a. GENETICĂ ȘI BIOLOGIE MOLECULARĂ

CONTENT

PROGRESS IN GENETIC PAIN STUDIES REGARDING ANALGESICS THERAPY - *A Systemic Review*
PART I - GENETIC MODULATION OF PAIN from genotype-phenotype

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Abstract: The intervention of the genetic factor in pain have a decisive importance not only for the effectiveness of the therapeutic strategy but also for avoiding the adverse (unwanted) effects of the drug molecules. The human genome assures by coding and synthesizing the functional protein structures participating in the mechanisms of receiving, conducting and projection of pain sensitivity in superior nerve centers as perception and interpretation of pro-nociceptive and anti-nociceptive molecules as well as modulation of pharmacokinetics and pharmacodynamics of analgesics. Genes with an indirect pain impact are a large number of genes, each having a little contribution to the interindividual variability of pain parameters in humans and to response type to analgesic therapy reflected by the required dose, administration time and efficiency. Epigenetic factors with effects on the mechanisms of pain and the patient's analgesic responses types are numerous and of wide diversity.

1.INTRODUCTION

Association between genetics and pain is not surprising, as it is to be expected, given that few are the fields of human pathology without the involvement of heredity as a predisposition, modulation, or causal determination. This is also the case for painful sensitivity, and for the last two decades substantial contributions have been made to the genetics of pain phenotypes. Pain is the most common symptom in the pathology, either in acute form associated with an underlying disease or as a self-standing disease - chronic pain. In cancer, for example, on average over 70% of patients accuse of continuous pain, being a major cause of healthcare and opioid treatment, given that the dose is of crucial importance, and the establishment of an ideal therapy (the "magic bullet ") is likely to become possible in a number of cases by genotyping. At the base of the inter-individual variability of the perception of painful stimuli as well as the different response to analgesics, there is a complex sum of contributing factors on which the pain phenotype profile is grouped into genetic factors of the individual (allelic variations in genomic DNA sequences) and environmental epigenetic factors.

This study bring into attention the latest achievements of pain genes involvement into pain treatment. A better understanding of the specific role of these genes in pain mechanism will give the possibility to made a genetic profile of the patients in order to improve the actual pharmacological treatment to an individualized one.

2. GENETIC MECHANISMS OF PAIN

Although acquisitions in medical genetics over the last 40 years (starting with DNA and proteins\sequencing - Sanger, 1975-double Laureate Nobel for Chemistry) are impressive, consistent data on gene contributions to pain mechanisms are recent and they deserve to be highlighted and known. In the same time, with all the performances currently achieved by the new generations of analgesics reaching the patients, they are still not in line with expectations, therapeutic satisfaction remains behind of speed of the emergence of new pharmacological agents, and despite intense studies regarding the genetic component of pain (only between 2005 and 2016 there are 846 studies in the field according to data published by the Human Pain Genes Database-HPGD, 2017). Difficulties encountered come from the heterogeneity of the studied human populations and from the "silent" way of the pressure of environmental epigenetic factors on genotypes that alter the phenotypic pain architecture, from differences in applied molecular technologies and some reproductive failures of experimental studies conducted by various research groups (Klepstad, 2011). Knowing in detail the intervention of the genetic factor in pain is of decisive importance not only for the effectiveness of the therapeutic strategy but also for avoiding the adverse (unwanted) effects of the drug molecules, the effects of which usually reach a significant value of 6,7%. This finding is particularly important in the treatment of major, long-term or high-dose analgesics, especially for pain types such as persistent pain (chronic pain), intense (tumor) and post-surgical (acute). The prevalence of chronic pain (the most

expensive in analgesic therapy) represents 15-20% of the adult population (Wilson, 2006), characterized by marked individuality, modest therapeutic efficiency, severe intensity and particular and important affective-emotional reactions which profoundly disturbs the professional, social and family relationships of the patient. Under these conditions, have become inevitable the concerning of the specialized clinics/centers in the therapy of chronic pain (especially oncological, rheumatological) about the need to determine the genetic profile of some patients in order to assess the risk of inappropriate therapy with opioids or other classes of analgesics, considered by many specialists as becoming in the near future routine stages (LaCroix-Fralisch, 2009; Trescot, 2014). Diagnosis and etiological pharmacology of pain by genotyping (gene therapy of pain) is extremely useful even though the proportion of genomic protein coding regions with important functional roles in pain mechanisms appears to be of minor importance, accounting for only 2% of the human genome, and the impact of deviation of gene polymorphisms has a contribution of only about 1% for painful phenotypic variants in general pathology. In addition, recent research shows the existence of genetic changes that influence pain that also occurs in RNA regions that do not encode proteins, which increases the size and importance of the impact of genetics involvement in painful suffering (Muralidharan, 2011).

The attempts to determine the causal relationship between genetic factors/epigenetic pain factors do not yet have a definite answer. In experimental animal studies, the contribution of genetic factors to the variability of nociception, hypo- and hypersensitivity to pain in different species (in mice, for example) varies between 25-60% or even 76% (Lariviere, 2002). In man, the contribution of genetic factors from all the factors involved in the pain phenomenon is variable, but some permanent and widespread diseases among the population have a strong hereditary character: 39-58% migraine, 21-67 % low back pain and cervical pain 50% shoulder and elbow pain, 55% menstrual pain (Kim, 2005).

The human genome assures by coding and synthesizing the functional protein structures participating in the mechanisms of receiving, conducting and projection of pain sensitivity in superior nerve centers as perception and interpretation of pro-nociceptive and anti-nociceptive molecules as well as modulation of pharmacokinetics and pharmacodynamics of analgesics. Epigenetic factors exert small but added pressures on environmental factors that act on the same sphere of patient pain and variable effectiveness of analgesic therapy plus age, gender, lifestyle, integrity of hepatic-renal functions, co-morbidities and associated medication (eg. co-analgesics and para-analgesics). The complete decode of the human genome within the well-known international project "The Human Genome" (1990-2003) was the time when molecular biology studies, implicitly those of pharmacogenetics, pharmacokinetics, pharmacodynamics, and pain-targeted pharmacotherapy, get soar on completion of the determination of all genomic DNA sequences (over 3.7 billion nucleotides) and their assembling modes in each chromosome, all human genes being identified physically and functionally (total number of human genes = 22,333 plus / minus 1000 genes after Data from the National Center for Biotechnological Information (NCBI-USA) (Pertea and Salzberg, 2010) of which about 19,000 genes are protein coding (Ezkurdia, 2014). For more genes, there is evidence that they are responsible for the mechanisms of different types of pain (HPGD, 2016), of which over 25 genes are confirmed to be directly involved in painful sensory mechanisms and associated with clinical systemically manifestations proven experimentally and clinically on homozygous and heterozygous, while other genes have the status of indirect participation in pain modulation. Genes with direct involvement in painful pathology, few in number, are responsible for congenital familial diseases with severe impairment of pain perception. Genes with an indirect pain impact are a large number of genes, each having a little contribution to the interindividual variability of pain parameters in humans and to response type to analgesic therapy reflected by the required dose, administration time and efficiency, gene modulation of pain being cumulative in this case, as a sum derived from the interactions of several genes, and on the other hand, from the association of these interactions with the pressure of the epigenetic factors. In analgesic therapy, the genome responds by its nucleotide sequences of gene expression, transcription and translation, with implications in both the enzymatic metabolism of the substrate (represented by the administered analgesic and the absorption, transport, distribution, synthesis of pharmacological receptor populations, binding on the receptor of the active drug molecule and the kinetics of the excretion of the resulting metabolites. The transport and processing of a drug substance through the body is accomplished with genetically engineered functional proteins. The least common genetic disorders of painful sensitivity (but also the most severe) are eredo-familial, and the most widespread types of gene changes among the population with less severe influence on pain are polymorphisms. The latter are represented by small variations in some individuals in the nucleotide structure of the DNA / RNA sequences, resulting in changes in the structure of the synthesized functional proteins. The most common allelic polymorphic modification is uninucleotidic polymorphism type (SNP- single nucleotide polymorphism) given by gene mutations by substitutions, duplications / replications, deletions or nucleotide insertions (e.g., substitution of an A, T, C or G nitrogen base from a single nucleotide, each nucleotide having 2 possible alleles). SNP variants represent 90% of all variations in the human genome (one variation per 1000 base pairs) (James, 2013), which can be found in exons (coding regions) or introns (non-coding regions) of the genes without phenotypic effect or with phenotypic effect (which is pathogenic). Existence of polymorphisms amplifies to the carrier individuals the risk of

developing pathogenic abnormal phenotypes by altering the transcription and translation of amino acids by mRNA in newly formed functional protein molecules. Depending on frequence, SNP polymorphisms may be common (present in some common diseases) or rare (in some rare diseases). The notions of *polymorphism* and *mutation* are used to have the same meaning, but generally the mutation refers to allelic variants with a frequency below 1% in the population, and the polymorphisms refer to allelic variations with a frequency above 1%.

SNP polymorphisms in the field of physiological and pathological pain can induce (not-necessary) coding modifications of neuro-functional proteins that are phenotypically finalized by affecting pain sensitivity and pharmacokinetic and pharmacodynamic modulations of the therapeutically administered analgesic (**Figure 1**).
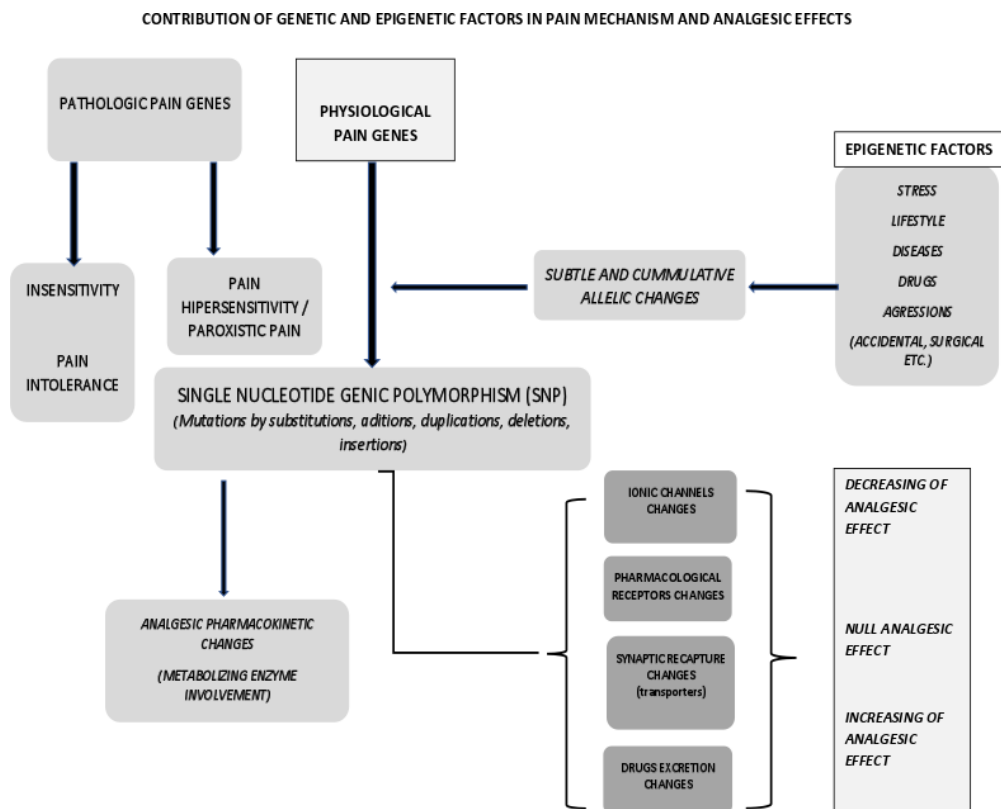
Figure 1

These changes outline the variability of interindividual response to therapy as efficacy, inducing to the carriers the susceptibility to respond unsatisfactorily (requiring an increase in the analgesic dose) or rather high (requiring lowering the dose), the variability ranging from treatment ineffectiveness to excessive toxicity manifested to a small part of the population carrier of a particular SNP.

2.1. **Monogenic modulation of pain**
        Pain genes
Over 25 functional genes are decisively involved in altering painful perceptions, of which at least 12 genes are responsible for either pain insensitivity or paroxysmal pain, hereditary transmitted. Each of the 25 genes listed below represents the genomic support of some clinical manifestations related to the pain phenomenon in man or animal (experimental pain) (HPGD, 2016):

- Genes with hereditary transmission of *pain insensitivity*: *SCN9A, SPTLC1, HSN2, IBKAP, NTRK1, NGFB;*

- Genes with hereditary transmission of *paroxysmal pain* (*pathological pain*): *CACNA1A* (familial hemiplegic migraine-MHF type I), *ATP1A2* (MHFtip II), *SCN1A* (MHF type III), *SCN9A;*
- SNP genes that *amplify pain*: *COMT* (low back pain, fibromyalgia, nociception), *KCNS1* and *SC9A* (sciatica, ghost pain), *HTR2A* and *SLC6A4* (fibromyalgia), *CACNG2* (post-operative pain), *CYP19A* (migraine), *GCH1* (pain, fibromyalgia, nociception), *TRPA1* (neuropathic pain), *TRPV1* (arthritic pain);
- Pain-reducing SNP genes: in human - *COMT, OPRM1, MC1R, GCH1, CYP2D6, TRPV1* and experimental animal-*TRPA1* (experimental pain).

Some of the above genes are part of both the group that amplifies the pain perception and the group that decrease this perception, depending on the allele/genotype variant and/or the SNP variant (*rs*) (for example, the allelic variant: the COMT gene *SNP rs 4680* genotype G induces analgesia, and G/G genotype low back pain). There are also individuals carrying genes that do not associate with clinical expressions of painful perception, but which in turn highlight an increased risk for the induction of pain (*SCN9A*-coding gene of Na^{2+} channels, *KCNS1*-coding gene of K^+, *CACNG2* - gamma 2 subunit coding gene from Ca^{2+} dependent channel) or a low risk of pain appearance (*CACNA2D3*-delta 2 encoded gene from the Ca^{2+} dependent channel) (Diatchenko, 2007).

Severe monogenic disorders of painful sensitivity

In humans there are two major serious impairment of the pain sensitivity generated by gene mutations, phenotypically represented either by the loss of sensitive function (pain insensitivity) or the increase in the activity of this function (painful hypersensitivity), both of which are rare but serious.

Congenital insensitivity to pain through non-functional gene mutations

The most pronounced genetic damage to painful sensitivity is highlighted in individuals with inherited insensitivity to pain involving several types of genes that have undergone mutations. The transmission of nociceptive signals is altered by disrupting the synthesis of proteins from a wide bio-functional range: enzymes, transcription factors, neurotrophins or ion channels of Na^+ and Ca^{2+} (canalopathies). All clinical forms are hereditary transmitted and show, among other signs, the inability to sense pain or indifference to pain. These mutations induce sensitive vegetative neuropathies (*Hereditary Sensory and Autonomic Neuropathy* - HSAN), due to aberrant protein encoding, thus becoming ineffective in the mechanisms of nerve influx conduction (LaCroix-Fralisch, 2009). Thus, the carriers of polymorphisms of the *SPTLC1* and *WNK1/HSN2* genes which affect the encoding of the key enzyme required for the synthesis of neuronal axonal sphinomyelin (serine-palmitoyl transferase and lysinkinase-1 respectively) transmit HSAN type I disease (autosomal dominant) and type II (autosomal recessive) characterized by decreased thermo-algesic sensitivity, acropathies and mutilations of the hands and feet. Mutations of the *IKBKAP* gene encoding the proteinic complex -1k B kinase which inhibit *kappa* opioid receptors which transmit HSAN type III neuropathy (*Autosomal Recessive Riley-Day* syndrome) manifested by weak reaction to painful and thermal stimulation, vegetative disorders, hyperhidrosis and alacrimia, and the *NRTK1* gene that modulates the structure of the neurotrophic tyrosine kinase receptor transduces HSAN type IV neuropathy (autosomal recessive) to descendants defined by the absence of response to painful stimuli, anhydrosis, cutaneous/corneal lesions and average mental retardation. Modification of nerve growth coding in *NGFB* gene carriers induces HSAN V disease (autosomal recessive) clinically evidenced by pain insensitivity and joint deformity. Ion channels for sodium types Na_v 1.3, 1.7, 1.8. and 1.9. type are also known as important structures involved in the transmission of pain. Individuals carrying SNPs with non-sense mutations in the SCN9A gene are insensitive to pain, with the exception of the Na_v 1.7 channel (Ahn, 2010). Instead, patients with pain indifference recognize the painful sensation, but have a marked decrease in the affective-motivational component, and the withdrawal reaction to painful excitatory application is absent. Pain-insensitivity frequently causes the death of these individuals in childhood, as they are not able to grasp the dangers of the external or internal environment of the body related to pain.

Hypersensitivity to pain (pathological pain) through hyperfunctional gene mutations

In individuals with manifestations of exacerbation of hereditary transmitted sensitive, have been identified genes that have undergone mutations that phenotypic induce powerful decreasing in pain threshold and marked increase in excitability by abnormal intensification of the activity of ion channels in nociceptive neurons of the spinal ganglia. The SNPs variant of the SCN9A gene (rs 6746030 allele A) coding the modification of the sodium channel protein structure (Nav1.7.) and the strong activation of the pain conducting C fibers (Reimann, 2010) induces rare familial pain syndromes evidenced by two disorders with intense pain: Hereditary erythromelalgia and Paroxysmal pain syndrome, the first showing signs of burning intermittent pain, and the second paroxysmal rectal, mandibular and ocular pains. Another condition characterized by painful episodes with genetic substrate is Familial Hemiplegic Migraine (MHF) with three forms of clinical manifestations. MHF type I is due to the *CACNA1A* mutant gene and the affected protein belongs to the Ca^{2+} P/Q channel being represented by the alpha 1 subunit from the $Ca_v2.1$ ionic channel. The condition is characterized by migraine attacks with aura,

hemiplegia and epilepsy. Type II MHF involves the *ATP1A2* gene with affinity of the Na^+ -K^+ -ATPase enzyme alpha 1 subunit, and in the Type III MHF mutation of the *SCN1A* gene that alters the alpha subunit protein structure belonging to Na_v1 dependent voltage Na^+ channels. The MHF type II and III MHF clinical signs are similar to MHF type I but without neurological associations.

Monogenic polymorphisms at risk for analgesic therapy

Therapeutical risk studies associated with genetic analysis have shown the existence of an appreciable potential in establishing linkages between certain gene polymorphisms and the variability of clinical efficacy of analgesics. This is the case for morphine, the first-line painkiller that is recommended as efficacy (World Health Organization, 1996), which explains that many studies target the mechanism of variability of response to this substance. The possibility of predicting optimal morphine doses (for example in cancerous pain) based on genetic testing has become of fundamental importance for adequate pain management treatment. The most important target of morphine is the *mu* receptor whose coding responds to the *OPRM1* gene located in the chromosome *bq24-q25*, the gene considered to be the first candidate to influence the analgesic efficacy of opioids. The *OPRM1* gene has a large number of polymorphisms (SNPs) identified in the promoter, but only some have relevance to opioid analgesia. The most common and investigated SNPs of this gene is substitution in nucleotide of adenine with guanine 118A> G (A118G) SNP in exon 1 which will induce the replacement of the asparagine amino acid with aspartic acid at position 40 (N40D) in the OPRM1 receptor protein with a frequency between 8-48% in population depending of ethnicity and geographical area (Tegeder, 2009). In the individuals with the mutant variant decrease the response to opioids, the morphine dose needs to be increased, for example in the GG variant with 93% in the therapy of these patients. Moreover, homozygous patients carrying two 118GG alleles decrease the potency of the most potent analgesic metabolite of morphine (morphine-6-glucuronide-M6G) compared to single-altered (heterozygous) alleles or wild-type 118AA alleles (unmodified) (Lotsch-2007). If in patients with renal insufficiency which take morphine, the M6G metabolite can accumulate up to the risk of opioid toxicity, in contrast G118-carrier patients (decrease in morphine metabolism) have no adverse effects (sedation, somnolence, decreased alertness) states which are present in morphine treated carriers of wild alleles and are at risk from this point of view (Stamer, 2007).

Another modulating gene of the *mu*-opioid system is the COMT gene encoding the catechol-oxymethyl transferase enzyme that catabolizes dopamine, adrenaline and noradrenaline neurotransmitters with key roles in modulating nociception, analgesia, and pain behavior. The COMT gene has a poorly functional polymorphic nucleotide (G472A) encoded variant (G472A) identified by Zubieta (2003) in which the *valine* amino acid of the COMT structure is replaced by *methionine* at position 158 (COMT Val158Met), which reduces COMT activity by 3-4 fold. Homozygous individuals carrier of the 158Met type exhibit an increased sensitivity to associated pain and a high rate of affective living of pain. Increasing levels of the dopamine neurotransmitter by chronic activation (due to its low metabolism / inactivation due to the COMT enzyme inefficiency) induces the reduction of endogenous opioid activity (evidenced by enkephalin depletion - a situation that is counteracted by activation of increasing of opioid receptors population *mu* active (*up-regulation*) in different regions of the brain. This makes that heterozygotes having only one SNP 158Met variant, but especially homozygotes with two 158Met variants, require much lower doses of morphine in the case of long-term analgesia (e.g. In the cancerous disease), while patients carrying wild alleles need daily high doses of morphine. The existence of the SNPs Val118Met polymorphic variant in the COMT gene has thus become a significant predictor of the required morphine dose in cancer pain therapy (Raakvag, 2005).

The synthesis of a major mediator of pain perception, nitrogen monoxide (NO), in the presence of the enzyme GTP cyclohydrolase (GCH1), is also genetically determined. GCH1 regulates the production of dihydroneopterin (BH2) and further of tetrahydrobiopterin (BH4), the latter molecule being an enzymatic cofactor essential for the synthesis of NO, serotonin and catecholamines. Excessive BH4 growth following various axonal aggressions contributes to neuropathic pain, but the existence of the SNP mutation that confronts the inactive, nonfunctional GCH1 enzyme gene is associated with a decrease in pain under conditions of BH2 and BH4 depletion and NO synthesis reduction. Batch studies on cancer patients carrying mutant polymorphism haplotypes showed that the time elapsed from the diagnosis of cancer to opioid treatment is double in homozygotes carrying the mutant alleles compared to the heterozygous carrier of mutant genes but no effect, and almost triple to non-carrier heterozygotes (Lotsch, 2010), the decrease of the GCH1 enzyme synthesis, determining the reducing of the duration of opioid therapy under the same conditions of therapeutic efficacy, hence resulting the prophylactic role of inhibiting the enzyme GCH1 in neuropathic pain therapy.

2.2. Polygenic modulation of pain (combined mechanisms)

A common and widespread situation in population regarding the genetic contribution to pain mechanisms is polygenic modulation, as pain represent a complex phenotypic traitfeature that involves the intervention of several genes, each bringing a small individual effect and influencing pain behavior more than one single gene. The concept of polygenics

explains the influence of genes on the variability of the analgesic response of individuals carrying altered alleles. For example, the A118G polymorphism of the *mu* receptor synthesizing OPRM1 gene has interrelated effects with the SNP polymorphism of the *COMT Val158Met* gene so that for the carriers of non-functional homozygous SNPs alleles of both *OPRM1* G/G genotype and *COMT* Val /Val genotype, are required high doses of morphine to treat pain, in contrast to *OPRM1* A/A and *COMT* Met/Met genotypes where pain is suspended at doses reduced in half, which is a tremendous advantage (Reyes-Gibby, 2007).

A receptor known as an analgesic modulator is also the melanocortin receptor MC1R (encoded by the gene *MC1R* from the distal end of chromosome 8 ($68_c/16_q24.3$), a specific receptor for coupling melanocito-stimulating adenohypophysis hormone (MSH). The MC1R receptor modulates analgesia by *kappa*-opioids, but only in females (sexually analgesic dimorphism) (Stamer, 2010). Normally, MC1R coupling with MSH have as a result in both sexes, synthesis, diffusion of melanin pigment and darkening of the skin and hair, that people with non-functional gene mutations for MC1R synthesis show reddish hair and light skin, a 75% of these individuals carrying two or more inactive variants of the *MC1R* gene. The most important such variants are 29*ins*A, 451C> T (coding the R151C MC1 receptors), *478C>T* (coding for R160w MC1 receptors) and *880G> C* (D294H receptors), all those listed being *variants* of MC1R receptors with low-function due to impairment of their coupling to G protein. In female patients carrying poorly functional variants, effective *kappa*-opioid analgesia is obtained with low therapeutic doses of *kappa* agonists (eg. Pentazocin) , aspect also proved in animals with *e/e* experimental deletions of the gene responsible for MC1R synthesis (Mogil, 2005). In individuals without *MC1R* mutations, *kappa* opioids are fixed in males only on *kappa* receptors and in women on both receptors (*kappa* and MC1R) activating, but the coupling with the functional MC1R receptor (in women) has anti-opioid effect. In contrast, in women carrying non-functional mutations of the *MC1R* gene, coupling of *kappa* agonists occurs exclusively on these latter receptors (present mainly in central nerve structures that play a major role in suppressing of pain - for example, cerebrospinal gray matter in the brainstem), that the effect is a robust analgesia, identical behavior to female mice with *e/e* provoked deletions. This latter finding is of particular importance not only to the need for a genetic test in certain groups of patients before analgesic therapy but also to the fact that, in some instances, the translation of animal-to-human experimental results is identical, operable and useful. The cause of this type of response only in women is due to the circulating estrogenic hormone, in experimental animal studies the phenomenon disappeared after ovariectomy and reinstalled after estrogen therapy (Rees, 1999).

There is another link involved in pain, this time sex-independent, between MC1R receptors and opioid *mu* receptors. Thus, the codeine metabolite (morphine-6-glucuronide) that selectively couples opioid *mu* receptors induces a strong analgesic effect in patients with mutant gene not functional for the MC1R receptor in both men and women, which is in contrast to the effect of *kappa* opioids (e.g., pentazocine), which produce strong analgesia, as shown above, exclusively in women carrying the mutant gene for MC1R receptors (Mogil, 2005). Researches has shown that not only the *kappa* receptors but also the active population of opioid receptor population may increase in the presence of high estrogen concentrations, situation in which women perceive less pain. The explanation seems to be due to increased secretion of endogenous opioid in conditions of increased estrogen, which activates multiple opioid receptors (*up-regulation*). Thus in the menstrual phase when estrogens are low, the risk of migraine is high. Estrogen administration blocks headache, but not bleeding, and progesterone blocks bleeding, but not headache. The existence of sexual dimorphism (as a totality of differentiation phenotypes between the two sexes) regarding the pain phenomenon and the effectiveness of analgesics is also supported by clinical evidence. For example, *kappa* agonists, such as nalbufine, reduce pain at birth more effectively than morphine (mu agonist), and in men effective morphine doses are 30-40% higher than in women for the same type of general pain pain, which adds to these findings of a high efficacy in severe pain, so the therapeutic recommendations are different (Wilson, 2006). These data require that when restrictions on specific treatments with *kappa* opioids (sex-independent) or *mu* (sex-dependent) are required, genetic testing is required.

Stress-induced analgesia (SIA) is another example of polygenic modulation of pain characterized by sexual dimorphism. SIA occurs in both sexes, but with different genetic mechanisms, mediation being made in males through the couple: opioid receptor *kappa*- NMDA receptor (N-methyl-D-Aspartate), involved in pain transmission, while women have a SIA system represented by the couple: *kappa* receptors and non-NMDA receptors that have been shown to be potentiated/activated by circulating estrogenic hormone (in female animal experiments, they are also insensitive to NMDA receptor antagonist therapy) (Butler, 2009). Determination of genic location by QTL technique (Quantitative Trait Locus-mapping) has revealed that DNA gene sequence which respond to SIA in women are transmitted in conjunction with the distal region of chromosome 8 - "*Si1fq1*" (absent linkage in males), place which also corresponds to the gene coding of the MC1R melanocortin receptor with a modulatory role along with plasma estrogen levels ("68_c *M"/16q24.3*), as mentioned previously (animal and human studies obtained by *mapping*, *linking* but also pharmacologically and clinically). In other words, although in both men and women stress analgesia is mediated through the *kappa*-opioid system, sexual dimorphism

on *kappa*-opioid analgesia mediation and stress analgesia has in women a neurochemical substrate similar to that of the MC1R receptor gene for the MSH hormone, a gene also present at the distal end of chromosome 8. Clinical consequences are important because women with normal genetic equipment on chromosome 8 require higher doses of *kappa* agonists, while women with mutant *MC1R* variants null functionally or with the pharmacological blockade of MC1R benefits by a strong analgesic effect of *kappa*-opioid agonists. It should be underlined that the problem of genetic sex-dependent differences in pain perception targets not only receptors, but also the existence of separate nerve circuits, neuro-secretions and neuro-processing distinct and separate in the brain in men and women.

A polygenic intervention is also present in low back pain syndrome, a widespread disease in the population due to disc degeneration or lumbar disc herniation or osteoporosis and which is estimated to be inherited in the proportion of 30-45%, while in the rest of cases intervening structural, psychosocial and occupational factors. Once known the genes encoding the extracellular matrix proteins in the bone and cartilages (*genome mapping*) have been identified and SNP polymorphisms associated with discopathies which frequently generating low back pain, the genetic variants being consistent with MRI and clinical data (Tegeder, 2009). Thus, polymorphisms of genes *COL9A2* and *COL9A3* encoding the alpha 2 and alpha 3 chains of heterotrimeric collagen IX (major component of the intervertebral disc) are associated with premature alteration of the mechanical properties of the disc and contribute to the predisposition of lumbar disc herniation, root compression and of low back pain especially in Finnish, Japanese and Chinese (Aladin, 2007) which present TRP2 alleles (Glu326Trp-exchange in the amino acid alpha 2 chain, glycine with tryptophan, the most hydrophobic amino acid). In German and Greek, *TRP2* was not detected, but the rate of relapse of discopathy after discectomy was high in the carriers of the SNP variant with the Glu326Arg gene of the COL9A2 gene (Kales, 2004). *Sp1* variant carriers of the *COL1A1* alpha1 chain have, in addition to the risk of disc degeneration and osteoporosis, at the postmenopausal women (Sp1 variant is a G/T polymorphism promoter of the *COL1A1* gene, carriers having reduced expression of this collagen) but other genes SNP may also contribute, such as beta-transforming growth factor, estrogen receptor and for vitamin D3, or the *ACAN* gene that synthesizes the aggrecan protein (Ralston, 2006).

Studies on genetically modulated pain pathways have indicated that the increased number of genetic variants corresponding to clinical pain phenotypes is confirmed by their multifactorial nature. There is no response yet on the cause of genetic abnormalities generating disturbances in pain sensitivity in the population and their elimination by natural selection. However, the explanations will be different for the two different cases of the genetic contribution to pain described above: rare disease/rare genetic variant (serious conditions such as insensitivity and hypersensitivity) and common disease/common gene variant (common monogenic and polygenic pain modulation) (Diatchenko, 2007). In the first case, of rare diseases with low population extent are involved rare genes encoding crucial elements for pain transmission but with high deletional penetration of familial genes and a minor contribution of environmental factors (not known yet if the genes causing the rare and extreme painful pathology are involved in a subtle form also to the pain associated with various common diseases). Instead, in the case of genetic modulation of pain from common diseases (for example, the variability of opioid receptor populations or their response patterns, the metabolism of analgesics or neurotransmitters transporters etc.) based on combinations of polymorphic gene variants (SNP) subtle, with no dramatic allelic deviations, with wider expansion in the population, but with low penetration, the phenotypic expression requires both intergenic and epigenetic factors interactions (favoring external and internal environmental factors).

3. EPIGENETIC FACTORS INVOLVED IN THE PAIN MECHANISM

For 60 years, the gene term is synonymous with the region of the genome encoding mRNAs that is translated into proteins. After coding of polymorphic mARNs (2% of the human genome) a large amount of RNA remains largely transcribed into non-coding protein RNAs (ncARNs – without translation in protein) consisting of short or long molecules of RNAs. Recently, extensive human genome studies have shown that it is transcriptible everywhere and has the ability to produce thousands of ncARNs (microARNs small interfering RNAs, and various long classes of RNAs) (Taft, 2010). It has now become clear that the types of ncARNs fulfill by such forms key roles of transcriptional and post-transcriptional regulators and guides chromatin-modifying complexes by facilitating development and physiological processes. One of the important functions of these RNAs is to be an *epigenetic regulator* of functional protein coding genes, implicitly those participating essentially in pain mechanisms such as receptors, ion channels or metabolic enzymes. Specific SNPs alleles present in microARNs (miARNs-variety of ncARNs) facilitate interactions between the *transcriptome - environment - epigenome*, so that genetic information can be modified in response to environmental changes. In this way, ncARNs regulate thousands of genes, perturbations of miARNs being involved in many diseases. From current research it follows that regulation of

gene-environment communication through silent RNA editing appears as a possible mechanism for fine post-transcriptional control of gene expression and represents a field of great interest for bio-medical pathology (Vaiman, 2016).

Epigenetic factors with effects on the mechanisms of pain and the patient's analgesic responses types are numerous and of wide diversity (environmental conditions, dietary structure and calorie intake in 24h, co-morbidity and various applied pharmacological therapies etc.) involves modifications of histone proteins associated with DNA sequences in the chromatin structure resulting in activation of the individual genetic patrimony (Moore, 2015). The phenotypic changes produced by this can be inherited without changes in subordinate DNA sequences ("*silent*" modifications), but they appear to play a key role, for example, in neuronal plasticity due to aggressions applied to peripheral nerves (Uchida, 2010). Such common epigenetic mechanisms involving functional proteins with a crucial role in pain modulation through their presence in opioid receptors (*mu*) and the Na^+ ($Na_v1.8$) and K^+ ($K_v4.3$) channels are reduced as long-term phenotypic expression due to the involvement of a intragenic *neuron restrictive silencer factor* acting as a repressor of *OPRM1*, *SCN10A* and *KCND3* gene transcription from dorsal spinal nerve neurons participating in the pathways of painful sensitivity (Zhang, 2015).

CONCLUSIONS

The analgesic suppression of acute and chronic pain represents one of the main goals of therapeutic management. The analgesic efficacy depends on the interindividual variability in differential pain sensitivity and also on the different response to analgesic medication.

The action of a single gene or more frequently the interaction of multiple genes, each of them with a small effect shows that pain more frequently is a complex phenotypic feature *involving intervention of multiple genes*.

Now it is possible to individualize the therapy for few categories of patients and the recommended dose is evaluated by genotyping. This allows both optimal therapeutic results and the prevention of adverse and/or side effects from inappropriate doses.

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STUDY OF BIOLOGICAL MARKERS IN ENDOMETRIOSIS

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Keywords: endometriosis infertilit, biological markers

Abstract. Endometriosis is an estrogen-dependent inflammatory disorder. Researches proved the fact that there are changes in serum marker concentrations that precede the occurrence of infertility symptoms, with endometriosis as the background. The objectives of the present study were to find out if the changes CA-125 and IL-6 correlate with the clinical and biochemical state of the patient with infertility and if they would be good predictors for infertility, in the case of the patients with endometriosis. We compared the values of the markers in the patients with infertility and ovarian endometriosis (n=94) with those of a control group with ovarian endometriosis (n=106) for which the diagnosis of infertility was not confirmed, in order to establish the prognostic factors of infertility. The values CA-125 and IL-6 were significantly higher for the group with infertility.

INTRODUCTION

Endometriosis is a disease that affects a woman in ten worldwide (Johnson, N.P., Hummelshoj, L., 2013).

Endometriosis – the growth of the stroma and endometrial glands outside the uterus, represents the third cause of gynaecologic consult for chronic pelvic pain, dysmenorrhea, dyspareunia and infertility (Giudice, L.C., 2010; Holicov-Lutuc, M., 2014; May, K.E., et al., 2010; Verkauf, B.S., 1987).

Patients with ovarian endometriosis face a lot of inconveniences daily, which causes deterioration in the quality of life, an increased stress due to infertility, depression and anxiety (Bulun, S.E., 2009; Burney, R.O. Giudice, L.C., 2012).

The data based on our cases is in accordance with the data in the specialized literature, which describes the fact that there is a prevalence of 30-50% for the women with endometriosis, who also suffer of infertility (Bulun, S.E., 2009; Streuli, I., et al., 2013).

Protective factors (Giudice, L.C., 2010; Holicov-Lutuc, M., 2014):
- ➢ multiparity
- ➢ COC (combined oral contraceptive)
- ➢ Regular physical exercise

Increased incidence (Giudice, L.C., 2010; Holicov-Lutuc, M., 2014):
- ➢ Pregnancy over 35 years old
- ➢ High socioeconomic level
- ➢ menstrual cycles ‹ 27 days
- ➢ abnormally long menstruations › 8 days
- ➢ malformations connected to Muller duct.

The results of our study come to confirm the importance of determining these markers for the diagnosis and for monitoring the patients with endometriosis and infertility.

PURPOSE AND OBJECTIVES

The main purpose is, on the one hand, a better understanding of the problems our patients have to face, by offering them more social and psychological support to overcome these obstacles (Barbieri, R.L., et al., 1986; Bedaiwy, M.A., et al., 2002, Bedaiwy, M.A. Falcone, T., 2004; Giudice, L.C., 2010; Holicov-Lutuc, M., 2014; May, K.E., et al., 2010; Pittaway, D.E., et al., 1989), and on the other hand, improved therapeutic options (Peltecu, Gh., 2014).

MATERIAL AND METHOD

In this study we assessed a number of 200 patients aged between 18 and 45 (the mean age being about 32) with endometriotic ovarian cysts, who were investigated at "Elena Doamna" Clinical Hospital of Obstetrics and Gynaecology in Iași, in the period of time 2012–2016, 94 of which infertility associated with endometriosis.
We performed:

- a Doppler endovaginal ultrasound examination (Giudice, L.C., 2010; Peltecu, Gh., 2014);
- the anatomo-pathologic exam (the patients who were performed ablation or tissue excision during surgery had an extemporaneous examination, and also a histologic examination for paraffin, in order to confirm or infirm endometriosis) (Giudice, L.C., 2010; Holicov-Lutuc, M., 2014; May, K.E., et al., 2010;).

The assessment of serum markers
We observed the changes of the serum markers in endometriosis, studying CA-125 and cytokines IL-6 and TNF-α, as indicators for diagnosing endometriosis (Barbieri, R.L., et al., 1986; Bedaiwy, M.A., et al., 2002, Bedaiwy, M.A. Falcone, T., 2004; Keenan, J.A., et al., 1995; Koyama, N., et al., 1993).

Statistical analysis
Data was loaded and processed with the help of statistical functions in SPSS 18. We applied: ANOVA significance tests – using the descriptive indicators of the monitored parameters, with 95% interval of trust; t-Student test – quantitative test applied to study the significant difference between three environments; χ^2 test – a qualitative test by which two or more frequencies of the same population were compared; Pearson correlation to determine the relation between the parameters considered in the study. By drawing the ROC curve, predictability of serum markers was determined in infertility determinism.

RESULTS AND DISCUSSIONS

The social status from the perspective of its association with the degree of addressability to the doctor for the patients showed the fact that, generally, they were part of families with an income above the average (94%).
The age of the patients varied from 18 to 45 years old, with a mean of 31.77±5.23 years old and a median of 35, which suggests the homogeneity of the series of values.
Marital status of the patients with endometriosis: 72.5% married; 15.34% single and 12.1% in a relationship.
Living environment was mainly urban 86%.
The location of endometriosis was unique or mixt:
- ovary 100%;
- anterior cul de sac or in the bladder, broad ligament, uterus, parietal peritoneum (44%);
- Douglas pouch and utero-saccharal ligaments (3%);
- Fallopian tube (2%):
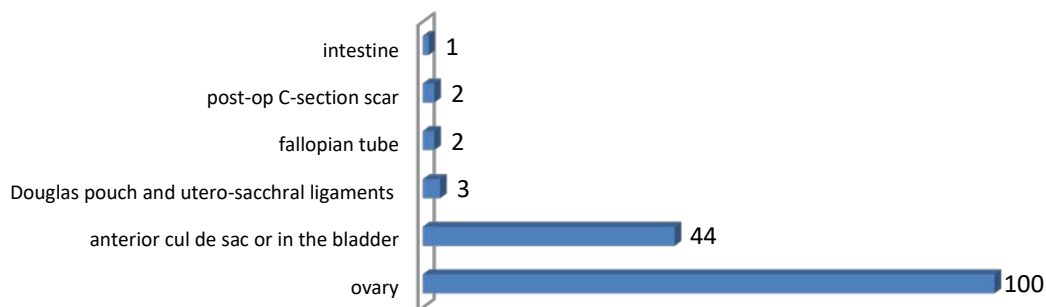- Post-op C-section scar (2%);
- intestine (1%).

Fig. 1. Share of cases depending on the location of endometriosis

Signs and symptoms

Among the main symptoms identified in the cases studied, we noticed: dysmenorrhea (90%), pelvic pain (87.5%), irregular menstrual cycles (67.5%). Gastrointestinal tract manifestations were predominantly constipation / diarrhea (28%), and from the urinary tract manifestations haematuria was identified more frequently (42.5%) (tab. I)

Table I. Signs and symptoms for the patients with ovarian endometriosis

Signs and symptoms	n	%
In the reproductive organs		
Dysmenorrhea	180	90.0
Pelvic pain	175	87.5
Lumbosacral pain	84	41.0
Irregular menstrual cycles	135	67.5
Torsion / rupture of the endometrium	44	22.0
Infertility	94	47.0
Gastro-intestinal tract		
Cyclic rectorragia	27	13.5
Rectal tenesmus	40	20.0
Alternant constipation/diarrhea	56	28.0
Urinary tract		
Haematuria	85	42.5
Dysuria	28	14.0
Ureteral obstruction	8	4.0

Based on the cases studied. infertility was associated in 47% of the cases with ovarian endometriosis, which allowed us to split the study group in two sub-groups:
- 94 patients with ovarian endometriosis and infertility;
- 106 patients with ovarian endometriosis without infertility.

Based on the demographic characteristics, we noted an increased frequency of infertility cases for the overweight patients (31.9%), with a mean age of about 35 years old, smoking (18.1%), and a sedentary life style (95.7%) (tab. II).

Table II. Characteristics of the group with endometriosis depending on the presence of infertility

Characteristics	Lot with infertility (n=94)	Lot without infertility (n=106)	p
Demographic data			
Mean age (years)	34.91±9.34	28.63±12.15	**0.001**
Obesity	30 (31.9%)	28 (26.4%)	0.484
Smoking	17 (18.1%)	3 (2.8%)	**0.001**
Alcohol consumption	7 (7.5%)	9 (8.5%)	0.991
Sedentary life style	90 (95.7%)	98 (92.5%)	0.496

Characteristics	Lot with infertility (n=94)	Lot without infertility (n=106)	p
Personal pathologic history			
Cardiovascular damage	92 (97.9%)	98 (92.5%)	0.153
Chronic kidney disease	54 (57.4%)	27 (25.5%)	**0.001**
Respiratory damage	13 (13.8%)	11 (10.4%)	0.595
Psychiatric damage	12 (12.8%)	22 (20.8%)	0.189
Neoplasia	7 (7.4%)	6 (5.7%)	0.823
Gastro-intestinal damage	4 (5.3%)	3 (2.8%)	0.593
Autoimmune diseases	1 (1.1%)	5 (4.7%)	0.273
Diagnostic			
Laparoscopy	63 (67.0%)	75 (70.9%)	0.680
Laparotomy	27 (28.7%)	13 (12.3%)	**0.006**
Transvaginal ultrasound exam	33 (35.1%)	22 (20.8%)	**0.035**
CT scan	19 (20.2%)	6 (5.7%)	**0.004**
MRI	46 (48.9%)	36 (34.0%)	**0.045**
Lab parameters			
Haemoglobin, g/dL	13.14±1.48	13.26±1.33	0.561
Total cholesterol, mg/dL	181.45±43.23	187.95±45.84	0.305
Serum triglycerides, mg/dL	116.86±46.66	115.13±55.63	0.853
Glycemia, mg/dL	115.33±49.89	100.99±19.92	**0.007**
Creatinine Clearance	67.96±32.07	92.01±29.30	**0.001**
Creatinine, mg/dL	1.44±0.52	0.88±0.34	**0.001**
Proteinuria, mg/dL	195.63±126.12	98.68±23.00	**0.050**
Urea, mg/dL	29.18±18.41	18.09±4.42	**0.005**
C-Reactive Protein, mg/dL	12.00±4.58	6.72±1.99	**0.002**
CA-125, U/mL	33.21±15.02	27.10±10.10	**0.021**
IL-6, pg/mL	1.99±0.15	0.95±0.10	**0.049**
TNF-α	16.15±9.90	14.13±8.71	0.625

Considering the *personal history*, we noted by frequency: cardiovascular damage (97.9%) and chronic renal disease (57.4%) which apparently induce a twice higher relative risk of atherosclerosis (RR=2.26; IC95%: 1.56-3.26).

The lab parameters showed significantly increased values of glycaemia for the patients with infertility (115.33 *vs* 100.99 mg/dl; p=0.007), also of creatinine (1.44 *vs* 0.88 mg/dl; p=0.001), proteinuria (195.63 *vs* 98.68 mg/dl; p=0.05), urea (29.18 *vs* 18.09 mg/dl; p=0.005) and **CRP** (12 *vs* 6.72 mg/dl; p=0.002) and the mean value of creatinine clearance was significantly reduced (67.96 *vs* 92.01; p=0.001).

CA-125 marker (33.21 vs 27.10 U/ml; p=0.021) and **IL-6** (1.99 *vs* 0.95 pg/ml; p=0.049) recorded mean values that were significantly higher for the patients with infertility. CA-125 is associated with ovarian cancer most of the times and there is a wrong impression that an increased level of CA-125 in the blood means cancer ovarian, or that a normal level means the absence of cancer (Barbieri, R.L., et al., 1986; Lucidi, R.S., et al., 2005).

By drawing ROC curve we noticed that CA-125 marker was a good predictor in identifying infertility (AUC=0.747; IC95%: 0.555–0.939). The analysis proved the fact that in the analysed group there is no significant correlation between the values of CA-125 and the patients' age (r=0.1006. p=0.618).

We assessed the size of the ovarian endometrium (cm) by performing an endovaginal or abdominal ultrasound exam, using a GE Voluson 750 ultrasound machine. The investigation proved the fact that, for the analysed group, there is a significant correlation between the values of CA-125 and the size of endometrium (r=0.41. p=0.038). In the presence of adhesions, significantly higher values were found for CA-125 (F=3.71. p=0.041). The analysis showed that in case of tubal damage (the uni- or bilateral hydrosalpinx) the values of CA-125 were significantly higheri (F=11.059. p=0.031). It was also proved that. in case of peritoneal damage through endometriotic implants. the values of CA-125 were significantly higher (F=7.118077; p=0.037).

The serum dosage of IL- 6 de 1.99 pg/mL have a sensitivity of 90% and a specificity of 67% in diagnosis and **TNF-α** dosed from the peritoneal fluid, with a value of over 16 pg/mL has 100% sensitivity and 89% specificity when establishing the diagnostic. These values are similar with those from the specialized literature (Keenan, J.A., et al., 1995; Pittaway, D.E., Douglas, J.W., 1989).
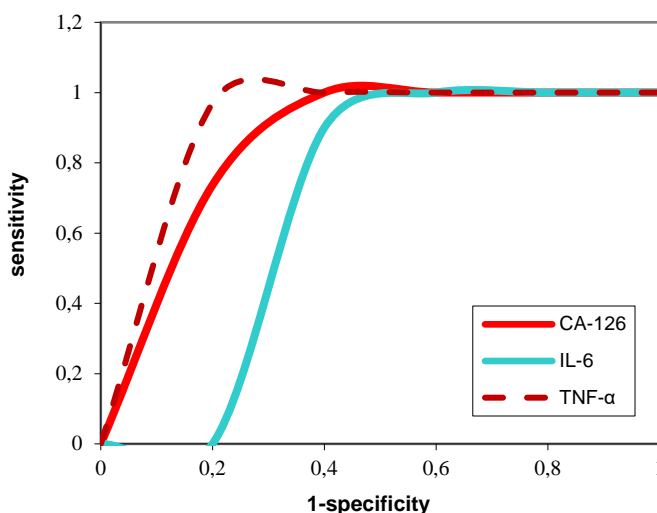

Fig. 2. Predictability of serum markers for detecting infertility

The mean values of haemoglobin, total cholesterol and serum triglycerides did not show significant differences depending on the absence or presence of infertility (p>0.05) (tab. II).

CONCLUSIONS

Endometriosis is considered a tabu disease, with serious consequences on the physical and mental health.
Endometriosis is the first cause of feminine infertility.

The disease is translated into insufferable pain, long term treatment and their side effects, many surgeries and infertility.

For the patients with ovarian endometriosis, CA-125 markers with a mean level of 33 U/ml and IL- 6 with a mean level of 2 pg/ml, proved to be good predictors of infertility.

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INQUIRY ON AMYLASE ISOZYMES IN BARLEY (*HORDEUM VULGARE*) SEEDS DURING GERMINATION

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Abstract. Barley is an important crop for both human and animal food use because of its high starch seed content. Germination, as a process of developing a new plant, or as part of making alcoholic beverages, make use of starch stored in barley seeds as food for the embryo. Amylases, who broke down the starch, are among the most important enzymes, mostly from human point of view. Analyzing and understanding the whole starch mobilizing process by amylases could be of high value for both the agricultural processes and for biotechnological ones. The amylases from germinating barley seeds have been investigated by electrophoresis for the presence of different isoenzymic forms. Both α and β amylases have been investigated and the results shows that there is more than one isoform in both types of amylases.

INTRODUCTION

Since immemorial times amylases and barley seeds are a part of human life, even if only looking to day to day work or to celebration. As a process taking place in plants, germination is one of the most important processes. Barley is the fourth main among grains, considering the quantity produced each year. Studies shows that it was among the first plants used by humans. Even if today its importance as human food had decreased it remains very important as animal food and base for malting and brewing. (Smith, 1998; Ulrich, 2011)

As substances all enzymes, the well-known biological catalysts, belong to the proteins. (Pratt et al., 2018) It was proved that an enzyme could have more than one form and that each of its many forms is an isozyme. (Markert, 1968; Scandalios, 1969; MacDonald et al., 1972; Soltis et al., 1989) The existence and the identification of different forms of the enzymes is a problem related with the genetic variability of each individual of a species and also could be a problem of localization within a cell compartment or even tissue (MacDonald et al., 1972; Daussant et al., 1994)

Amylases have been well studied from many point of view. It is well known that there are more than one type and in each specific type there are isoforms. (van Onckelen et al., 1969; MacDonald et al., 1972; Brown et al., 1982; Daussant et al., 1994)

The main purpose of the paper was to find if there are any different isozymes of both, α and β amylase, in the extracts made with specific extraction solutions from the probes collected during germination of the seeds from barley (*Hordeum vulgare*).

MATERIALS AND METHODS

For our study we used barley (*Hordeum vulgare*) seeds that have been produced in farms from our region (Nord-Eastern part of Romania). Three kinds of seeds (noted as A, B and C) have been chosen to be subjected to germination. Germination was performed in Petri dishes on sterile material and prior to germination seeds have been checked for integrity then washed thoroughly with water. (Akinyosoye et al., 2014) To ensure that almost no fungi will grow alongside germinating seeds, those were cleaned with 30% H_2O_2 for 10 minutes then washed again, few times, with sterile water. (Deno, 1993) Samples have been collected on a day to day basis. Germination was considered over when newly formed leaves become green. (Bewley et al., 1994; Bewley, 1997) All the samples have been stored in a freezer at -25°C until analysis was done.

To extract amylases, probes have been taken from each sample and extracted 1/10 after they were crushed using a mortar and pestle. Extraction of α-amylase has been done with distilled water. β-amylase was extracted with a pH 8 buffer solution, 50 mM Tris-HCl with added 1mM EDTA. (Artenie et al., 2008) Centrifugation of those extracts was performed in two steps, first at 4000 rpm for 25 minutes, then with smaller samples, at 13000 rpm, both in cold, 4°C. The clear extracts were used for electrophoresis.

Electrophoresis was performed according to Laemli system using native conditions. The migrating gel was 8%. (Sambroock et al., 2001) Following the electrophoresis gels have been incubated for 1 hour in a 2% starch solution at room temperature. Then gels were washed with distilled water and incubated with N/3000 iodine solution. Starch gives a blue color when mixed with iodine and gels become blueish with clear spots where amylase is present, and starch has been hydrolyzed.

(Artenie et al., 2008) Because the color is not stable in time photos have been taken immediately using a transilluminator and a DSLR. Further analysis of gels was done by comparing the Rf of clear spots representing different isoforms of amylase(s).

RESULTS AND DISCUSSIONS

Following the experiments, it is very interesting that each of our type of barley seeds expressed a different time needed for germination. From only 4 days in A variant to 6 days in B variant and 9 days in C variant.

Profiles of α-amylase isozymes from barley (*Hordeum vulgare*) seeds during germination are presented in Figure 1 A, B and C.

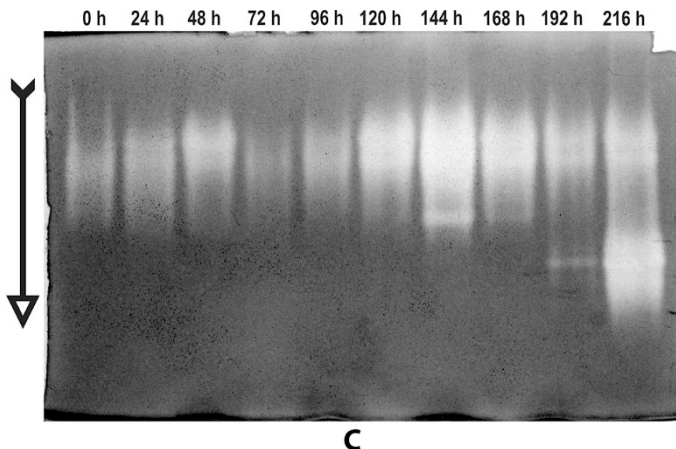Even if it had the shortest germination period A variant exhibit many isozymes compared with variant B and C.
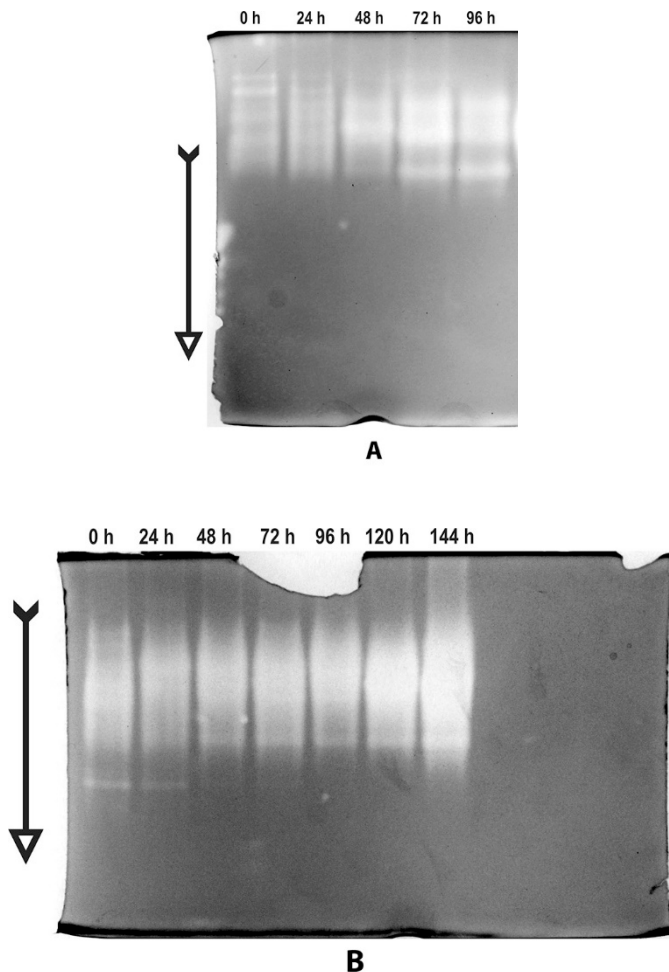
A

B

Figure 1 α-Amylase isozymes identified in extracts from barley (*Hordeum vulgare*)
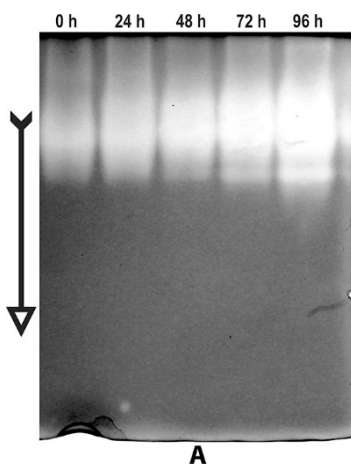seeds during germination

In variant A spots with Rf around 0,26 present an increase in activity during germination (reflected in spot dimensions) and are present from dry seed until the end of germination. Spots with smaller Rf tend to disappear but at the end of process (72 hours and 96 hours) a new spot appear with Rf 0,35.

The simplest profile is found in barley seeds variant B where only a very big spot is clearly visible throughout the entire germination.

Almost same situation is to be found in variant C but here, at the end of germination, maybe because of the newly formed plant, there is some variation.

Looking to all identified spots corresponding to different isozymes of α-amylase form those three variants analyzed we could conclude that there is more than one isozyme in each variant and that during germination profile change.

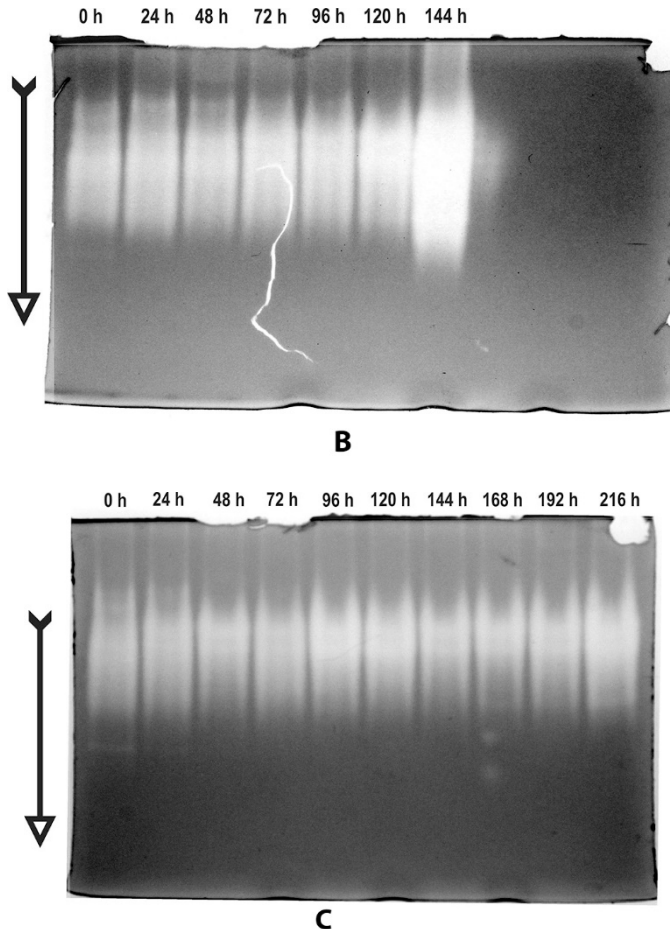For β-amylase there have been detected far less spots no matter the variant (A, B or C) of the seeds.

Figure 2 β-Amylase isozymes identified in extracts from barley (*Hordeum vulgare*) seeds during germination

In each case there is a very big spot which somehow masks almost all other (if any) spots. Though, at the beginning of at the end of the process there are some small spots, corresponding to different isoforms of β-amylase.

Future analysis using different acrylamide / bisacrylamide concentrations will be needed to further enhance the separations to achieve a better image over the spectrum of isozymes of amylases during germination of barley seeds.

CONCLUSIONS

There have been detected several isozymes of α-amylase in the extracts made from germinating barley seeds.

The profile of those isozymes is changing through the entire germination process.

β-Amylase shows less diversity concerning the presence of isozymes.

More investigations are needed to find the slightest differences between different isozymes, both in α-amylase but also in β-amylase during germination in barley (*Hordeum vulgare*) seeds.

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