care ar putea fi formulate pe scurt astfel: 1) *legea segregării unei perechi de caractere diferențiabile*; 2) *legea segregării independente și combinării libere a perechilor de caractere diferențiabile*; 3) *legea corespondenței tipurilor de gameți ai hibrizilor cu formele constante din descendența lor*. Într-adevăr, aceste formulări diferă întrucâtva de cele pe care le întâlnim în prezent prin mai toate manualele și cărțile de profil unde, în loc de trei legi se vorbește, în general, de două și anume: 1) *legea segregării caracterelor în* $F_2$ sau *legea purității gameților* (rezultată în urma experiențelor de monohibridare, n.n.) și 2) *legea*

doctrine in trend, promoted by Lorenz and supported by Goethe and Hegel, who was anti-reductionist and non-transformist), and Christian Doppler - about the manner he conceived the hybridization experiments, and explaining the segregation of characters in the second generation (a phenomenon which would resemble with white light who passes through a prism, which ensures its separation in components). That time was dominated by the idea of heredity by mixture in hybrids. Or, just a priest (ironically!), gifted with a different method of working and very precisely in quantifying the hybridization results, comes and shows that the contrasting features of a character come together in the hybrid, but secede, segregate in descendants.The same J. L. Serre considered it is possible that the years spent in Vienna have armed Mendel with other useful information, such as the ones provided by the cytologists Hoffmeister and Amici, who showed that pollen and ovule have a role strictly equal in fertilization at plants with flowers. In addition, as I already mentioned, Mendel knew all the Hybridology works published until him, by botanists, agronomists, hybridologists, and especially the results of J. G. Kölreuter and C. Gärtner, often quoted in his work, who have served in the design of his hybridization experiences and have guided Mendel on the main biological material used in his experiments - the peas.

For Professor Ștefănescu the impulse to write this book was the restoring of Mendel, to present him exactly how he was, as he thought and worked etc, to restore his work, starting from the observation that in different manuals and books of Genetics published in Romania (but not only) over the years, there are some inadvertencies regarding his work, there is no consensus even on the number of the laws discovered by him, about formulating these laws etc, fact which shows that various authors did not read Mendel, but were "inspired" from the comments of the others. In this context, the author of the book specifies that after the hybridization experiments conducted mainly on peas, but also on beans and other species, Mendel established three laws, which may be formulated briefly, as follows: 1) *the segregation law of one pair of differentiable characters*; 2) *the law of independent segregation and free combination of differentiable character pairs*; 3) *the correspondance law of gamete types of hybrids, with the constant forms from their descendence* (which no longer segregate). Indeed, these formulations differ somewhat from those we

*segregării independente şi combinării libere a caracterelor/a factorilor ereditari* (rezultată din experienţele de polihibridare, n. n.). [Uniformitatea hibrizilor din prima generaţie, datorată dominanţei unui caracter sau a interacţiunii între caractere/gene (ereditatea de tip *Pisum* sau *Zea*), nu este propriu-zis o lege a eredităţii, aşa cum au înţeles unii, şi nu-i aparţine lui Mendel, ea fiind observată de mai mulţi hibridologi cu mult înaintea lui Mendel (prima dată fiind sesizată în 1761, de către Kölreuter)]. Cum precizează autorul cărţii de faţă, legea a 3-a a lui Mendel (care ar echivala cu puritatea gameţilor) ”*reprezintă suportul explicativ al legilor a I-a şi a II-a ale eredităţii hibrizilor*”. Iată ce spunea Mendel despre legea a II-a: ”*Legea combinării caracterelor diferenţiabile, după care se desfăşoară dezvoltarea hibrizilor, îşi găseşte explicaţia în principiul demonstrat, după care hibrizii formează în cantităţi egale celule germinale şi polenice corespunzând tuturor formelor constante, obţinute din combinarea caracterelor asociate pe calea fecundării*”. Iar în privinţa legii a treia, Mendel arăta că: ”*Hibrizii formează celule germinale* (gameţi femeli, nota noastră) *şi polenice* (gameţi masculi, n. n.) *care, prin capacităţile lor şi în număr egal, corespund tuturor formelor constante care se obţin în combinaţiile de caractere asociate prin fecundare*”.

Notând cu simboluri caracterele urmărite în descendenţă (cu literă mare caracterele dominante şi cu literă mică pe cele recesive), Mendel a constatat că în cazul hibridării unor forme de mazăre pure biologic, ce difereau printr-o pereche de caractere, hibrizii din prima generaţie exprimau doar un caracter (cel dominant). Prin autofecundarea hibrizilor din prima generaţie ($F_1$), a observat că în a doua generaţie reapărea caracterul recesiv, într-un raport de 3:1, potrivit expresiei de dezvoltare ”A + 2Aa + a”. Urmărind comportarea plantelor obţinute în $F_2$ în generaţia următoare ($F_3$) a constatat că jumătate din ele nu mai segregau (reveneau la caracterele/formele iniţiale), iar jumătate segregau în acelaşi raport de 3:1 (dominant/recesiv), precum hibrizii din $F_1$. De reţinut faptul că şi alţi autori până la Mendel ajunseseră să cuantifice rezultatele unor hibridări de acest fel. Bunăoară Gärtner, în experienţe de hibridare la porumb, a găsit că în $F_2$ rezultatul a fost într-un caz de 224 seminţe gri şi 64 roşii, iar în alt caz de 104 seminţe gri şi 39 roşii, dar nu a reuşit să generalizeze şi să deducă raportul de segregare a caracterelor de 3:1 în cazul unor genitori ce diferă printr-o pereche de caractere (de fapt e vorba de un caracter cu două trăsături

meet today in almost all the manuals and books in this area where, instead of three laws, it is spoken, generally, of two, namely: 1) *the segregation law of characters in F2 or the purity law of gametes* (resulted from the experiences of monohybridization) and 2) *the law of independent segregation and free combination of characters/hereditary factors* (resulted from the experiences of polihybridization). [The uniformity of hybrids from the first generation, due to the dominance of a character, or the interaction between characters/genes (*Pisum* or *Zea* heredity) is not actually a law of heredity, as some have understood it, and it does not belong to Mendel, it being seen by many hybridologists, long time before Mendel (the first time being notified in 1761 by Kölreuter)]. As it is specified by the author of the present book, the third law of Mendel (which would equate to purity of gametes) „*represents the explanatory support of laws I and II of hybrids heredity*". Here is what Mendel's second law says: "*The law of combination of differentiable characters, after which it take place the development of hybrids, finds its explanation in the demonstrated principle, after which the hybrids form germ cells and pollen in equal amounts corresponding to all constant forms, obtained from the combining of characters associated by fertilization*". And about the third law, Mendel show that: "*Hybrids form the germ cells* (female gametes, our note) *and pollen* (male gametes, o. n.) *which, by their capabilities, and in equal numbers, correspond to all constant forms that are obtained in combinations of characters associated by fertilization*".

Noting with symbols the characters traced in descendence (with uppercase the dominant characters, and with lowercase the recessive ones), Mendel found that in case of hybridization of some biologically pure forms of pea, that differed by a pair of characters, hybrids from the first generation expressed only one character (the dominant one). By self-fertilization of hybrids from the first generation (F1), he observed that in the second generation the recessive character reappears in a ratio of 3:1, according to the developed expression "A + 2Aa + a". Watching the behavior of plants obtained in F2 in the next generation (F3), he found that half of them did not segregated (they came back to initial characters/forms), half of them segregated in the same ratio of 3:1 (dominant/recessive), like hybrids from F1. It is to note that other authors until Mendel reached the results quantification of this

contrastante, n. n.) – cum a precizat Mendel. După cum subliniază domnul Ștefănescu, Mendel a mers mai departe și a făcut distincție între aspectul indivizilor (plante sau semințe) obținuți în experiențele de hibridare și constituția, natura lor ereditară (genetică, n.n.), între *fenotip* și *genotip*, între *homo-* și *heterozigot*, cu alte cuvinte, dacă folosim termenii apăruți după 1900. Urmărind modul cum se transmit caracterele analizate în generații succesive, Mendel a sesizat și faptul că pe măsura scurgerii generațiilor de autofecundare: ”*numărul formelor hibride, apărute din încrucișările între genitori care prezintă o pereche de caractere diferențiabile, scade în mod considerabil, din generație în generație, în raport cu numărul formelor constante sau pure, dar* (fapt foarte important de subliniat!) ***hibrizii*** (heterozigoții, n. n.) ***nu pot să dispară cu totul***”. El ajunge chiar să generalizeze această observație, pentru cazul a ”n” generații, astfel: $2^n$ – 1A : 2Aa : $2^n$ – 1a, dând spre exemplu care va fi rezultatul după 10 generații succesive (”*din 2048 de plante care apar în această generație, 1023 de plante au caracterul constant dominant, 1023 au caracterul constant recesiv și doar două sunt hibride*”).

Autorul cărții consideră de asemenea incorect și faptul că unii autori îi atribuie lui Mendel stabilirea raporturilor de segregare fenotipică a caracterelor în $F_2$ de *9: 3: 3: 1*, în cazul încrucișării hibrizilor din $F_1$ între plante ce diferă prin două perechi de caractere diferențiabile (dihibridare, n. n.). Ba mai mult, sunt autori care i-au atribuit în mod eronat lui Mendel calcule probabilistice pe care el nu le-a făcut, și anume că, plecând de la faptul că în monohibridare, în $F_2$, se obțin 2 clase fenotipice (¾ din indivizi manifestând caracter dominant și ¼ caracter recesiv), pot fi deduse numărul de clase fenotipice în cazul dihibridării. Domnul Ștefănescu arată că pe Mendel nu l-au interesat aceste raporturi, ci mai degrabă natura ereditară a indivizilor din a doua generație (raportul dintre plantele constante, semihibride și hibride). Seria de dezvoltare, în acest caz, a constat din 9 membri: AB + Ab + aB + ab +2Abb + 2aBb + 2AaB + 2Aab + 4AaBb, care rezultă de altfel din combinarea expresiilor de dezvoltare specifice fiecăruia din cele două perechi de caractere diferențiabile, adică: *A + 2Aa + a* și respectiv *B +2Bb + b*. Chiar dacă Mendel nu a calculat raportul dintre cele patru categorii de indivizi (sub aspect fenotipic) în $F_2$ (9:3:3:1), acesta putea fi lesne dedus din seria de dezvoltare prezentată mai sus, și o vor face cei care îl vor

hybridization. For instance Gärtner, in hybridization experiments on maize, found that in F2 the result was, in one case, 224 grey seeds and 64 red seeds, and in another case 104 grey seeds and 39 red seeds, but he failed to generalize and to deduct the segregation ratio of characters, of 3:1, in the case of some genitors that differed by a pair of characters (in fact, it's about a character with two contrasting features, o.n) - as Mendel said. As it is pointed out by Mr. Ștefănescu, Mendel went ahead and made the distinction between the aspect of the individuals (plants or seeds) obtained in hybridization experiments and the constitution, their hereditary nature (genetic, o. n.), between phenotype and genotype, homo- and heterozygous, in other words, if we use the terms appeared after 1900. Following the way how the analyzed characters are transmitted in successive generations, Mendel noticed that as long as the generations of self-fertilization succeed "*the number of hybrid forms, arising from crosses between genitors with a pair of differentiable characters, decreases considerably, from generation to generation, in relation to the number of constant or pure forms, but* (a fact very important to note!) *the hybrids* (heterozygotes, o. n.) *can not disappear entirely*”. He even generalizes this observation, for the case of "n" generation, as follows: 2n-1A: 2Aa: 2n-1a, indicating, for example, which will be the result after 10 successive generations ("*from 2048 plants that appeared in this generation, 1023 plants have the constant dominant character, 1023 have the constant recessive character, and only two are hybrid*").

The author of the book considered also incorrect the fact that some authors assign to Mendel the determination of phenotypic segregation ratio of characters in F2, of 9: 3: 3: 1, in the case of crossbreeding of hybrids from F1 between plants that differ by two pairs of differentiable characters (dihybridization, o. n.).Moreover, some authors have erroneously attributed to Mendel the probabilistic calculations that were not done by him, namely that, starting from the fact that in monohybridation, in F2, are obtained two phenotypic classes (¾ of the individuals manifesting the dominant character and ¼ the recessive character), it can be deducted the number of phenotypic classes in the dihybridation case. Mr. Stefanescu shows that Mendel was not interested of these ratios, but the heredity nature of individuals from the second generation (the ratio between constant plants, semi-hybrid and hybrid). The

redescoperi pe Mendel peste trei decenii şi jumătate (în 1900). La fel procedează marele savant şi în cazul hibridării între plante ce diferă prin trei perechi de caractere (notate cu *ABC* şi respectiv *abc*). Ba mai mult, şi de data asta generalizează, şi arată care va fi rezultatul în hibridări în care genitorii iniţiali se vor deosebi prin ”n” caractere diferenţiabile, situaţie în care ”*numărul membrilor seriei combinative*” va fi de $3^n$ (numărul de clase genotipice în generaţia $F_2$ , n. n.), ”*numărul indivizilor aparţinând seriei*” va fi de $4^n$ (numărul de combinaţii posibile), iar ”*numărul combinaţiilor care rămân constante”* va fi de $2^n$ (de clase fenotipice, n. n.). Şi de data asta exemplifică, arătând că: în cazul când genitorii diferă prin 4 caractere, în $F_2$ *seria va conţine* $3^4 = 81$ *membri* (clase genotipice, n. n.), $4^4 = 256$ *indivizi* (combinaţii posibile, n. n.) *şi* $2^4 = 16$ *forme constante* (clase fenotipice, n. n.). Mendel explică şi cum stau lucrurile în cazul încrucişărilor reciproce dintre genitorii iniţiali, ce se întâmplă în situaţia în care hibridul este supus polenizării repetate cu polen provenit de la unul din partenerii iniţiali (comentând unele din experienţele de hibridare ale lui Kölreuter şi Gärtner) etc.

Mendel ajunge la formularea legilor enumerate printr-o muncă migăloasă şi deosebit de meticuloasă, metodică, desfăşurată timp de ani de zile (între 1854 şi 1863), pe mii de plante (indivizi), în care şi-a pus în valoare nu numai calităţile unui cercetător iscusit, ci şi geniul care l-a ajutat să aibă intuiţie şi să vadă acolo unde nu era încă nimic de văzut. El şi-a depăşit contemporanii nu numai în privinţa modului în care a conceput şi realizat experienţele de hibridare, a felului cum a cuantificat şi interpretat observaţiile (rezultatele), ci şi prin aceea că a intuit prezenţa în celule a unor ”*elemente*” (*factori*) responsabile de transmiterea caracterelor analizate. Mendel arată că, la formarea celulelor sexuale de către hibrizi, ”*elementele prezente*” în acestea (adică factorii ereditari, n. n.), ”*se distribuie în grupuri independente şi egale şi doar elementele diferenţiabile se exclud reciproc din aceste grupări*”, că astfel ”*este posibilă apariţia atâtor celule ovulare şi polenice, câte combinaţii diferite se admite că pot forma elementele*”, iar când discută celulele germinative ale hibrizilor, consideră că: ”*pretutindeni se confirmă faptul că descendenţii constanţi se formează doar atunci când celulele germinative şi polenul fertilizator au o natură identică, adică ambele sunt dotate cu aptitudini capabile să formeze indivizi perfect identici, aşa*

development series, in this case consisted of 9 members: AB + Ab + aB + ab +2Abb + 2aBb + 2AaB + 2Aab + 4AaBb, which otherwise results from the combination of development expressions specific to each of the two pairs of differentiable characters, meaning: A + 2Aa + a, and B +2Bb + b, respectively. Although Mendel did not calculate the ratio between the four categories of individuals (phenotypically) in F2 (9: 3: 3: 1), it can be easily deduced from the development series presented above, and it will be done by those who will rediscover Mendel over three and half decades (in 1900). The great scientist does the same also in case of hybridization between plants that differ by three pairs of characters (noted with ABC and abc, respectively). Moreover, he also generalizes, and shows the result in case of hybridizations in which the primary genitors will be differentiated by "n" differentiable characters, a situation in which "*the number of members of the combinative series*" will be $3^n$ (number of genotypic classes in the F2 generation, o. n.), "*the number of individuals belonging to the series*" will be $4^n$ (number of possible combinations) and "*the number of combinations that remain constant*" will be $2^n$ (phenotypic classes, o. n.). He also exemplifies, showing that: if genitors differ by 4 characters, in the F2 series will contain $3^4 = 81$ members (genotypic classes, o. n.), $4^4 = 256$ individuals (possible combinations, o. n.) and $2^4$ constant forms (phenotypic classes, o. n.). Mendel also explains how things stand in case of reciprocal crosses between primary genitors, which happens when the hybrid is subjected to repeated pollination with pollen from one of the initial partners (commenting some of the hybridization experiences of Kölreuter and Gärtner) etc.

Mendel reaches the formulation of the listed laws by a thorough and very meticulous work, performed for many years (between 1854 and 1863), on thousands of plants (individuals), in which he put in value not only his qualities of a skillful researcher, but also the genius who helped him to have intuition and to see where there was still nothing to see. He has surpassed his contemporaries not only about how he designed and conducted the hybridization experiments, the way he quantified and interpreted the observations (the results), but also how he intuited the presence of "*elements*" (factors) responsible for the transmition of the analyzed characters, in cells. Mendel shows that, to the formation of sex cells by the hybrids, "*the*

*cum se întâmplă şi în fecundarea normală la specii pure. De aceea, trebuie să admitem cu necesitate că la apariţia formelor constante* (homozigote, n. n.) *de la plantele hibride* (heterozigote, n. n.) *are loc unirea unor* ***factori*** *complet identici*”. Despre rolul egal al celulelor germinale şi polenice în formarea noului embrion şi al acestor factori (elemente) ereditari aflăm modul cum vedea Mendel lucrurile dintr-o notă infra-paginală din lucrarea sa, în care precizează: ”*La Pisum, dincolo de orice îndoială, s-a stabilit că la formarea noului embrion trebuie să aibă loc o unire completă a elementelor din ambele celule de reproducere. Cum s-ar putea altfel explica faptul că printre descendenţii hibrizilor se întâlnesc din nou ambele forme iniţiale, în acelaşi număr şi cu toate particularităţile lor?*” În capitolul din lucrare, ”*Consideraţii concluzive*” , Mendel adaugă ”*.... trebuie să admitem că elementele deosebite între ele reuşesc să iasă din asocierea obligatorie doar odată cu dezvoltarea celulelor de reproducere. La formarea acestor celule, toate elementele existente se distribuie în grupări complet independente şi egale, grupări în care doar elementele deosebite se exclud reciproc unul pe altul. Pe această cale este posibilă apariţia atâtor celule germinative şi polenice câte combinaţii se admite că sunt capabile să formeze elementele*”.

Lectura acestei cărţi mi-a produs o mare bucurie şi satisfacţie, fiind un mare admirator al genialului savant. Autorul şi-a asumat o misiune defel uşoară şi nelipsită de riscuri. Consider că şi-a atins pe deplin ţelul. Deşi a făcut-o într-un anumită măsură, mi-aş fi dorit totuşi să scoată mai mult în evidenţă importanţa operei lui Mendel, având în vedere că în perioada în care a lucrat şi a publicat nu se ştia nimic despre diviziunea celulelor, despre existenţa cromosomilor, despre faptul că nucleul este sediul ”factorilor ereditari”, nu erau cunoscute noţiunile de homo- şi heterozigot, de genotip şi fenotip etc. S-a dovedit totuşi atât de ingenios, atât de inspirat, atât de savant, acest onest călugăr – J. Gr. Mendel, încât prin ceea ce a făcut (experimentat) aparţinea veacului său, al XIX-lea, dar prin interpretările date rezultatelor sale, prin intuiţia sa, aparţinea deja secolului al XX-lea. Deşi contemporanii nu l-au înţeles, el era conştient că făcuse o treabă bună, un lucru temeinic, şi ne convinge de aceasta din consemnările lui în calendarul zilnic la data de 1 octombrie 1883 (cu cca 3 luni înainte de a muri): ”*... Lucrările mele ştiinţifice mi-au prilejuit profunde satisfacţii şi sunt convins că nu va trece multă vreme şi întreaga lume*

*elements present*" in them (meaning the hereditary factors, o.n.) "*are distributing in independent and equal groups and only the differentiable elements are reciprocally excluded from these groups*", that in this way "*it is possible the appearance of so many ovules and pollen, how many different combinations are to be capable to form the elements"*, and when he discusses about the germ cells of hybrids, he considered that *"everywhere it is confirmed that constant descendants are forming only when germ cells and the fertilizing pollen have an identical nature, meaning both are equipped with skills able to form perfectly identical individuals, as it happens in normal fertilization at pure species". That is why, we must admit with necessity that at the emergence of the constant forms* (homozygous, o.n.) *from the hybrid plants* (heterozygous, o.n.) *it take place the union of some completely identical* ***factors***. About the equal role of germ cells and pollen in the formation of the new embryo and of these hereditary factors (elements), we find the way in which Mendel saw things from an infra-page note in his work, stating: "*In Pisum, beyond any doubt, it was determined that to the formation of the new embryo it must take place a complete union of the elements from both reproductive cells. Otherwise, how it might be explained the fact that among the descendants of the hybrids are meeting again the two initial forms, in the same number and with all their particularities*?" In the chapter named "*Conclusive considerations*" of the work, Mendel added "*.... we must admit that the elements different between them succeed to emerge from the compulsory association only with the development of reproductive cells. To the formation of these cells, all the existing elements are distributed in groups completely independent and equal, groups in which only the different elements are mutually excluded, each other. In this way, it is possible the emergence of so many germ cells and pollen, how many combinations are admitted to be capable to form the elements*".

The reading of this book produced me a great pleasure and satisfaction, being a great admirer of this brilliant scientist. The author has assumed a mission at all easy and risky. I believe that he has fully achieved his goal. Although he did it to a certain degree, however I would have wanted to highlight more the importance of Mendel's work, considering that in the time he worked and published, no one knew anything about cell division, the existence of chromosomes, the fact that

*va recunoaşte rezultatele acestor lucrări*”. Au trecut 35 de ani (în 1900) până la confirmarea şi reconsiderarea lucrărilor lui Mendel, mult prea târziu pentru ca autorul lor să guste din cupa unui succes binemeritat. Mă situez pe aceeaşi poziţie cu autorul cărţii de faţă şi consider că anul în care s-a născut genetica ca ştiinţă a fost 1865 - odată cu prezentarea de către Mendel a rezultatelor sale în cadrul Societăţii Naturaliştilor din Brno, şi nu 1900 - când Hugo De Vries, Carl Correns şi Erich Tschermak au ajuns la concluzii asemănătoare cu marele lor predecesor.

Apreciez în mod deosebit efortul profesorului Ştefănescu de a nu lăsa nimic la voia întâmplării în cartea sa, de a se apleca cu atenţie şi pasiune asupra fiecărui amănunt din viaţa, lucrările şi scrisorile lui Mendel, de a prelua şi prelucra critic ideile marelui savant, de a debarasa opera acestuia de ceea ce nu-i aparţine, de a interpreta în manieră personală unele din consideraţiile lui Mendel, de a racorda unii termeni şi fapte ale lui Mendel la cuceririle de după 1900 în domeniul geneticii (ceea ce demonstrează că, în ciuda criticilor pe care le aduce unor autori de manuale şi cărţi de genetică, este conştient de faptul că nu-l mai poţi prezenta pe Mendel în ”hainele” de la 1865). Sunt convins că lucrarea D-Sale va stârni un interes legitim, că acţiunea de a-l restitui pe Mendel cititorilor din România va fi una reuşită şi că misiunea asumată de autor va fi una remarcată şi salutată de către confraţii noştri în ale geneticii.

the nucleus is the center of the "*hereditary factors*", there were not known the notions of homo- and heterozygote, genotype and phenotype etc. However, it proved to be so intelligent, ingenious and inspired this honest monk - J. Gr. Mendel – so that by what he did (experimented), he belonged to his time (the XIX century), but by the interpretations given to his results, by his intuition, he already belonged to the twentieth century. Although the contemporaries did not understand him, he knew that he had done a good job, a solid thing, and he convinces us of this from his notes in the daily calendar on October 1, 1883 (about three months before he died): "...*My scientific papers have occasioned me deep satisfaction and I am sure it will not take long, and the whole world will recognize the results of these works*". Thirty five years have passed untill the confirmation (in 1900) and reconsideration of Mendel's work, much too late for their author to taste the cup of an well-deserved succes. I stand on the same position with the author of this book and I think the year in which Genetics was borne as science, it was 1865 – along with the presentation of its results by Mendel within the Society of Naturalists in Brno, and not 1900 - when Hugo de Vries, Carl Correns and Erich Tschermak reached to similar conclusions with their great predecessor.

I particularly appreciate the effort of Professor Ştefănescu of not letting anything to chance in his book, of presenting carefully and passionately every detail of life, work and Mendel's letters, to retrieve and process in a critical manner the ideas of the great scientist, to release his work of what it does not belong to it, to interpret in a personnel way some of Mendel's considerations, to connect some of Mendel's terms and facts to the achievements in the field of Genetics after 1900 (which proves that, despite criticism brought to the authors of Genetics manuals and books, he is aware that Mendel can no longer be presented in "the clothes" of 1865). I am convinced that this book will arouse a legitimate interest, that the action of returning Mendel to the readers in Romania will be a success and that the mission assumed by the author will be noticed and welcomed by our compeers in the Genetics.

Professor Gogu Ghiorghiţă, PhD
Romanian Academy of Scientists

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