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REVIEW

THE HISTORY OF HLA AND ITS RELATED DISEASES

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INTRODUCTION

Allogeneic tumor transplantation was often used in early experiments to study tumor biology. In the early 1900s, two US geneticists, Ernest E. Tyzzer and Clarence C. Little, performed some crucial tumor transplantations in the offspring of crosses between mice that were susceptible or resistant to an allogeneic tumor. An antigen responsible for rejection was first discovered by the British physician and pathologist Peter A. Gorer in 1936, working at that time in the Lister Institute for Preventive Medicine in London. Following a suggestion from the British geneticist J.B.S. Haldane, he studied whether resistance factors to the growth of allogeneic tumors might be associated with some blood group antigens. First, he found that his own serum contained 'natural' antibodies that could distinguish between erythrocytes of three inbred strains of mice (1). Snell was studying tumor resistance genes, which he called *histocompatibility* or *H genes*.

Three papers appeared in 1958 by Jean Dausset, Jon van Rood and Rose Payne and their associates, respectively (2-4), which laid the foundation of what was later to become the description of the first human leukocyte antigens – the *HLA complex*. The discovery of the major histocompatibility antigens as so-called strong transplantation antigens, which play a major role in graft rejection after transplantation, has its origin in the animal experimental analysis of tissue transplantations with mice.

In 1960, Payne, together with Julia and Walter Bodmer, also using sera from multiparous women, not only detected two leucocyte antigens, LA1 (later HLA-A1) and LA2 (later HLA-A2), apparently controlled by alleles but also postulated at least one additional antigen, LA3, determined by an additional allele at the same locus (5).

A reversal point in the history of tissue typing was the first Workshop and Conference on Histocompatibility, which was organized by Bernhard Amos (Durham, NC) in 1964. During this workshop the participants compared the reactivity of their sera with various techniques. The results were incompatible, and could not be published.

During the second Workshop, which took place in 1965 in Leiden, the results were more consistent. Hereon Paul Terasaki and John McClelland at UCLA introduced the complement-dependent microlymphocytoxicity technique. This method has remained the standard serological test for HLA typing.

Sheehy et al. (6) reported that LD (or Dw) determinants could also be identified by priming lymphocytes against given LD determinants, which was called *primed LD typing*. Later studies showed that the provisional HLA-D locus consisted of several different closely linked loci, which encoded three different series of determinants, DR, DQ (previously called DC) and DP (previously called SB).

In 1968, an HLA Nomenclature Committee was established (sponsored by World Health Organization, (WHO)) consisting of leading investigators in the field. This committee, which still exists, is responsible for giving official names to HLA specificities and loci.

Kissmeyer-Nielsen and associates established that there were two HLA loci (7). They called the two loci 'LA' (adapted from the LA antigens of Payne and coworkers) and '4' or 'four' (adapted from the 4a and 4b antigens of van Rood).

In the early 1980s, the overall picture was that the HLA chromosomal region, found to be present on the short arm of chromosome number 6, encoded six different very polymorphic series of determinants, A, B and C that were present on most nucleated cells and DR, DQ and DP that remained present on B cells, monocytes and dendritic cells.

In 1967, Ceppellini had introduced the term HLA haplotype for the genetic information carried by each of the two HLA chromosomal regions of an individual. Klein (8) introduced the terms class I to describe the A, B and C antigens and class II to describe the DR, DQ and DP antigens (and the corresponding antigens in other species), a nomenclature that has since been followed. Furthermore, after the discovery of additional class I antigens, HLA-G, -E and -F, with a more limited tissue distribution, the latter were named non-classical HLA class I antigens, while the HLA-A, -B and -C antigens were named classical HLA class I antigens.

NOMENCLATURE OF HLA GENES AND ALLELES

The nomenclature of the HLA antigens is split by the information content of immunological methods (binding of antibodies,

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cellular methods) and DNA based typing methods. A number of HLA gene fragments have been reported and named. These are HLA-T previously known as HLA-16, HLA-U previously known as HLA-21, HLA-V previously known as HLA-75, HLA-W, HLA-80, HLA-P, HLA-90, HLA-Y, and HLA-BEL/COQ/DEL respectively (10).

a. Conditions for acceptance of new allele sequences (10)

As emphasized in previous reports, there are required conditions for acceptance of new sequences for official names.

1. Where a sequence is obtained from cDNA, or where PCR products are sub-cloned prior to sequencing, several clones should have been sequenced.

2. If direct sequencing of PCR amplified material is performed, products from at least two separate PCR reactions must have been sequenced.

3. In individuals who are heterozygous for a locus, and where one of the alleles is novel, the novel allele must be sequenced in isolation from the second allele.

4. A novel sequence should be confirmed by typing of genomic DNA using PCR-SSOP or PCR-SSP method. Where a new sequence contains either a novel mutation or a previously unseen combination of nucleotides, this must be confirmed by a DNA typing technique.

5. An accession number in a databank should have been obtained. Sequences may be submitted to the databases online at the following addresses:

EMBL: www.ebi.ac.uk/Submissions/index.html

GenBank: www.ncbi.nlm.nih.gov/Genbank/submit.html

DDBJ: www.ddbj.nig.ac.jp/sub-e.html

6. Full-length sequences are preferable though not essential; the minimum requirements are complete exons 2 and 3 for an HLA class I sequence and complete exon 2 for an HLA class II sequence.

7. Where a novel sequence differs only within an intron or other non-coding part of the gene, a full-length sequence must be obtained, which covers all coding and non-coding regions. In the absence of a full-length genomic sequence from the most closely related allele that is identical in its exon sequence, it may be required that this also be sequenced and submitted before a name can be assigned to the novel sequence.

8. Sequences derived solely from tumour material will not be considered for nomenclature.

9. The complete HLA type for the *HLA-A*, *-B* and *-DRB1* genes should be submitted for the material in which a novel allele has been defined.

10. Submission of a sequence to the WHO Nomenclature Committee should be performed using the online submission tool available at www.ebi.ac.uk/imgt/hla/subs/submit.html.

A list of the newly reported alleles is published each month in nomenclature updates in the journals Tissue Antigens, Human Immunology and the International Journal of Immunogenetics. The listing of references to new sequences does not imply priority of publication (10).

b. New Allele Sequences

A total of 2558 HLA alleles have been named since the last report. For HLA class I, 616 *HLA-A*, 913 *HLA-B*, 446 *HLA-C*, four *HLA-E*, 19 *HLA-F*, 31 *HLA-G*, 12 *HLA-H*, nine *HLA-J*, six *HLA-K*, five *HLA-L*, four *HLA-P* and three *HLA-V* alleles were named, making a total of 3249 class I alleles with official names. For HLA class II, 368 *HLA-DRB1*, 12 *HLA-DRB3*, one *HLA-DRB4*, one *HLA-DRB5*, seven *HLA-DQA1*, 45 *HLADQB1*, six *HLA-DPA1*, 22 *HLA-DPB1*, one *HLA-DMB* and four *HLA-DOA* alleles were named, making a total of 1198 class II alleles with official names (10).

INTRODUCTION OF COLON DELIMITED HLA ALLELE NAMES

The convention of using a four-digit code to distinguish HLA alleles that differ in the proteins they encode was introduced in the 1987 Nomenclature Report (11). Since that time additional digits have been added, and currently an allele name may be composed of four, six or eight digits dependent on its sequence. The first two digits describe the allele family, which often corresponds to the serological antigen carried by the allotype. The third and fourth digits are assigned in the order in which the sequences have been determined. Alleles whose numbers differ in the first four digits must differ by one or more nucleotide substitutions that change the amino-acid sequence of the encoded protein. Alleles that differ only by synonymous nucleotide substitutions within the coding sequence are distinguished by the use of the fifth and sixth digits. Alleles that only differ by sequence polymorphisms in introns or in the 5' and 3' untranslated regions that flank the exons and introns are distinguished by the use of the seventh and eight digits (10).

In 2002, the *A*02* and *B*15* allele families had more than 100 alleles (12). At that time the decision taken was to name further alleles in these families in the rollover allele families *A*92* and *B*95* respectively. For *HLA-DPB1* alleles, it was decided to assign new alleles within the existing system, hence once *DPB1*9901* had been assigned, the next allele would be assigned *DPB1*0102*, followed by *DPB1*0203*, *DPB1*0302* etc.

With the ever increasing number of HLA alleles described it has been decided to introduce colons (:) into the allele names to act as delimiters of the separate fields. To facilitate the transition from the old to the new nomenclature, a single leading zero must be added to all fields containing the values 1 to 9 but beyond that no leading zeros are allowed. This will help to lessen any confusion in the conversion to the new style of nomenclature (10).

Hence

*A*01010101* becomes *A*01:01:01:01*

*A*02010102L* becomes *A*02:01:01:02L*

*A*260101* becomes *A*26:01:01*

*A*3301* becomes *A*33:01*

*B*0808N* becomes *B*08:08N*

*DRB1*01010101* becomes *DRB1*01:01:01:01*

For allele families that have more than 100 alleles such as the *A*02* and *B*15* groups it will be possible to encode these in a single series. Thus the *A*92* and *B*95* alleles have now been

renamed in to the *A*02* and *B*15* allele series (10).

For example:

*A*9201* becomes *A*02:101*

*A*9202* becomes *A*02:102*

*A*9203* becomes *A*02:103* etc

*B*9501* becomes *B*15:101*

*B*9502* becomes *B*15:102*

*B*9503* becomes *B*15:103* etc

The names *A*02:100* and *B*15:100* will not be assigned (10).

In cases of other allele families where the number of alleles reaches 100 these will be numbered sequentially, for example *A*24:99* will be followed by *A*24:100* (10).

The *DPB1* allele names that have been previously assigned names within the existing system have also be renamed, for example (10):

*DPB1*0102* becomes *DPB1*100:01*

*DPB1*0203* becomes *DPB1*101:01*

*DPB1*0302* becomes *DPB1*102:01*

*DPB1*0403* becomes *DPB1*103:01*

*DPB1*0502* becomes *DPB1*104:01* etc

The 'w' will be removed from the *HLA-C* allele names, but will be retained in the HLA-C antigen names, to avoid confusion with the factors of the complement system and epitopes on the HLA-C molecule often termed C1 and C2 that act as ligands for the Killer-cell Immunoglobulin-like Receptors (10).

*Cw*0103* becomes *C*01:03*

*Cw*020201* becomes *C*02:02:01*

*Cw*07020101* becomes *C*07:02:01:01* etc

These changes to the HLA Nomenclature were officially introduced in April 2010. A full listing of old and new HLA allele names was made available through the IMGT/HLA Database (www.ebi.ac.uk/imgt/hla) (13) and implemented with the April 2010 release of the database (10).

MOLECULAR STRUCTURE AND FUNCTION OF HLA MOLECULES

The extended collection of genes on the short arm of human chromosome 6 at 6p21.3 is called Major Histocompatibility Complex (MHC). This region is very gene dense and highly polymorphic. It was originally identified and named because of its role in tissue rejection after transplantation. Furthermore, many of the genes in this region show an important role in the biology of the immune system. The MHC is subdivided into three classes. The classification is based on main functional characteristics of the genes within each of the classes. The human MHC class I and class II are also called HLA class I and class II. Genes in the HLA code for HLA molecules. The main role of the HLA molecules has little to do with transplantation. Originally these molecules are receptors, which capture peptides or protein fragments of antigens. They are present on cell surfaces where they can be recognized by appropriate T cells. With foreign antigens displaying to cells, HLA molecules evoke cytotoxic T lymphocyte (CTL). CTL is helper for a T cell response, which then regulates specific immunity (14, 15).

HLA class I

Telomeric region of the MHC have the genes of the MHC class I. This class contains genes which encode the α-chain of HLA class I molecules. The region of the MHC class I expand over 2 Mb. These genes are also members of the immunoglobulin gene family. The molecules of the MHC class I genes are involved in the presentation of peptides predominantly derived from intracellular proteins, to CD8+ cytotoxic T-cells. The α-chains form heterodimers with the non-MHC coded β_2-microglobulin (β_2m). The heavy α-chain (45 kDa) is non-covalently associated with the β_2-microglobulin (12 kDa) a polypeptide which is also found free in serum. The HLA class I α-chain contains three extracellular domains, a transmembrane region and the cytoplasmic C-terminus. The extracellular domains are α_1, α_2 and α_3. The domains α_1 (N-terminal) and α_2 together create the peptide-binding groove (14).

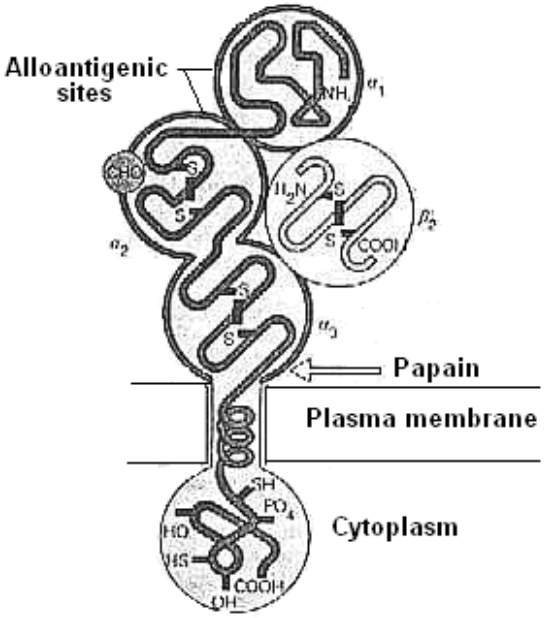

Fig. 1. Schematic representation of a class I antigen

In human β_2-microglobulin is a non-polymorphic protein, which is encoded on chromosome 15. It has the structure of an immunoglobulin C domain. β_2m is also associated with a number of other class I-related molecules, for instance the products of the CD1 genes on chromosome 1. The HLA-molecules of the MHC class I region are expressed ubiquitously (14).

HLA class II

HLA class II molecules are membrane glycoprotein formed through the non-covalent association of a 32 kDa α-chain and a 28 kDa β-chain. The α- and β-chains of all traditional HLA class II molecules have the same overall structure (15).

The structure contains two extracellular domains, which are referred to as α_1-α_2, and β_1-β_2, respectively. The domains distal of the membrane, α_1 and β_1 together form a peptide-binding groove (Figure 2) (15).

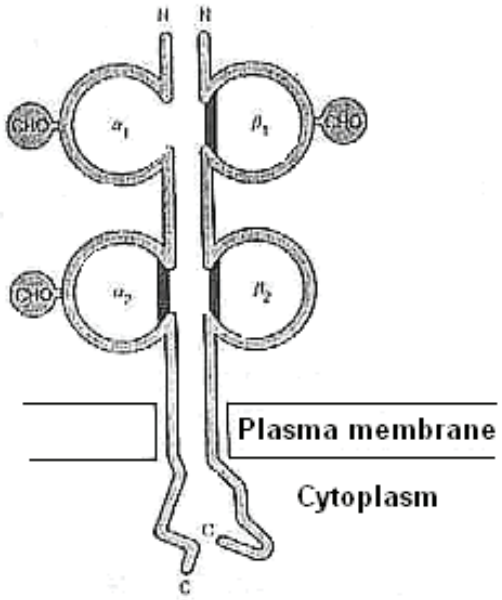

Fig. 2. Schematic representation of a class II antigen

HLA class II molecules have a more limited tissue distribution than HLA class I molecules. Class II molecules are mainly expressed in the cells of the immune system such as B-cells, dendritic cells, macrophages and activated T-lymphocytes (15).

MHC class II is the most centromeric class of the MHC. This class contains the genes HLA-DP, HLA-DQ and HLA-DR, as the so called classical MHC II genes, as well as HLA-DM and HLA-DO as non-classical MHC II genes. Genes of the MHC class II are found in pairs, encoding the α- and β-chains, which form the heterodimers of the MHC class II protein molecules (15).

CURRENT METHODS FOR HLA TYPING

Since the mid-80s serological typing methods, which were carried out exclusively (16), are complemented by analysis at the DNA level. During the 9th IHCW in 1984, several papers described the use of restriction fragment length polymorphisms method to study HLA at the DNA level (17). The introduction of the PCR (18) allowed the development of a variety of simple and rapid DNA sequence based typing methods (19, 20). Nowadays several methods, which describe the HLA at DNA level, are standard methods in HLA typing laboratories. The most frequently used methods in the HLA typing laboratories are "Sequence Specific Oligonucleotide Probe Hybridization" (SSOP), "Sequence Specific Primers Amplification" (SSP), "Reverse Line Blot" and "Sequence Based Typing" (SBT). The latter method is in principle sequencing of the different HLA genes. It is the only technique which allows identification of unknown HLA alleles immediately. Another high resolution technique which is used in quite a few HLA typing laboratories is "Reference Strand Conformation Analysis". All these DNA based methods have in common the use of polymorphisms in the DNA sequence to identify the HLA type.

Nevertheless serological typing methods are still standard procedures in many HLA typing laboratories. For example, many laboratories use serological typing as a pre-typing method before SBT.

HLA ANTIGENS ARE ASSOCIATED WITH DISEASES

The human leukocyte antigen (HLA) region has been associated with hundreds of human diseases, including most of autoimmune diseases (21). HLA-associated diseases can be the result of the combination of different HLA molecules expressed at various loci (class-I and/or class-II) rather than the result of one HLA variant only. Almost all HLA-associated diseases are multifactorial polygenic diseases in which HLA allele(s), in combination with other genetic variants and environmental factors, is involved in disease susceptibility. Human autoimmune diseases are phenotypically heterogeneous, in terms of clinical presentation, age at onset, association with other autoimmune disorders, and severity or rapidity of evolution, and it is likely that different alleles, or allelic combinations at different loci, will predispose to different forms of the disease (22).

The molecular mechanisms underlying HLA associations have been determined in few autoimmune diseases in which both autoantigen(s) and specific disease-associated HLA variants were unambiguously identified, such as celiac disease (CD) and type 1 diabetes (T1D). A number of hypotheses have been proposed on the basis of comparison HLA alleles at the sequence level and theoretical, but in the absence of known autoantigen, no molecular or functional analysis has been able to confirm any hypothesis. This is particularly well illustrated in two diseases – ankylosing spondylitis (AS) and narcolepsy – in which HLA associations are very strong and perfectly defined at the allelic level, but the mechanisms underlying these associations are still unknown (22). In 1973, Brewerton et al. (23) and Schlosstein et al. (24) independently reported a very strong association between HLA-B27 and ankylosing spondylitis. The studies found that 88%–96% of the patients carried HLA-B27 compared with 8%–4% of healthy controls, respectively. Later, the same year, Jersild et al. (25) first reported a strong disease association to an HLA class II antigen. Multiple sclerosis (MS) was found to be more strongly associated to a determinant as established by MLC typing, LD-7a (later to become HLA-Dw2, now HLA-DR2), than HLA-B7. The association of HLA-B27 with AS is one of the strongest; 90–95% of patients with AS are positive for HLA-B27 compared with less than 10% of healthy subjects, which confers B27 a high relative risk (>100). Given the disease prevalence (between 0.1% and 1.5%), the absolute risk of AS developing is about 5% in HLA B27-positive individuals, and most HLA B27-positive individuals remain healthy. Not all HLA-B27 allotypes have a same predisposing effect: while B*2702, B*2704, and B*2705 (and probably B*2707) are associated with AS, B*2706 and B*2709 are not. It has been shown that the HLA-B*2705 and B*2709 subtypes (that differ in only a single amino acid) can bind a shared peptide with indistinguishable conformation, whereas other peptides are bound in two extremely different conformations and with different stability.

Narcolepsy is strongly positively associated with the DQB1*0602 allele and 90–100% of patients with definite cataplexy carry this allele. The closely related DQB1*0601 allele, which differs at only nine

residues, protects against developing narcolepsy, suggesting that peptide-binding differences between these two alleles determine whether they predispose to or protect against narcolepsy. Because of the strong DQB1*0602 association, narcolepsy has long been suspected to be an autoimmune disease like virtually the other entire major histocompatibility complex (MHC)-associated disorders.

Regarding celiac disease (CD) more than 90% of patients express the HLA-DQ2 molecule, encoded by HLA-DQA1*0501-DQB1*0201, and most of the remaining patients carry DQ8, encoded by DQA1*0301-DQB1*0302. Thus, HLADQ2 and HLA-DQ8 are necessary, but there are not sufficient to predispose to CD (most DQ2- or DQ8-positive individuals remain healthy). A large number of non-HLA genes likely contribute to the pathogenesis of CD, but the participation of a single predisposing non-HLA gene might be quite modest.

T1D is a multifactorial and polygenic disease, which affects about 1% of the population. Approximately 50% of the genetic risk of T1D can be attributed to HLA genes, and a large number of studies have shown that specific alleles at the DRB1, DQA1, and DQB1 loci are strongly associated with T1D.

CONCLUDING REMARKS

It is now more than 50 years since Dausset first discovered a leukocyte antigen in man, which became the first HLA antigen, HLA-A2. Since then, the field has moved from *histocompatibility* to become one of the most central fields in basic and clinical immunology in general. The term MHC for the HLA complex and similar genetic complexes in animals should be considered a misnomer because the role of the HLA class I and II molecules as histocompatibility antigens is more a side-effect of their immunobiological function (25).

There are several reasons for the quick and extensive developments of the field. First are the instrumental contributions by its many pioneers. Some of them have received the Nobel Prize (Snell, Dausset, Benacerraf, Zinkernagel and Doherty). That such a relatively large number of Nobel Prizes has gone to pioneers in this field witnesses its importance. But another factor that must not be underestimated is the extensive international collaboration, which has taken place since the early days of HLA, in particular the IHWSs. Together, the pioneers and the extensive international collaboration are responsible for the giant progress we have seen in this field during the past 50 years (25).

Statistic studies proved that the frequency of an HLA antigen is abnormally increased in individuals with certain diseases (susceptibility genes) or decreased (protective genes) (26)

HLA-disease association may be generated by a connection which requires an intra-familial study, or a connection which translates in a relative risk between the sick subjects and the normal ones of the same population (26).

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REVIEW

SMALL INTERFERING RNAs AND THE RNA INTERFERENCE PHENOMENON

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ABSTRACT

The RNA interference phenomenon's applicability both in functional genomics and as a therapeutic tool for different disease including cancer has gained increased attention during the last few years. As a result the siRNA technology has rapidly evolved and currently several suppliers offer ready to use siRNA designs that target the mRNAs of a high number of genes. At the same time discovery of a high number of miRNA involved both in normal developmental stages but also in different pathological processes have gained a lot of interest in its practical use as a prognostic marker. This review presents, based on a rich documentation, the main aspects of RNAi mechanism, miRNA and siRNA pathways and their therapeutic applicability in cancer.

Key words: RNA interference, miRNA, siRNA, cancer

1. RNA interference mechanism

RNA interference (RNAi) is a phenomenon within the living organisms that controls gene expression by post-transcriptional gene silencing mechanism. The RNAi is an evolutionarily conserved gene silencing mechanism whereby small sequences of extrinsic dsRNA or intrinsic microRNA inhibit complementary post-transcriptional mRNA (35). The ancestral function of the RNAi system is generally agreed to have been immune defense against exogenous genetic elements such as transposoms and viral genomes (8, 32).

First pointed out by Fire et. al. 1998, RNAi occurs through a series of steps involving generation of small interfering RNAs fragments through the action of a specific RNAaseIII endonuclease. The small molecules resulted are able to mediate degradation of their complementary mRNA by association with a nuclease complex called the RNA-induced silencing Complex (RISC) (49). The dsRNAs (double stranded RNA) can be endogenous (originating in the cell) as pri-microRNAs expressed from microRNAs encoded genes within the genome. When the dsRNA is exogenous (coming from infection by a virus with a RNA genome or laboratory manipulations), the RNA is imported directly into the cytoplasm and cleaved to short fragments so called small interfering RNAs (siRNA) by the enzyme Dicer (53).

The RNA interference pathway has become very useful in experimental biology to study the function of genes both in vitro, in cell culture and in vivo, in animal model. Routinely double-stranded RNAs are synthesized with a sequence complementary to a gene of interest and introduced into a cell culture or organism, where once recognized as exogenous genetic material activates the RNAi pathway. Using this mechanism, a drastic decrease in the expression of a targeted gene can be induced. The RNAi mechanism may only partially abolish expression of the target gene. For this reason the RNAi silencing using siRNA technique is sometimes referred as a "knockdown", to make distinguish from "knockout" procedures in which expression of a gene is entirely eliminated (42). As methods of mediating the RNA interference effect two types of molecules have been developed: chemically syntethized **small double stranded RNAs (siRNAs)** and **short hairpin RNAs (shRNAs)**.

Fig.1 MiRNAs and siRNAs implication in RNA interference phenomenon schematic representation

2. MicroRNAs (miRNAs)

MicroRNAs (miRNAs) are short endogenous RNA molecules that have been shown to regulate, together with the epigenetic mechanisms the genes expression in plants and animals. Is being estimated that miRNAs could regulate 33% of the human genome (1). Since the discovery of the founding members of the class, *let-7* and *lin-4* miRNAs in *Caenorhabditis elegans* the number of discovered miRNA has considerably increase. Currently a total of 940 human, 590 mouse and 171 *D. melanogaster* miRNA sequences are already registered at the miRNA registry web site (52) and the list is expanding with every new miRNA identified.

Even if only a small number of miRNAs are known for their biological functions they are considered to have important roles in diverse regulatory pathways like: development timing, cells differentiation and proliferation, and cell death (31). Abnormal expression of miRNAs has been associated with different types

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of cancers, and also, germline and somatic mutations in human miRNAs were recently identified (39).

The fast rate of miRNAs discovery requires a strict evidence of the miRNAs identity within the scientific community. In order to avoid unintended overlap, it is important for researchers around the world to have free access to an independent arbiter of gene names and permanent up-to-date repository for published miRNA sequences.

3. MicroRNA pathway

Although the first miRNA was identified over ten years ago, it is only recently that scientists have begun to understand the role and diversity of these fascinating molecules (11). MicroRNAs are double stranded RNAs of 21-25 nucleotides derived from endogenously expressed transcripts with characteristic hairpin structures. In humans, the majority of miRNAs (70%) are transcribed from introns and/or exons, and approximately 30% are located in intergenes regions (50). Bradley and colleagues showed that ~70% of mammalian miRNA genes are located in defined transcription units. Interestingly, 117 miRNA genes were found in the introns in the sense orientation. Of these 117 intronic miRNAs, 90 miRNAs are in the introns of protein-coding genes, whereas 27 miRNAs are in the introns of non-coding RNAs (ncRNAs).

The miRNA biogenesis pathway starts in nucleus with the transcription of a primary miRNA (**pri-miRNA**) from a miRNA coding gene. The pri-miRNA has a hairpin structure and contains 70 up to 100 nucleotides. The primary miRNA is process in the nucleus by the ribonuclease *Drosha* to become miRNA precursor (**pre-miRNA**). After processing, the pre-miRNA is transported in the cytoplasm by exportin 5 where a second ribonuclease, *Dicer*, digests the pre-miRNAs resulting in a 21-25 nucleotide miRNA. *Dicer* is a highly conserved protein that is found in almost all eukaryotic organisms such as microorganisms, plants and animals (27). At this stage, the miRNA binds the *RNA-Induced Silencing Complex (RISC)*, and aligns with the mRNA. Based on the complementary level between the miRNA and the target sequence, the mRNA can either be translational repressed (partial complementary) or cleaved (identical complementary) (19). In Figure 2 is presented the schematic representation of miRNA pathway.

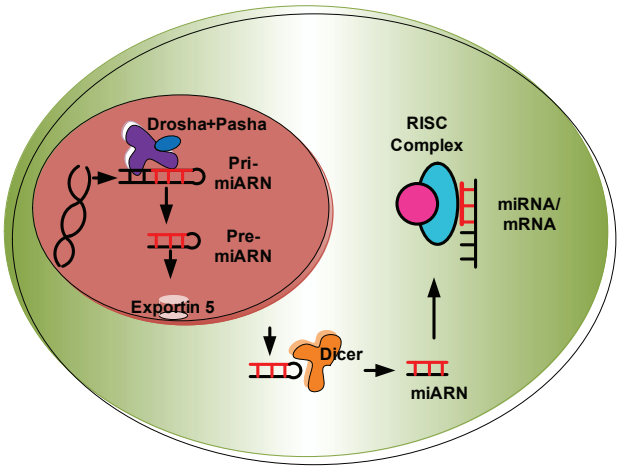

Fig. 2. MiRNA pathway

4. siRNA pathway

The siRNA pathway is considered an evolutionarily conserved response triggered by an externally introduced double stranded RNA (dsRNA). The **dsRNA** detected within the cell activates Dicer ribonuclease, which will cleave it into small interfering RNAs (siRNA) that are approximately 21-23 nucleotides (19).

The siRNA is successively loaded into an RNA-Induced Silencing Complex (RISC) which facilitates the separation of the two strands and alignment of the siRNA with its appropriate target mRNA. Based on siRNA complementarities to its target mRNA will repress or cleave the miRNA. In figure 2 is presented the siRNA biogenesis.

Scientists have used the siRNA pathway by artificially introducing either dsRNA or siRNA designed to degrade targeted mRNAs. These synthetic silencing reagents are used as molecular biology tools for novel gene identification, gene functional analysis, and biological pathways screening (*23*). In order to be able to synthesize a siRNA a number of parameters have been suggested: selection of a target cDNA region 50-100 nucleotides, downstream of the start codon, selection of a 5'-AA(N19)UU target mRNA sequence where N is any nucleotide, 50% G/C content in the target sequence, avoidance of 5' or 3' untranslated regions and high G-rich areas, and confirmation of exclusive target-specific sequences (15). Presently, custom siRNA synthesis service is available through a number of companies, including Dharmacon, Qiagen, and Ambion (5).

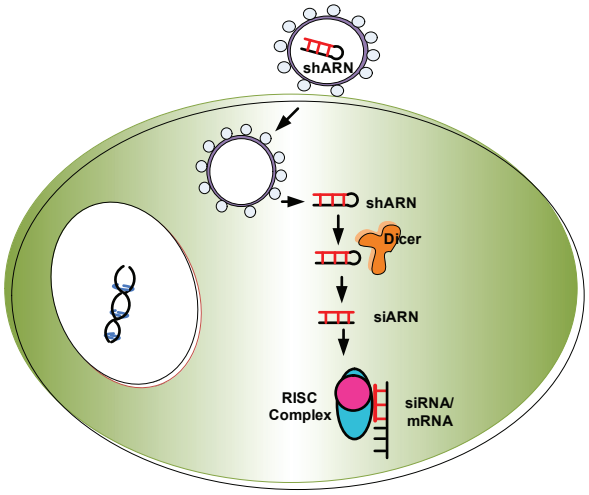

Fig. 3. siRNa biogenesis

In order to succeed siRNA internalization within the cells different approaches have been used like: electroporation (30), soaking in siRNAs (28), feeding bacteria carrying dsRNA (4), transfection with commercial reagents (15), and vector-based strategies (26, 34). The vector-based strategies involve either DNA or viral vector- mediated RNAi. In the case of DNA vector based strategy, RNA Polymerase II or III promoters have been incorporated into DNA vectors along with siRNA expression cassettes (3, 34, 43, 45). These cassettes have included either sense and antisense siRNA strands expressed from tandem promoters or a short hairpin (shRNA) cassette whereby the two

siRNA strands are separated by a short spacer (12). Use of RNA Polymerase II promoters to generate shRNAs has the advantage that they allow easier adaptation of inducible/repressible tissue- or cell-specific siRNA expression (47).

The plasmid-based siRNA technique has a low efficiency. In order to substitute this deficiency viral vectors have been developed. In several published papers adenoviral vectors and various retroviral vectors (e.g. lentivirus-based) have proven effective delivery systems in numerous cell types including non-cycling cells, stem cells, and zygotes (26, 37, 42).

3. MiRNA/siRNAs applications in cancer

The extensive studies made during the last few years have indicated that many miRNAs are associated with primary human tumors, and more than 50% of human miRNAs genes are located at genomic regions implicated in cancers, such as common breakpoint regions and fragile sites (*3*). Several miRNA have been found to have increase expression in different type of cancers. According to *Heneghan 2010*, in breast cancer have been observed two types of miRNA (miR-21, miR-29b-2) with increase expression, while other three miRNA are observed to have decreased expression (miR-125b, miR-145 miR-10b). Similar situations where observed for different types of tumors like ovarian, thyroidal, testicular and so on.

Phenotyping and molecular profiling of human cancers have greatly enhanced the diagnosis and biological classification of several tumors, in particular breast cancers where the miRNA technology has enhanced disease classification beyond single-gene markers (21).

A comprehensive screening of the breast cancer subclasses via miRNA expression profiling could further characterize the molecular basis underlying these subtypes, perhaps define more precise subsets of breast cancer, and provide opportunities for the identification of novel targets that can be exploited for targeted therapy. In the studies taken by *Iorio et al. 2005*, were analyzed 76 breast tumor and 34 normal specimens, and succeed to identified 29 miRNAs that were differentially expressed in breast cancer tissue compared to normal, and a further set of 15 miRNAs that could correctly discriminate between tumor and normal.

Because of the high incidents of cancers disease there is great need for the identification of sensitive, reliable and acceptable markers of response to neoadjuvant and adjuvant therapies. To date no work has been published on the role of circulating miRNAs in breast cancer—an area where, if feasible, their use as novel minimally invasive biomarkers would be an incredible breakthrough in the management of this disease (21). In order to be extensively used a biomarker must accomplish several characteristics like: easily accessible, it can be sampled relatively non-invasively, sensitive enough to detect early presence of tumors' in almost all patients and absent or minimal in healthy tumor-free individuals.

MiRNAs are considered to have enormous potential to serve as an **ideal class of cancer biomarkers** for the following considerate: they expression profiles is tissue-specific and is known to be aberrant in cancer; are stable molecules that are well preserved after different modalities of sample preservation were applied: formalin fixed, paraffin embedded tissues as well as fresh snap frozen specimens.

siRNA pathways is consider to be an important gene silencing mediating technique. siRNAs constructs can be easily synthesized to triggered mRNAs of chosen genes considered to be responsible of different pathologic processes. As a result was observed 80-90% down-regulation of the target gene expression. This approached produce a transient effect, the mRNA of the target gene was observed to return to normal after 3-7 days (44). In order to be suitable as a therapeutic tool an effective delivery mechanism to assure integration into target tissues remains a major impediment for RNAi therapy before they can be tried clinically (40). Different strategies have been elaborated and comprise different delivery system including synthetic small RNA molecules by complexing or covalently linking with lipids and/or delivery proteins (16, 12). Besides nonspecific agent like cationic liposome and cholesterol (41,51), novel nanotechnology-based conjungation of bacterial phage packaging RNA with therapeutic molecules, antibody-protamine, etc, have been designed to deliver small RNA assembly to target cells (41,17). Conjugation with homing signals for tissue/cell-type specific delivery has recently progressed (29). All development in this aspect plus the research on local delivery may eventually expand the therapeutic opportunities for miRNA targeting.

Currently, there are several biotechnology companies developing clinical applications of siRNA in various human diseases like: solid tumors, hepatitis C, HIV, Spinal Cord Injury, Parkinson and Huntington disease (20). The current testing is in preclinical or phase 1 clinical stage. In addition of low production costs and ease synthesis (compared with protein or antibody therapies), data indicates that siRNA has favorable pharmacokinetic properties and can be delivered to a wide range of organs (2). However, blood stability, delivery, poor intracellular uptake, and nonspecific immune stimulation still present significant challenges for the development of RNAi reagents for clinical use (24).

4. Methods for screening miRNA

The explosion of interest in miRNAs over the past few years requires effective tools for detecting their presence, quantification and functional analysis. High-throughput profiling techniques such as miRNA microarrays and bead-based miRNA flow cytometric approach have facilitated miRNA expression profiling (7). According to Dharmacon 2005, TaqMan quantitative qRT-PCR and sequencing miRNAs are the most widely used methods, for the identification of new miRNAs, while microarray analysis is a high throughput method that typically only reveals the expression of known miRNAs.

Because of miRNA exceptional stability within the visceral tissue efforts have been made to establish several techniques for detection, preservation and quantification of circulate miRNA within the bodily fluids (blood, urine, saliva, etc). Until now successfully results have been obtain by isolating small RNA fraction of size typical of miRNAs within the blood plasma. Mitchell et

al., 2008 have made experiments in order to point out miRNAs presence in clinical samples of plasma and serum in a stable form. They isolated total RNA from human plasma from healthy. The RNA obtained was amplified using 18-24 nucleotides primers provided by Dharmacon and gel purified by electrophoresis through denaturing polyacrilamide gel. They research might be considered the foundation for the development of miRNAs as a novel class of blood-based cancer biomarkers and raise provocative questions regarding the mechanism of stability and potential biological function of circulating miRNAs.

5. Future perspectives

Since its initial discovery in 1998 RNAi has brought a lot of excitement and hope in the scientific community. The promise of RNAi screening has attracted many researchers but their practical applications still require new technical expertise. Even if it has been intensively studied within the last years there are still many unknowns that remain to be revel within the next years. Development of more efficient delivery methods and regulated tissue-specific or differentiation-dependent expression of siRNA/shRNA are critical issues for transgenic studies and gene therapy.

Computer assisted sequence predictions and more complex understanding of miRNA mechanisms will promote the development of artificial miRNAs. The miRNA signature in susceptible individuals including their expression profiles, dynamics, and even miRNA target variants (single-nucleotide polymorphisms) may eventually enable the miRNA-based individual-specific therapy, as well as disease diagnosis and prognosis. In addition, siRNA/miRNA specific delivery to target cell populations using approaches of nanobiotechnology has begun and shows increased potential. With the development in miRNA field, these tiny molecules might become invaluable tools for various areas of basic and applied research and eventually for therapeutic intervention (50).

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ARN-URI MICI DE INTERFERENTA SI FENOMENUL ARN INTERFERENTEI

REZUMAT

Aplicabilitatea fenomenului ARN interferenţei atât în genetica funcţională cât şi în scop terapeutic pentru tratarea diferitelor tipuri de boli, inclusiv cancer se bucură de tot mai mare interes. Ca rezultat tehnologia siARN s-a dezvoltat rapid şi în prezent diferiţi furnizori oferă designuri siARN pentru unu număr mare de mARN aparţinând diferitelor gene ţintă. În acelaşi timp, identificarea unui număr mare de miARN implicate atât în stadiile de dezvoltare normale, dar şi în diferite procese patologice se bucură de un interes ridicat privind utilizarea acestora în practică ca şi marker de prognostic. Acest articol prezintă, pe baza unei documentaţii bogate, principlalele aspecte ale mecanismului ARNi, calea de sinteză a miARN şi siARN şi aplicabilitatea terapeutica a acestora în cancer.
Cuvinte cheie: interferenta ARN, miRNA, siRNA, cancer

ACL (ANTERIOR CRUCIATE LIGAMENT) RECONSTRUCTION USING HAMSTRING TENDON GRAFT - A CASE REPORT

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ABSTRACT

ACL rupture is in the most cases a sporting accident, due to the patient feels pain in knee joint, accompanied by regional deformation, enlarging joint volume and experiencing partial functional impotence. In our case, both tests for the instability of the knee joint were positive. After laboratory investigations consisting in sagittal radiography of the knee joint to rule out any bone lesions, MRI is done, putting it accurate diagnosis of ACL injury, more precisely an ACL rupture. For therapeutic purpose is decided arthroscopy, ACL reconstruction was done using semitendinosus muscle tendon. Functional recovery takes between 6-8 months, mainly consisting of physiotherapy. Evolution is very good.
Keywords: ACL, semitendinosus muscle, reconstruction, tendon.

INTRODUCTION

Seen as a stabilizer of the knee joint, anterior cruciate ligament (ACL) is to limit the movement of the tibia in relation to the femur from the posterior toward anterior and at the same time to prevent an anterior movement of the tibia to the femur, and limiting an internal rotation of tibia in relation to the femur.. In ACL rupture the ligament heads distance themselves from one another and are not returning to the original site as might happen to the other ligaments of the knee joint (tibial collateral ligament and fibular collateral ligament), where surgical reconstruction is necessary (1).

The causes of ACL rupture are represented in majority of injuries sports: skiing, soccer, and so on.

MATERIALS AND METHODS

I. Anamnesis

C.F. 27 years old, male, suffered a skiing accident by falling ,patient accused a right knee joint pain and felt a cracment sensation because of ACL rupture. In an attempt to support his weight on his right leg the patient found a great instability of the joint. It is reported that the patient is clinically healthy, showing no acute or chronic disease.

II. Clinical examination data

In our emergency department the patient had his right knee joint with an increase of its volume, accusing a severe pain and functional impotence in the joint. The joint presented also a purple eritema, the tegument being in the same time hot. Drawer sign (used to detect rupture of the cruciate ligaments in the knee joint, with the knee flexed to 90^0) was positive, as well as Lachman test (the knee flexed to 30^0).

III. Laboratory data

Being a young patient without a history of pathological, have made only a complete blood count (CBC) and coagulation profile, which were normal, no setting of medical problems, both, anesthetic and intraoperator.

IV. Laboratory investigations

In the emergency was made an X-ray of right knee joint, but no bone lesions were detected. Coupled with the clinical examination was suspected a ligament damage. It was recommended to perform a MRI exam, ligament injuries due to objectivity and reliability with a diagnosis of over 90%. MRI in conjunction with clinical examination gives a diagnosis of certainty, supporting the indication for surgery in the arthroscopic right ACL reconstruction (Figures 1 and 2).

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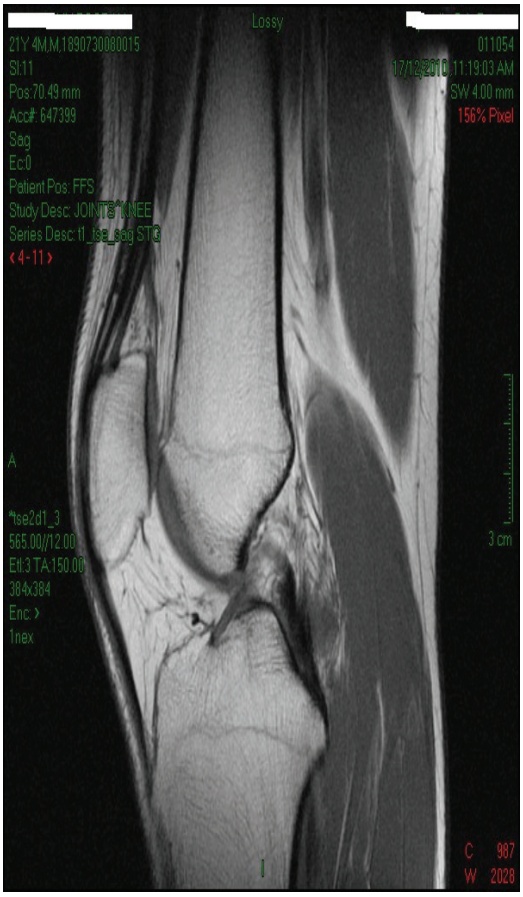

Fig. 1. Sagittal MRI shows a broken ACL

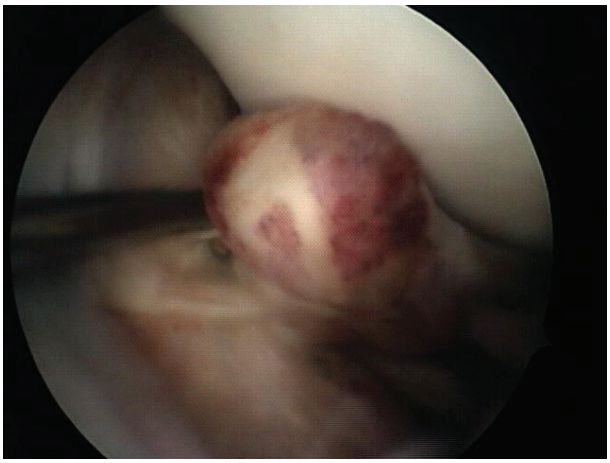

Fig. 2. MRI, ACL is broken, picture shows an entrapment ligament stump

V. Treatment and evolution

Arthroscopic surgery is the correct treatment. Semitendinosus muscle tendon is taken from the pes anserinus level for the autograft preparation (Figure 3). If semitendinosus muscle tendon is not thick enough, minimum 7 mm, can be taken a gracilis muscle tendon. In our case the patient with an athletic build, the semitendinosus muscle tendon was enough. It was prepared an autograft having dimensions as 10 cm in length and 8 mm in diameter (Figures 4 and 5).

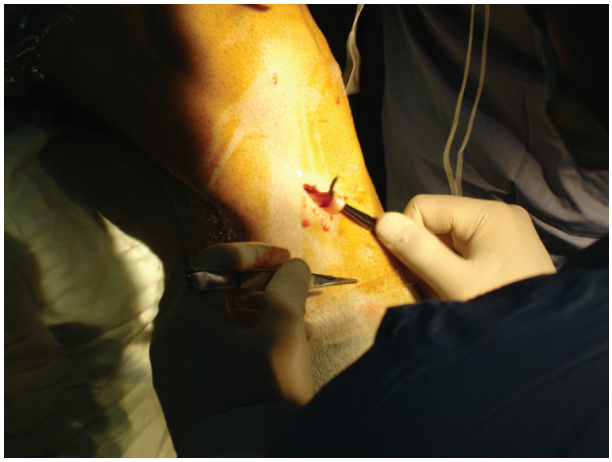

Fig.3. Harvesting tendons

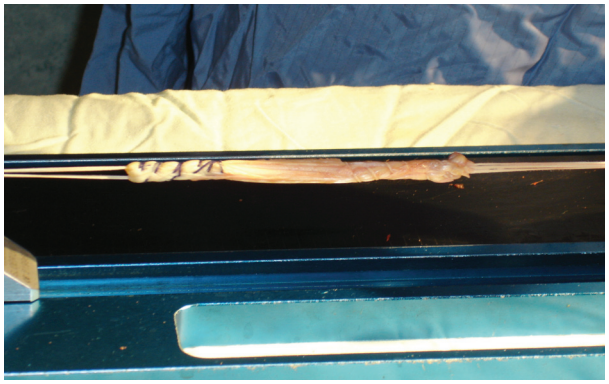

Fig.4. Preparation autograft

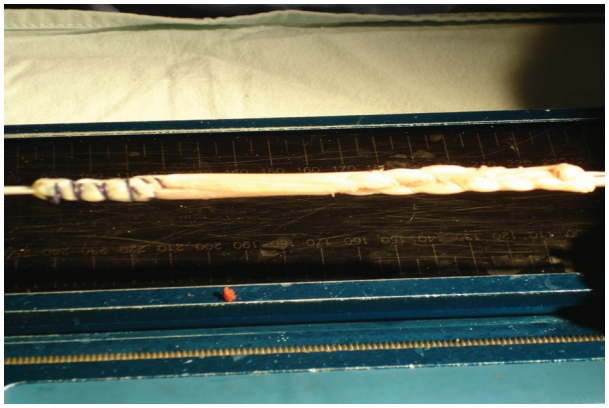

Fig.5. Preparation autograft

Surgical Technique

The patient is positioned on the operating table in supine position with the knee at 90 °, right leg is painted with iodine solution, is isolated with sterile fields. Right semitendinosus muscle tendon has been harvested through an oblique incision centered on right pes anserinus (2). The autograft was prepared on workbench at optimal length and thickness, femur and tibia were being tunneled in order to place the autograft, taking to account its size and in a stable position with specific kit tools for ligament reconstruction, in order to realize a quasi-anatomical reconstruction of the anterior cruciate ligament (5). After position-

ing the autograft, it was fitted with two femoral transfix and tibial interference with resorbable screw (Figure 6).

Evolution is the favorable in most cases and our case is no exception to the rule, considering the patient's age (young one) and because there is no intraoperative events in conjunction with an appropriate program of functional recovery of the right knee joint using kinetotherapy. Recovery for sport performance lasts about 6 months, depending on the patient's functional ability to perform physical exercises.

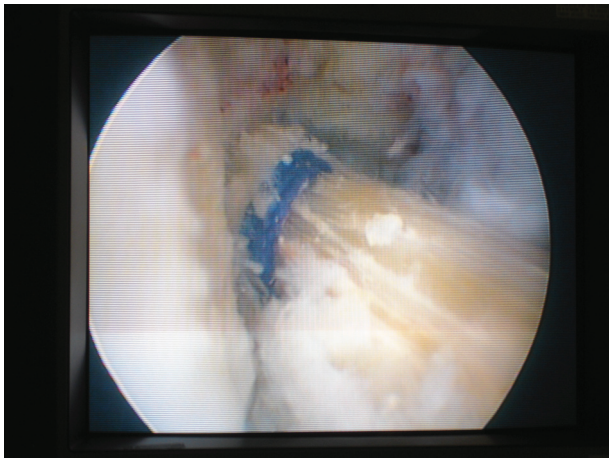

Fig.6. Fixing autograft

DISCUSSION

Anterior cruciate ligament reconstruction is the only way to save the function of the knee which suffered rupture of this important anatomical structure (3). In classical literature ACL rupture is considered "the beginning of the end of the knee." In the absence of function of this ligament the joint degradation continues through joint menisci lesions, cartilage lesions, and finally the gonarthrosis disease appearance, whose treatment is only articular endoprosthetic replacement (4).

We believe that for physically active individuals with age around 50-60; this type of ligament reconstruction is indicated in the presence of ACL ruptures.

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RECONSTRUCTIA LIGAMENTULUI INCRUCISAT LATERAL (ACL) UTILIZAND GREFON TENDINOS

REZUMAT

Ruptura ACL este de cele mai multe ori rezultatul accidentelor sportive, pacientul prezentand durere la nivelul articulatiei genunchiului, insotita de deformare articulara, edem si impotenta functionala partiala. In cazul de fata, ambele teste efectuate pentru instabilitatea articulatiei genunchiului au fost positive. Dupa efectuarea investigatiilor de laborator, care au constat in radiografii sagitale ale articulatiei genunchiului, pentru excluderea afectarii osoase, s-a efectuat RMN, in urma caruia a fost stabilit diagnosticul cu precizie, si anume lezarea ACL – ruptura ACL. In scop terapeutic s-a decis efectuarea artroscopiei, iar reconstructia ACL a fost efectuata prin utilizarea tendonului muschiului semitendinos. Refacerea functionala dureaza intre 6-8 luni si consta in proceduri fizioterapice. Evolutia post operatorie este favorabila.

Keywords: ACL, muschi semitendinos, reconstructie, tendon

INTERRELATION BETWEEN DIABETES MELLITUS -THYROID DISEASES

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ABSTRACT

Diabetes mellitus and thyroid diseases represents two endocrinopathies frecquently in general population. Because insulin and thyroid hormones are implicated in celular methabolism, excess or deficiency of one of these determinated functional disorders of another. The purpose of this study is to determinate influence of thyroid diseases on diabetes mellitus or influence of diabetes mellitus on associated thyroid diseases. The studied group was of 733 cases, with an age between 7-79 years.

The studied group was subdivided according age in 2 subgroups: children subgroup and adults subgroup. We used clinical, imagistic, biochemistry, hormones, imunological parameters.

Keywords: diabetes mellitus, thyroid diseases, interrelations

INTRODUCTION

Influence of thyroid disease on diabetes mellitus

Diabetes mellitus (DM) and thyroid disorders are two endocrinopathies frequently in the general population. Since insulin and thyroid hormones are involved in cellular metabolism, excess or deficit of one of them determines the other functional disorders.

Thyroid disorders are commonly associated with DM in old age, especially in type 2 diabetes, and with autoimmune diseases in type 1 diabetes (9). Thyroid disorders complicate diabetes treatment and diagnosis of diabetes complications. For example, 6.6% of the general population has thyroid disease, compared with 10.8% and 13.4% of people with diabetes. The high prevalence occurs in women with type 1 diabetes (because of the autoimmune DM type) and because thyroid disorders are more common in women. Also, postpartum thyroid disorders are three times more common in women with diabetes than in non-diabetic (5).

In terms of clinical, thyroid disorders may influence glycemic balance. For example, hyperthyroidism can worsen glycemic balance and increase insulin requirement. Also, thyrotoxicosis can mask a subclinical DM.

A number of studies (16) agree with the following aspects:

-hyperthyroidism may determinate worsening of glycemic control

-hyperglycemia may improve under thyrotoxicosis treatment

-uunexplained worsening hyperglycemia may be due to uncompensated hyperthyroidism

Graves-Basedow disease is the most common cause of hyperthyroidism. While Graves-Basedow disease may be associated with type 1 diabetes in the poliglandular autoimmune syndrome, thyrotoxicosis itself is diabetogenic. Glucose intolerance occurs in 50% of patients with Graves-Basedow disease and clinical diabetes only at 2-3%. In patients with known diabetes causes worsening glycemic control (3).

Disturbances that occur as a result of hyperthyroidism and contribute to the deterioration of glycemic control are:

■ **Gastro-intestinal system.** In hyperthyroidism occurs accelerating gastric emptying, increase intestinal absorption of glucose and increase portal venous flow (6).

■ **Insulin secretion.** While some studies show decreased insulin secretion in hyperthyroidism (3), most studies report a normal or increased insulin secretion in peripheral blood and portal (3). It is possible that the exacerbation of insulin secretion is masked by increased insulin degradation. In hyperthyroidism, insulin clearance rate is increased about 40% (3). Thyrotoxicosis causes long-term beta-cell dysfunction, characterized by reduced secretion of pancreatic insulin, inadequate insulin response to glucose and decrease insulin secretion rate (3).

■ **Endogenous glucose production.** In hyperthyroidism, endogenous glucose production is increased by various mechanisms:

- increasing the availability of gluconeogenetic precursors by lactate, glutamine and alanine form in skeletal muscle and by glycerol in adipose tissue

- elevated serum free fatty acids (AF) which stimulates gluconeogenesis

- increase in glycogenolysis caused by inhibition of glycogen synthesis

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- increased expression of glucose transporter protein
- increased secretion and exaggerated effects of glucagon and adrenaline on liver cells (14)

■ **Use of glucose**

Adipose tissue

In adipocytes isolated from rats or from patients with hyperthyroidism, sensitivity of glucose transport and insulin use is normal, increased or decreased (14).

Skeletal muscle

In skeletal muscle, there is an increased intake of glucose and lactate formation. This occurs from stimulation of basal and stimulates insulin by the transporters GLUT-1 and GLUT-4, increased glycogenolysis responsiveness to beta-adrenergic stimulation, increased hexokinase and 5-fosfofructokinase activity and decreased sensitivity glycogen synthesis to insulin (14).

Influence of diabetes on associated thyroid diseases

Malignant ophthalmopathy induced blindness may be the most feared complication of Graves-Basedow disease. Is determined by optic nerve compression through increasing exrtraocular muscle at the orbital apex. Its incidence in patients with diabetes is increased approximately 10 times. High prevalence is explained by the reduction of optic nerve oxygenation in diabetic patients because of vasculopathy (16).

When diabetes occurs in euthyroid subjects, causes altered thyroid function tests, but not clinically manifest disorders. In patients with preexisting ophthalmopathy of Graves' disease, the risk of vision loss is increased and recovery of sight is limited (16).

MATERIAL AND METHODS

Investigated population

The study included subjects with diabetes mellitus which in time present thyroid disease or subjects with thyroid disease who subsequently present diabetes mellitus

The study group comprised 733 cases aged 7-79 years. Subjects were divided as follows:

■ group of children that included 83 children and adolescents aged 7-17 years (14.57 ± 2.25 years), with a ratio F / M of 5.9 / 1.

■ group of adults that included 650 adults aged 18-79 years (52.03 ± 12.46 years), with a ratio F / M of 9.48 / 1.

Methods of investigation

Methods of investigation were the clinical data - history, present status, imaging - ultrasound thyroid, biochemistry - carbohydrate metabolism parameters: fasting glycemia, glycosuria, glycosylated hemoglobin and thyroid hormone investigations and some immunological parameters.

Glucose determination was performed by enzymatic techniques with glucozooxidase. Were considered normal fasting blood glucose between 70-110 mg%, diabetes mellitus - fasting blood glucose values above 126 mg%, impaired glucose tolerance - fasting glucose values between 110-126 mg% and oral glucose tolerance test (OGTT) at 2 h between 140-200 mg% and fasting impaired glucose tolerance - fasting glucose values between 110-126 mg% and OGTT at 2 h under 140 mg%.

Determination of glycated hemoglobin (HbA_{1c}) was achieved through the DiaStat program for glycated hemoglobin HbA_{1c} who measures the ratio of glycated hemoglobin to total HbA.

Determination of serum levels of TSH, free fraction of triiodothyronine (FT3), free fraction of thyroxin (FT4) were ARCHITECT quantitative method, which is an imunodetermination by chemiluminescence's with small Chemilumnescent Microparticle Immunoassay (CMIA). The following values were considered normal: TSH = 0.465 to 4.68 mIU / ml, FT3 = 3.69 -10.4 pmol / l, FT4 = 10 to 28.2 pmol / l.

Immunological parameters were represented by some markers of thyroid autoimmunity - antiTPO and antiTg antibodies.

To determine the serum titers Atc antiTPO Axsym antiTPO kit was used, the method is enzyme immunoassay with micro particles, Meia (Microparticle Enzyme Immunoassay). Was considered normal: Atc. antiTPO (<35 IU / ml).

To determine the serum titers Atc antiTg Axsym antiTg kit was used, the method is enzyme immunoassay with micro particles, Meia (Microparticle Enzyme Immunoassay). It was considered normal: Atc. antiTg (<55 IU / ml).

Thyroid ultrasound performed in all cases is a non-invasive method of exploration that allows measurement of thyroid volume, thyroid study report with cervical anatomical structures, thyroid parenchyma changes etc.

The appearance of normal thyroid parenchyma is characterized by a high intensity echogenic, homogeneous, easily distinguishable from the neck muscles which look hypoecogenic.

Inflammatory and autoimmune processes are hypoecogenic. Degree of thyroid hypoecogenity was assessed as: discreet +, moderate + + and marked + + +.

In autoimmune thyroid disease is found hipoecogenity of thyroid parenchyma.

Graves' disease appears: thyroid volume generally increased and hipoecogenity with different intensities with variable homogeneity.

Chronic autoimmune thyroiditis appears: hipoecogenity generally uneven and normal or increased thyroid volume.

RESULTS AND DISCUSSION

The group of children and adolescents was represented by 83 subjects, aged 7-17 years (Table I). All children take in the study had type 1 diabetes.

Table I. Distribution by age and sex of children and adolescents group

Age	Number of cases		Female		Male	
	n	%	n	%	n	%
0 - 4 years	-	-	-	-	-	-
5 - 9 years	2	2.4	2	100	-	-
10 – 14 years	32	38.56	22	68.75	10	31.25
15 – 17 years	49	59.04	47	95.92	2	4.08

Regarding the type of thyroid disease, the first priority was in the ACT, followed by diffuse goiter with euthyroidism and Graves-Basedow disease (Table II).

Adult group consisted of 650 people, young adults and elderly adults, aged between 17 and 79 years (Table III).

Table II. Distribution by type of thyroid disease in the lot of children and adolescents

Type of thyroid disease	Number of cases		The average age group (years)	Female		Male	
	n	%	medie ± DS	n	%	n	%
ACT	54	65.06	14 ± 2.4	47	87.03	7	12.97
Diffuse goiter with euthyroidism	25	30.12	14 ± 1.93	20	80	5	20
Graves-Basedow disease	4	4.82	16 ± 0.5	4	100	-	-

Legend: ACT = chronic autoimmune thyroiditis

Table III. Distribution by age and sex of adult group

Age	Number of cases		Female		Male	
	n	%	n	%	n	%
18 – 19 years	11	1.7	10	90.9	1	9.1
20 – 29 years	29	4.46	27	93.1	2	6.9
30 – 39 years	48	7.38	43	89.58	5	10.42
40 – 49 years	168	25.84	141	83.93	27	16.07
50 – 59 years	219	33.7	209	95.43	10	4.57
60 – 69 years	118	18.15	112	94.91	6	5.09
70 – 79 years	57	8.77	46	80.7	11	19.3

Adult group was subdivided in function of the type at glycemic balance in 4 subgroups (Figure 1):

- group with type 1 diabetes represented by 60 cases (9.23%)
- group with type 2 diabetes accounted for 290 cases (44.61%)
- group with impaired glucose tolerance (IGT) accounted for 183 cases (28.15%)
- group with fasting impaired glucose tolerance (IFG) accounted for 117 cases (18%)

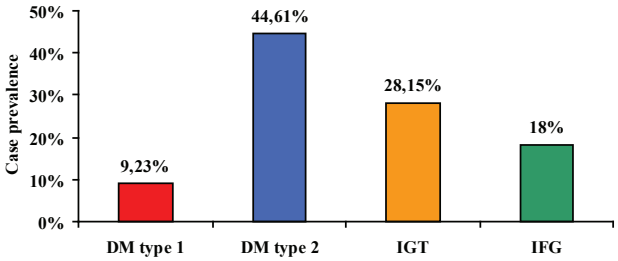

Fig. 1. Distribution of cases by type of changes in glycemic balance

Regarding the type of thyroid disease, in the adult group we obtained the results shown in Tab.IV. In type 1 diabetes case the principal prevalence was with ACT (83.33%), followed by Graves-Basedow disease (10%), thyroid carcinoma (5%) and diffuse goiter with euthyroidism (1.66%).

In the group with type 2 diabetes, the main prevalence was diffuse goiter with euthyroidism (43.1%), followed by ACT (26.55%) and Graves-Basedow disease (20.69%).

In the group with IGT, the main prevalence was diffuse goiter with euthyroidism (44.26%), followed by ACT (28.41%) and Graves-Basedow disease (23.49%).

In IFG group the main prevalence was with diffuse goiter with euthyroidism (33.33%), followed by Graves-Basedow disease (32.47%) and ACT (24.78%).

Table IV. The prevalence of thyroid disease in subjects with changes in glycemic balance

Type of thyroid disease	Total		DM type 1		DM type 2		IGT		IFG	
	n	%	n	%	n	%	n	%	n	%
Graves -Basedow disease	147	22.61	6	10	60	20.69	43	23.49	38	32.47
Autonomous thyroid adenoma	8	1.23	-	-	3	1.03	3	1.64	2	1.71
Amiodarone-induced hyperthyroidism	4	0.61	-	-	4	1.38	-	-	-	-
Differentiated thyroid carcinoma (papillary, follicular)	21	3.23	3	5	10	3.49	2	1.09	6	5.12
Nodular goiter with euthyroidism	11	1.7	-	-	7	2.41	2	1.09	2	1.71
Diffuse goiter with euthyroidism	246	37.84	1	1.66	125	43.1	81	44.26	39	33.33
ACT (with goiter, atrophic)	208	32	50	83.33	77	26.55	52	28.41	29	24.78
Sub acute thyroiditis	5	0.77	-	-	4	1.38	-	-	1	0.85

Depending on "fasting" glucose, we assess the level of glycemic balance in diabetic patients (Tables V and VI).

Table V. Glycemic balance in children and adolescents studied group

Very good		Good		Satisfactory		Unsatisfactory	
n	%	n	%	n	%	n	%
26	31.32	12	14.45	9	10.84	36	43.37

Table VI. Glycemic status in the studied group of adults

Glycemic balance	DM type 1 (n = 60)		DM type 2 (n = 290)		IGT (n = 183)		IFG (n = 117)	
	n	%	n	%	n	%	n	%
Very good	8	13.33	151	52.06	151	82.52	78	66.66
Good	7	11.66	59	20.34	29	15.84	35	29.91
Satisfactory	10	16.66	25	8.62	3	1.64	4	3.42
Unsatisfactory	35	58.33	55	18.96	-	-	-	-

The results of comparison between subgroups of lot of adults are found in TableVII.

Table VII. Comparison (p) in blood glucose between sub lots of lot of adults

Glycemic balance	Very good	Good	Satisfactory	Unsatisfactory
DM type 1 vs. DM type 2	< 0.001	0.1177	0.0586	< 0.001
DM type 1 vs. IGT	< 0.001	0.4289	< 0.001	< 0.001
DM type 1 vs. IFG	< 0.001	0.0069	0.0019	< 0.001
DM type 2 vs. IGT	< 0.001	0.2208	0.0017	< 0.001
DM type 2 vs. IFG	0.0072	0.0381	0.0648	< 0.001
IGT vs. IFG	0.0016	0.0037	0.3193	-

We observed highly significant differences ($p<0.001$) between the unsatisfactory glycemic balance who predominate in cases with type 1 diabetes toward type 2 diabetes and other changes in glycemic balance and between very good glycemic balance who predominate to other changes in glycemic balance toward type 1 diabetes.

If the study group, **Graves-Basedow disease** was discovered in 4.82% cases in the group of children and in 22.61% cases in the group of adults.

In the lot of children, in two cases the influence on the DM was marked: one case was presented with advanced ketoacidosis ("fasting" glycemia 765 mg %) and 1 case in moderate ketoacidosis ("fasting" glycemia 418 mg %). The rest had values for blood glucose between 200-250 mg%. Under the treatment of Graves-Basedow disease, there was an improvement in glycemic balance with decreased initial insulin requirements.

In the case of type 1 diabetes in adults, in one case met early ketoacidosis, in the remaining cases blood glucose values being between 200-250 mg%. Under the treatment of thyroid disease, glycemic balance was restored, with lower initial insulin requirements.

In the case of type 2 diabetes in adults, only 4 cases had glucose values between 200-250 mg%. Initially, they require changing the therapeutic regimen, with dose escalation of oral antidiabetic (OAD), the combination of two OAD, OAD establishment in patients with diet and insulin treatment in 2 cases. After balance of Graves-Basedow disease, was restored original scheme of treatment, and in those under insulin therapy were low doses of insulin.

Changes in glycemic balance were diagnosed by OGTT and required treatment with diet.

Between TSH and HbA_{1c} value we found a very weak correlation ($r = 0.22$, $p = 0.001$) in all cases with diabetes and Graves-Basedow disease.

Correlation between serum TSH and HbA_{1c} in all cases of type 2 diabetes and Graves - Basedow disease was also very weak ($r = -0.10$, $p <0.001$).

A number of studies show the influence of type of thyroid disease on the metabolic balance. Thus, studies in Spain, Japan and the U.S. highlights the role of thyrotoxicosis in the development of diabetic ketoacidosis, it is meeting in these conditions not only in patients with type 1 diabetes but also in those with type 2 diabetes (4,13, 15, 17, 19).

Patients with Graves-Basedow disease should be closely monitored both in terms of response to antithyroid therapy and in terms of glycemic balance.

Once stabilized disease, may decrease insulin requirements. Also after stabilization of disease, blood glucose monitoring is necessary to avoid hypoglycemia that can occur as a result of lower insulin requirements.

In case of labile Graves-Basedow disease, which frequently causing the glycemic decompensation, it is indicating surgery treatment of the disease or therapy with I^{131}.

Autonomous thyroid adenoma was found only at 8 cases in adults. We had not cases of autonomous thyroid adenoma and type 1 diabetes, only with type 2 diabetes and other glycemic changes. He manifested less severe thyrotoxicosis than Graves-Basedow disease. In the group with type 2 diabetes, a single case presented glucose values above 200 mg% All three cases were balanced with insulin. Changes in glycemic balance were diagnosed by OGTT and required treatment with diet. All cases of thyroid adenoma were operated independently.

Differentiated thyroid carcinoma (papillary, follicular) was discovered in 21 cases. All cases were operated, treated with I^{131}, being then under suppression therapy with LT_4. Because high doses of LT_4, in one case of type 1 diabetes glucose values were above 200 mg%, requiring a marked increase in insulin doses. In other cases there was slight increase in insulin dose, the dose of OAD and the establishment of the dietary in other glycemic changes. It is recommended that doses of LT_4 (suppressive therapy) remain unchanged, even worse glycemic control, because if the dose of thyroid hormones decrease it is the risk of developing systemic metastasis.

Amiodarone-induced hyperthyroidism was found only in 4 cases with type 2 diabetes. Poor glucose control was easily, with blood sugar below 200 mg%, 2 cases were treated with diet and 2 cases with OAD.

In the case of hypothyroidism, it causes impaired carbohydrate metabolism, these changes were rarely clinically significant. In hypothyroidism, the synthesis and release of insulin is low. They cause a decrease in gluconeogenesis, decreased glucose utilization in the periphery (14). Following is an increased risk of hypoglycemia in individuals with diabetes (7). May decrease exogenous insulin requirements. Sometimes produce dyslipidemia, including increased plasma triglycerides and LDL cholesterol. Also, hypothyroidism may exacerbate atherogenic manifestations and coexisting dyslipidemia in type 2 diabetes. Treatment with thyroxin causes reversal of lipid metabolism disorders

Chronic autoimmune thyroiditis (ACT) met in 65.06% cases in children. In ACT with euthyroidism, without treatment, glycemic balance has not changed. ACT with hypothyroidism determinate an improvement in glycemic control with decreased insulin requirements. The treatment with thyroid hormones in

some cases led to increased insulin requirements, even obtaining an unsatisfactory glycemic balance in 21 cases.

In the case of the group of adults ACT met in the 32% cases. In ACT with euthyroidism, without treatment, glycemic balance has not changed. ACT with hypothyroidism determinate an improvement in glycemic control, with decreased insulin dose and the necessary OAD. The treatment with thyroid hormone determinate an unsatisfactory glycemic balance in 31 patients with type 1 diabetes and in 11 cases with type 2 diabetes.

As a result, we increased dose of insulin and OAD. ACT with hypothyroidism (sub clinical and clinical) is frequently associated with dyslipidemia, leading to an increased risk of atherosclerosis and cardiovascular disease.

Between the value of TSH and HbA_{1c} we found a very weak correlation ($r < 0.05$, $p < 0.05$) in all cases of diabetes and the ACT.

The correlation coefficient between the serum TSH concentration and HbA_{1c} in all cases with type 1 diabetes and ACT was insignificant ($r = -0.17$, $p = 0.40$).

Also, the correlation coefficient between serum TSH and HbA_{1c} in all cases with type 2 diabetes and ACT was insignificant ($r = -0.046$, $p = 0.30$).

Dyslipidemia were seen in the group of children in five cases (four with hypercholesterolemia and 1 with hypertriglyceridemia), in the group of adults with type 1 diabetes in 25 cases (24 with hypercholesterolemia and 1 with mixed dyslipidemia), in the group of adults with type 2 diabetes in 62 cases (34 with mixed dyslipidemia, 23 with hypercholesterolemia and 7 with hypertriglyceridemia), in the group of adults with IGT in 36 cases (17 with mixed dyslipidemia, 16 with hypercholesterolemia and 3 with hypertriglyceridemia) and in the group of adults with IFG in 24 cases (7 with mixed dyslipidemia and 17 with hypercholesterolemia).

Rotterdam study also showed that sub clinical hypothyroidism is a risk factor for cardiovascular disease (7). Other studies have shown contrary results, sub clinical hypothyroidism is not associated with an increased risk of death from cardiovascular disease (2)

Other authors have also shown the benefit of sub clinical hypothyroidism treatment, besides achieving balance thyroid function and decreased LDL-cholesterol (14).

Studies from Czech also show the influence of the association of various endocrine disorders on metabolic balance. The most serious consequences are increased frequency of hypoglycemia in hypothyroidism and occurrence of diabetic ketoacidosis in case of thyrotoxicosis (20).

In hypothyroidism is an increased risk of mental retardation in children of hypothyroid mothers, and in pregnant women with diabetes is frequently associated production of hypoglycemia by increasing insulin secretion, decreased insulin requirements and by reducing liver glucose production (10).

Also, some authors suggest that the diagnosis of thyroid diseases can be difficult. For example, poor glucose control produces similar symptoms with hyperthyroidism as weight loss despite increased appetite, marked weakness, fatigue, poliuropolydipsia syndrome.

Clinicians must be careful to not confuse hypothyroidism with severe diabetic nephropathy: produce edema, fatigue, pallor and weight gain.

In the study group, only in type 1 diabetes at two patients, initial diagnosis was established Graves-Basedow disease, symptoms are weight loss despite increased appetite, marked fatigue, asthenia. After, they were diagnosed through blood glucose determination also with diabetes type 1.

A particular association is the association of type 1 diabetes with autoimmune thyroid disease, particularly chronic autoimmune thyroiditis. In patients with ACT predominates hypothyroidism (clinical and sub clinical), while hyperthyroidism is rare (1).

American Thyroid Association (ATA) recommends testing thyroid function in all patients over 35 years and thyroid function revaluation every five years.

More commonly, testing is indicated in individuals at high risk or those symptomatic. American Association of clinical Endocrinology (AACE) recommends TSH screening in women before pregnancy and during the first semester (19).

In 2005, AACE, ATA and Endocrinology Society (TES) published a consensus about thyroid dysfunction (1). They recommended determination of anti-TPO antibodies in patients with sub clinical hypothyroidism because those with positive antibodies have an increased risk of developing clinical thyroid disease (8).

It is therefore recommended in patients with diabetes to determine the TSH to assess thyroid function screening. In patients with type 1 diabetes must determine antiTPO antibodies as predictors of autoimmune thyroid diseases.

Treatment is similar as in non-diabetic population. The authors point out that inappropriate treatment with L-thyroxin may exacerbate angina pectoris and may increase myocardial contractility and heart rate. Also recommend sub clinical hypothyroidism treatment if patients have elevated LDL-cholesterol exacerbated by hypothyroidism or antiTPO antibodies detectable in serum. The authors concluded that thyroid disorders are common in patients with diabetes and may determinate metabolic disorders. It is therefore recommended regular screening of patients with diabetes, which will allow early treatment. In patients with type 1 diabetes is recommended determination of anti-TPO antibodies at diagnosis. In those with positive TPO antibodies is recommended annual screening of TSH.

In patients with type 1 diabetes with antiTPO antibodies negative TSH should be evaluated every 2-3 years. In patients with type 2 diabetes should be evaluated TSH at diagnosis and then at least at 5 years (11).

In the study group met severe ophthalmopathy in Graves-Basedow disease in cohort of children in four cases, in adults with type 1 diabetes in two cases, in patients with type 2 diabetes in 21 cases, in those with IGT in 15 cases and at those with IFG in 16 cases. A study in Greece shows in Graves-Basedow disease that children are the same or even higher risk than adults to develop infiltrative ophthalmopathy (12).

CONCLUSIONS

If thyroid disease was first developing, excess of thyroid hormone or administering thyroid hormone lied to an imbalance of a pre-existing type 2 diabetes or unmasking latent one, and the emergence of IGT and IFG.

Type 2 diabetes that appeared was easy, requiring only diet therapy.

If the DM preceded thyroid disease with hyper function, its appearance has led to imbalance of DM. Thus, cases of type 2 diabetes initially treated with diet requiring treatment with OAD and those who received treatment with OAD required combination of OAD or even treatment with insulin.

In the case of type 1 diabetes, was a major imbalance, manifested by diabetic ketoacidosis. Hyperthyroid syndrome characteristic Graves-Basedow disease has led to a more severe metabolic disturbances of diabetes mellitus type 1 compared to that caused by chronic autoimmune thyroiditis and diffuse goiter with euthyroidism. Thus, thyrotoxicosis has required increasing doses of insulin, in parallel with the establishment of an energetic antithyroid treatment.

In children and adolescents were observed thyrotoxicosis resistant to usual therapy with antithyroid treatment, with incomplete and late onset of remission. Association between type 1 diabetes and Graves-Basedow disease requires systematic dispensary of these patients due to the high risk of relapse thyrotoxic syndrome.

Graves-Basedow disease and diabetes is a particular pathogenic entity, the presence of decompensate thyrotoxicosis adversely affecting the evolution and prognosis of diabetes mellitus. Because Graves-Basedow disease has a chronic evolution, being relatively difficult to stabilize even antithyroid treatment, particularly its association with type 1 diabetes, requires definitive correction of hyperthyroidism by surgical thyroidectomy or radiation.

Dispensary of diabetic patients with associated thyroid disease (including those with euthyroidism) covers annual TSH monitoring for early detection of hypothyroidism. If thyroid hypo function has no major implications for glycemic balance, atherosclerotic complications of mixedema adversely affect long-term prognosis of the association between diabetes mellitus and hypothyroidism.

Therapeutic maneuvers required by the diagnosis of thyroid cancer (total, treatment with I^{131} and suppressive therapy with high doses of thyroid hormones) can unbalance previously compensated diabetes. With damage to the metabolic balance of diabetes, because high doses of thyroxin, it is recommended higher doses of insulin, oral hypoglycemic agents respectively, and not decrease the thyroxin dosage to avoid potential local recurrence.

Diffuse and nodular goiter with euthyroidism required a suppressive treatment with thyroxin up to the induction of sub clinical hyperthyroidism. In case of a difficult control of diabetes requires thyroidectomy.

In conclusion, the main changes that occur as a result of interrelation between diabetes - thyroid diseases are found in TableVIII.

Table VIII. Interrelation between DM-thyroid diseases (modif.17)

Clinical Condition	Effects on glycemic balance	Effects on thyroid function
DM at euthyroid subjects	-	Decreased T3 Increased rT3 **Decreased TSH response to TRH** Decreased nocturnal peak of TSH
DM in subjects with hyperthyroidism	Worsening of glycemic control	Increasing severity of optic neuropathy
Hyperthyroidism in the normoglycemic subjects	Glucose intolerance in 50% of cases DM in 2-3% cases	-
Hyperthyroidism in subjects with diabetes	Damage of diabetes control	-
Hypothyroidism in subjects with diabetes	Predisposition to recurrent hypoglycemia **Multiple atherosclerotic manifestations**	-
Subjects with type 1 autoimmune diabetes	-	Increased prevalence of thyroid diseases

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INTERRELATIILE DINTRE DIABETUL ZAHARAT SI AFECTIUNILE TIROIDIENE

REZUMAT

Diabetul zaharat şi afecţiunile tiroidiene reprezintă două endocrinopatii frecvent întâlnite în populaţia generală. Deoarece insulina şi hormonii tiroidieni sunt implicaţi în metabolismul celular, excesul sau deficitul unuia dintre aceştia determină tulburări funcţionale ale celuilalt. Scopul acestui studiu a fost de a determina influenţa afecţiunilor tiroidiene asupra diabetului zaharat, respectiv a diabetului zaharat asupra afecţiunilor tiroidiene asociate. Lotul general studiat a fost reprezentat de 733 cazuri, cu vârste cuprinse între 7-79 ani.
Lotul studiat a fost subîmpărţit după criteriul vârstei în 2 loturi: lotul de copii şi lotul de adulţi. S-au folosit parametrii clinici, imagistici, biochimici, hormonali, imunologici.
Cuvinte cheie: diabet zaharat, afecţiuni tiroidiene, interrelaţie

THE EFFECT OF EPILEPSY ON THE AGE-RELATED PROLONGATION OF THE P300 WAVE

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ABSTRACT

Background and purpose: Event related evoked potentials (ERPs), according to the literature, present disturbances in epilepsies. It is known that P300 latency shows an age-related prolongation also. This study was designed to evaluate if the age-related prolongation of the latency of the auditory P300 is influenced by the presence of epilepsy.
Methods: 54 patients were included, and four groups were formed, a control group for the 20-40 age group, one for the 40-60 age group, and two epilepsy groups divided using the same age criteria. Auditory P300 evaluation was performed.
Results: P300 latencies were significantly prolonged in the presence of epilepsy in both age groups, when compared with the matched controls, for all the investigated derivations. P300 latencies were significantly prolonged in the 40-60 group compared with the 20-40 group for controls and epileptics as well. Concomitant presence of the two altering conditions, epilepsy and age, has no augmenting effect, it presents only a linear additive effect on the prolongation.
Conclusion: The velocity of the age-related prolongation of the P300 latency is not influenced by the presence of epilepsy, under proper, but heterogenous treatment, meaning that the cognitive decline is linear with age, both in controls and epileptics.
Keywords: event-related evoked potential, P300, latency, age, epilepsy

INTRODUCTION

Evoked potentials, as EEG techniques, are recorded over diverse cortical areas, generated as a response to external stimuli, or as an image of a cognitive response induced by these stimuli. Considering the parameters influencing the characteristics of the evoked potentials, these are classified in two major categories: stimulus related potentials (SRPs) and event related potentials (ERPs).

Among the studies investigating epilepsy, there are several researches performed for the evaluation of ERPs, but there is little consideration about the influence of the epilepsy on the age related prolongation of P300.

The latency of the P300 component reflects the temporal definition of post-stimulus attentional processes (1). Cognitive evoked potentials in epilepsies were stated to be a form of measure for the associated cognitive deficits. Postictal changes of these potentials were reported in temporal lobe epilepsy (2), without interictal persistence (3). Increased latency of the P300 was reported also in children with occipital paroxysms (4). Other studies performed on patients with idiopathic generalized epilepsy support the prolongation of the P300 latency in epilepsy (5). Convergent results were reported, showing only slight differences regarding the type of epilepsy (6,7), treatment options (8), seizure duration and frequency (9) and the morphology of the P300 (10). The prolongation also differs according to the stimulus used to evoke the P300 wave, auditory P300 being more sensitive, than the visual one (11). There are, on the other hand, authors affirming that P300 latencies are not influenced by the presence of epilepsy (12), but these opinions are still in a minority. Others are underlining the importance of older antiepileptic drugs, mainly valproic acid, as an important factor contributing to the prolongation (13).

Even if there are so many different factors possibly contributing, there's a need to further investigate the effect of epilepsy and its treatment on congnition, mirrored by the parameters of the cognitive evoked potential.

METHODS

The present study was performed, after informed consent was obtained from the participants, on adults, between 20-60 years, admitted to the Neurology Clinic of Cluj-Napoca. The four groups were: group I of healthy controls, between 20-40 years (n=13), group II, patients with epilepsy between 20-40 years (n=17), group III of healthy controls between 40-60 years (n=10), and group IV (n=15) of subjects between 40-60 with positive diagnosis for epilepsy. Patients with cognitive deficits, diabetes mellitus, dyselectrolytemias and psychiatric comorbidities were excluded. The presence of any hearing disturbance was also

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an exclusion criterion.

The groups were formed, after applying the inclusion and exclusion criteria. Group I will be referred as Control 1 in the followings. Group II showed a positive diagnosis for epilepsy, with ongoing treatment for it, and it was named Epi 1. Group III, named Control 2, and was composed of healthy adults between 40-60, and Group IV, Epi 2, epileptic patients from the same age group, under antiepileptic treatment. P300 evaluation was performed on the selected patients. The aim of the study was to determine the effect induced by epilepsy, as a symptom, or its treatment, on the velocity of the age-related prolongation of the P300 wave. According to this, the epilepsy groups were heterogenous for the type of epilepsy and for the applied treatment also.

A Medtronic Keypoint 4 device was used to record the P300 wave, stimuli being presented through earphones, according to the oddball paradigm, using a resolution of 100 ms, 50 μV per division, low pass frequency at 0.2 Hz, high pass at 0.1 kHz, maximum tolerated impedance of the electrodes 1 kOhm, variable stimulation frequency, between 0.3 and 1 Hz, percentage of the odd stimuli 15%, the frequency for odd stimuli of 2000 Hz and the frequent ones of 1000 Hz. Recordings were made with electrodes placed at Fz, Cz and Pz of the 10-20 system, reference electrode in A1 and A2, and the ground at Fpz. Two evaluations were performed for each subject, with a number of 150 averages, 22-23 odd sounds/evaluation. The recorded parameter was the latency of the P300, mainly because this is the most robust measure that can be determined with this technique. The amplitude presents a high intra- and interindividual variabity, this being the reason it was not taken into account.

Statistical analysis was performed using SPSS version number 17, after the calculation of means and standard errors ($\bar{x} \pm s.e$), implementing ANOVA and post-hoc Scheffè test, and afterwards two-way ANOVA, with a treshold for significance of $p<0.05$.

RESULTS

The P300 wave was recorded in Fz, Cz and Pz of the 10-20 system. The latencies were compared at first between the three derivations, without significant differences (not shown).

Age becomes a grouping factor, by ordering patients according to it; then paired comparisons were made between Epi 1 and Control 1, and Epi 2 and Control 2, without significant differences (see below, Table I), so the control groups can be considered as age-matched. On the other hand, the distribution of the patients using as a criterion their sex is also matched between the age-defined groups (not shown).

The latencies of the P300 component presented a difference with a high global significance (ANOVA), when all the groups were compared, and at the post-hoc evaluation (Scheffè) the latencies were found significantly prolonged for each derivation on the epilepsy groups, versus the control groups; the two control groups, and the two epilepsy groups showed also significant differences at the paired analysis (Table I).

Table I. Means, standard errors, and significance thresholds, obtained from the statistical analysis performed on the groups

		Age	Fz	Cz	Pz
		$\bar{x} \pm s.e$	$\bar{x} \pm s.e$	$\bar{x} \pm s.e$	$\bar{x} \pm s.e$
	Epi 1	27.38 ± 1.38	353 ± 8.23	357 ± 8.49	360 ± 8.38
	Control 1	28.00 ± 1.51	326 ± 3.58	328 ± 3.56	330 ± 3.47
	Epi 2	50.93 ± 1.93	389 ± 9.08	392 ± 8.80	395 ± 8.69
	Control 2	50.33 ± 2.07	356 ± 6.01	358 ± 5.98	361 ± 5.96
P	**ANOVA**				
	Epi 1-Control 1-Epi 2-Control2	0.000	0.000	0.000	0.000
	Scheffè				
	Epi 1 vs Control 1	0.976	0.048	0.033	0.027
	Epi 1 vs Epi 2	0.000	0.006	0.009	0.006
	Control 1 vs Control 2	0.000	0.005	0.007	0.007
	Epi 2 vs Control 2	0.887	0.020	0.019	0.012

The second part of the study investigated if the presence of epilepsy has an effect on the age related prolongation of the P300 wave. We've used the two-way ANOVA test, and determined that even if both epilepsy and age, taken alone, has a significant prolonging effect on the auditory P300 wave, there is no significant effect of the interaction of the two factors (Tables II, III and IV).

Table II. The combined effect between age and epilepsy on the prolongation of the P300 wave registered in Fz: the interaction has no significant effect.

Dependent Variable: Fz

Source	Type III Sum of Squares	df	Mean Square	F	Sig.	Partial Eta Squared
Corrected Model	.006[a]	3	.002	14.657	.000	.463
Intercept	46.285	1	46.285	318736.764	.000	1.000
Epilepsy	.003	1	.003	20.409	.000	.286
Age_interval	.003	1	.003	20.937	.000	.291
Epilepsy * Age_interval	1.410E-5	1	1.410E-5	.097	**0.757**	.002
Error	.007	51	.000			
Total	48.186	55				
Corrected Total	.014	54				

a. R Squared = .463 (Adjusted R Squared = .431)

Table III. The combined effect between age and epilepsy on the prolongation of the P300 wave registered in Cz: the interaction has no significant effect.
Dependent Variable: Cz

Source	Type III Sum of Squares	df	Mean Square	F	Sig.	Partial Eta Squared
Corrected Model	.006[a]	3	.002	14.644	.000	.463
Intercept	46.424	1	46.424	322267.232	.000	1.000
Epilepsy	.003	1	.003	21.496	.000	.297
Age_interval	.003	1	.003	19.944	.000	.281
Epilepsy * Age_interval	7.631E-6	1	7.631E-6	.053	**0.819**	.001
Error	.007	51	.000			
Total	48.334	55				
Corrected Total	.014	54				

a. R Squared = .463 (Adjusted R Squared = .431)

Table IV. The combined effect between age and epilepsy on the prolongation of the P300 wave registered in Pz: the interaction has no significant effect
Dependent Variable: Pz

Source	Type III Sum of Squares	df	Mean Square	F	Sig.	Partial Eta Squared
Corrected Model	.006[a]	3	.002	15.756	.000	.481
Intercept	46.556	1	46.556	339028.480	.000	1.000
Epilepsy	.003	1	.003	23.384	.000	.314
Age_interval	.003	1	.003	21.160	.000	.293
Epilepsy * Age_interval	1.110E-5	1	1.110E-5	.081	**0.777**	.002
Error	.007	51	.000			
Total	48.473	55				
Corrected Total	.013	54				

a. R Squared = .481 (Adjusted R Squared = .450)

DISCUSSION

For the beginning, the possibility that the obtained results could be more significantly validated in case of higher number of subjects should be emphasized.

The P300 wave appeared with a prolongation of the latency in the presence of epilepsy, regardless the investigated age interval, 20-40, respectively 40-60. The prolongation is in accordance with most of the studies found in the recent literature. This increase of the latency should be cautiously interpreted when the source of the prolongation is investigated, because it can be influenced not only by the presence of epilepsy, but also by the length of the exposure to antiepileptic treatment, and the type of this treatment.

As it was already stated, the P300 wave shows an approximate prolongation rate with age, of 1.6 ms/year (14). Our study concludes that this rate is not influenced by the presence of epilepsy, although the overall values in every time point are higher for epileptics, than for normal controls. This is the reason we think epilepsy, or it's treatment, or both, have a static role on the velocity of the age-related prolongation of P300, when epilepsy is controlled, maintaining the linearity of the effect induced by age.

While in other diseases placebo is used in situations requiring discrimination between different variable effects, epilepsy has the property of causing potentially life-threatening states if left untreated, so it is an ethical issue to be solved in order to define the primary role of either the epilepsy or its treatment is causing the investigated effect on the evoked potential. Also in such situation we might be able to have healthy controls under antiepileptic treatment, which is also not acceptable for such study conditions. If epilepsy alone has the effect, than it should be emphasized that a proper treatment is needed for a better control, less seizures meaning better long-term cognitive performances; if treatment contributes, than newer agents, with less side effects, should be recommended.

CONCLUSIONS

The presence of epilepsy, in both investigated age groups, causes a highly significant prolongation of the P300 wave, possibly related to either a cognitive deficit, or at least partly a consequence of the ongoing treatment. The age related prolongation's velocity is not increased by the presence of epilepsy, maintaining its linearity regardless of the length of the disease or the length of exposure to antiepileptic drugs.

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THE EFFECT OF EPILEPSY ON THE AGE-RELATED PROLONGATION OF THE P300 WAVE

REZUMAT

Introducere si scop: Conform datelor din literatura, potentialele evocate relationate evenimentului (ERP) prezinta anumite neregularitati in epilepsie. Este cunoscut faptul ca latenta P300 prezinta, de asemenea, alungire relationata varstei. Acest studiu evalueaza influenta prezentei epilepsiei asupra alungirii latentei P300 relationata varstei.
Metode: Au fost inclusi in acest studiu 54 de pacienti si au fost formate 4 grupuri de studiu, un grup de control cu varsta cuprinsa intre 20 si 40 ani, un alt grup control cu varsta intre 40 si 60 ani si doua grupuri de pacienti cu epilepsie, stabilite conform acelorasi criteria de varsta. S-a efectuat evaluarea auditiva a P300.
Rezultate: Latentele P300 au fost semnificativ prelungite in prezenta epilepsiei pentru ambele grupuri de varsta, comparativ cu grupurile de control corespunzatoare, in toate derivatiile investigate. Latentele P300 au fost semnificativ prelungite in grupul de varsta 40-60 ani, comparativ cu grupul de varsta 20-40 ani, atat pentru control, cat si pentru grupul cu epilepsie. Prezenta simultana a celor doua conditii determinante, epilepsie si varsta inaintata, nu a avut un efect augmentativ, prezentand doar un efect aditiv liniar asupra prelungirii.
Concluzie: Viteza de alungire a latentei P300 relationata varstei nu este influentata de prezenta epilepsiei, in cadrul unui tratament adecvat, heterogen, ceea ce demonstraza ca declinul cognitiv este linear cu varsta, atat la grupul de control, cat si la cel cu epilepsie.
Cuvinte cheie: potential evocat relationat evenimentului, P300, latenta, varsta, epilepsie

OXIDANTS-ANTIOXIDANTS BALANCE IN ORAL LICHEN PLANUS

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ABSTRACT

Lichen planus is a chronic inflammation of unknown etiology. The disease has been most often reported to affect the oral mucosa. The most dangerous complication of oral lichen planus (OLP) is oral squamous cell carcinoma. Recently, oxidative stress has been implicated in the pathogenesis of OLP. The aim of this study is to evaluate the status of oxidative stress and the antioxidant defense system in patients with OLP. We evaluated and compared the levels of a series of oxidative stress markers in patients with OLP with that of normal controls. Serum levels of malondialdehyde, protein carbonyl, sulphydryl groups, hydrogen donor and glutathione were measured. Malondialdehyde (MDA), carbonyl protein (PC) and glutathione were increased and sulphydryl groups (SH) and hydrogen donor (HD) were decreased in patients compared to controls. In conclusion our results point to a disturbance in the prooxidants-antioxidants balance in OLP and demonstrate the involvement of oxidative stress in the pathogenesis of this disease.
Key words: prooxidants-antioxidants balance, oxidative stress, oral lichen planus

INTRODUCTION

Lichen planus is a chronic inflammation of unknown etiology. It involves the skin, mucous membranes, genitalia, nails, and scalp. The prevalence of LP is 1-2% and it is seen most frequently in middle aged patients more commonly in women than in men (31). The disease has been most often reported to affect the oral mucosa (29).

Although the exact etiology is unknown OLP is recognized as an autoimmune disease in which activated T cell release cytokines leading to the attraction of inflammatory cells and the destruction of keratinocytes by cell mediated cytotoxicity (9,21). Recently oxidative stress has been implicated in the pathogenesis of OLP (3).

The probability of malignant transformation is low still the most dangerous complication of OLP is oral squamous cell carcinoma (OSCC) (22,24). The accumulation of 8-nitroguanine and 8-oxoG in the tissue of patients with OLP and OSCC (6) leaded to the conclusion that cell mediated DNA alteration via oxidative stress may play a key role in carcinogenesis of OLP (16-7).

The aim of this study is to evaluate the status of oxidative stress and the antioxidant defence system in patients with OLP.

MATERIALS AND METHODS

This study included 9 patients with OLP and 4 healthy volunteers. All subjects were retrieved from The Department of Oral, Craniomaxillary and Cervicofacial Surgery of "Iuliu Hatieganu" University of Medicine and Pharmacy, Cluj Napoca, Romania. This work has been approved by the ethical committee of "Iuliu Hatieganu" University of Medicine and Pharmacy and informed consent was taken from all the participants in this study. All patients were subjected to a thorough history regarding dietary habits and addiction and clinical examination.

Blood samples (5ml) were obtain from patients and controls by venous arm puncture and were centrifugated for 5 minutes at 3500 rpm. After the plasma was obtained it was immediately frozen and kept at a temperature of -80^0 C until it was processed. Serum MDA was established by fluorescein dosage, according to Conti (7). The concentration values were expressed in nmol/ml. PC determination was based on Reznick method (26). The results were expressed in nmol/mg protein. The assessment of ability of hydrogen donor was measured by Janaszewska method and expressed in inhibition% (14). Total SH groups of serum were assayed according to the method of Hu (13). The concentration was expressed in micromol/ml. The serum GSH level was examined using Ellman method and expressed in nmol/ml.

Statistical analysis

Data normality was assessed using Kolmogorov-Smirnov test. Data were analyzed using the a nonparametric Kruskall-Wallis test for overall groups comparison, Mann-Whitney U Test/ Student for the comparison of two groups. The correlations between the qualitative variables were analyzed by Chi square test. Adjustments were used using stratification of groups to exclude the effect of possible error factors. SPSS 17.0 (Chicago, Il,USA) statistical package was used to analyze all data.

RESULTS

General characteristics and correlations

The average age of the participants was 47.27 ± 17.83 years

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old, significantly higher in patient's group (p = 0.007) (Table 1). Subjects in the control group have reported to consume one toxic substance (alcohol/nicotine/caffeine) while subjects in the tested group consume 1-3 types of drugs. The correlation between the use of drugs and the group origin was not statistically significant (p = 0.246) (Table I). White reticular pattern of OLP was found in all the subjects in the test group (Figure 1).

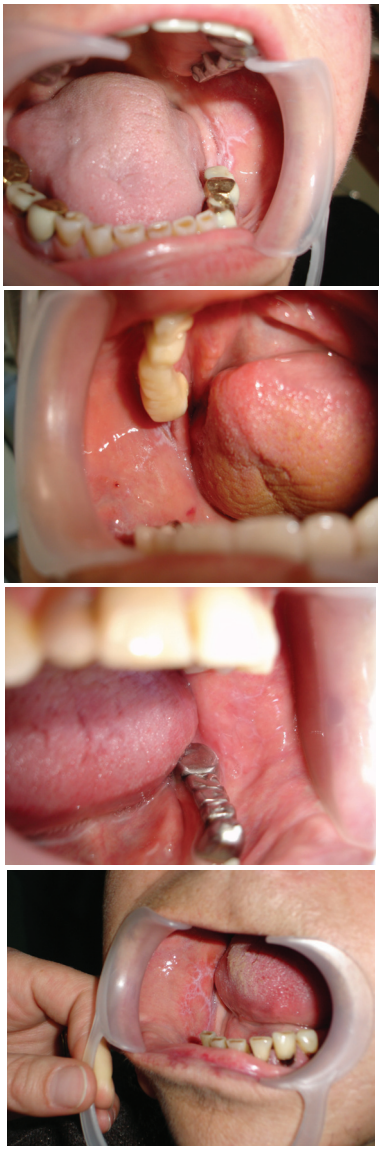

Fig.1. Clinical aspect of oral lichen planus

Table I. Characteristics of subjects

Variable	Controls	Tested group
Age (median ± SD)	28.25±2.98	54.18±15.67
Toxic substances, no. (%)		
Absent	0(0%)	2(16.7%)
1 substance	4(100%)	5(41.7%)
2 substances	0(0%)	1(8.3%)
3 substances	0(0%)	4(33.3%)

Serum

Median MDA levels were increased in the tested group 3.0 (1.10-6.70) versus control group 1.8 (1.2-2.10). The simple comparison between groups has given values of 0.150 (not significant). However, after the age adjustment, the difference was highly significant (p < 0.0001) (Figure 2).

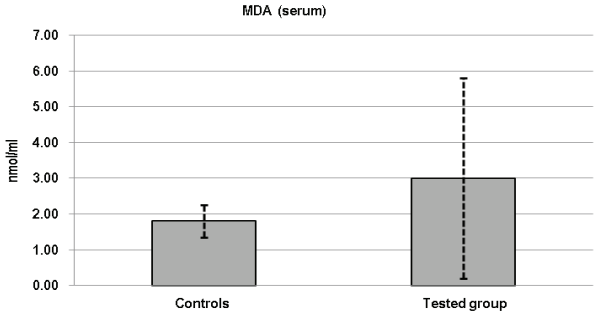

Fig. 2. Serum MDA levels in tested group and controls

Similar results were obtained for the PC determination. Statistical data showed a value of 1.7 (1.0-3.2) in the patient's group and 1.2 (1.0-1.6) in the healthy group. Non-adjusted significance was p = 0.089 (marginally significant). After age adjustment, the difference was highly significant p < 0.0001 (Figure 3).

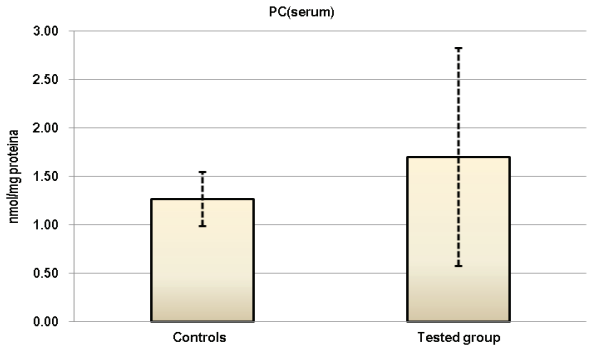

Fig. 3. Serum PC levels in tested group and controls

HD median level was decreased in the tested group 47.7 (38.5-58.2) as compared to control group 53.2 (47.7-58.3). The difference was not statistically significant (p = 0.133). However, after age adjustment it became highly significant (Figure 4).

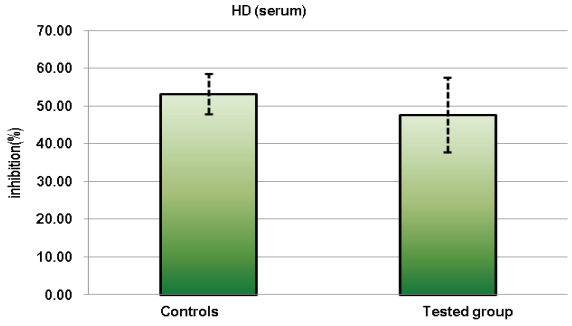

Fig. 4. Serum HD levels in tested group and controls

Median serum SH was lower in patients with OLP - 0.32 (0.15-0.54) - then in healthy participants - 0.39 (0.37-0.51). The unadjusted level of significance was p = 0.026, while after adjustment, the alpha level was p < 0.0001 (Figure 5).

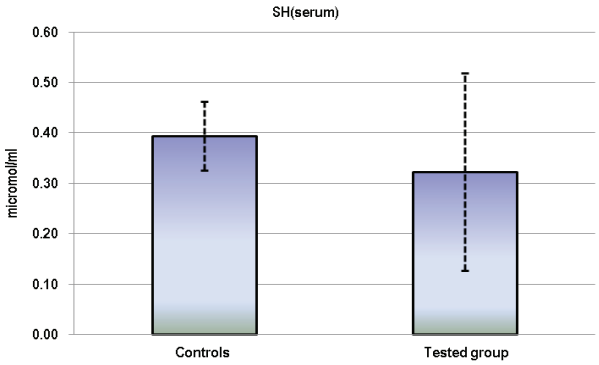

Fig. 5. Serum SH levels in tested group and controls

The median level of glutathione (GSH) was higher in patient's serum as compared to control group - 7.12 (3.40-11.90) versus 4.17 (3.05-6.60). Statistical parameters, such as p value, were different in the absence or after age adjustment - p = 0.090 and p < 0.0001, respectively (Figure 6).

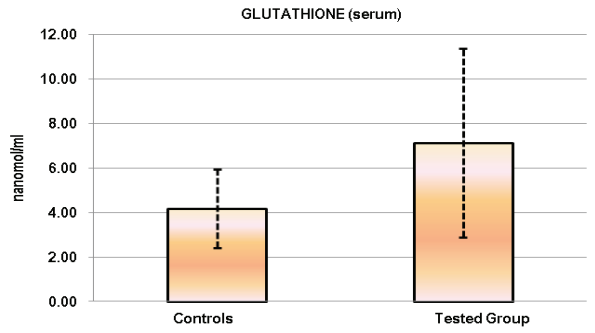

Fig. 6. Serum glutathione levels in tested group and controls

DISCUSSION

Oxidative stress is a disturbance of the prooxidants-antioxidants balance in favor of the prooxidants (8,12,30). This usually results in the production of free radicals that can damage cell membranes through lipid peroxidation, as well as numerous cellular molecules such as proteins, nucleic acids, amino acids, carbohydrates, and vitamins (2,23,28).

MDA, the final product of lipid peroxidation is considered a good biomarker of oxidative stress. We found an increased serum level of MDA in patients versus healthy subjects. Our results indicate the implication of oxidative stress in the pathogenesis of OLP. These findings are in corcondance with several studies that demonstrated a significant increase in lipid peroxidation product MDA, increased oxidative DNA damage, increased protein oxidation, and disturbed enzymatic antioxidant defenses in lichen sclerosis vulvae tissue specimens. In addition another study reported higher serum levels of MDA in LP patient group than the control group and high MDA levels in LP, leukoplakia, and cancer (1).

Generation of carbonyl groups on amino acid side chains is one of the most commonly studied and used markers for protein oxidation (20). In our study the values of PC in patient's serum were higher than in controls. This point out to the involvement of oxidative stress in the pathogenesis of the disease we studied. Other works showed increased protein oxidation, DNA damage, lipid peroxidation product MDA, and disturbed enzymatic antioxidant defenses in lichen sclerosis vulvae tissue specimens (27).

Serum hydrogen donor capacity (HD) is an expression of the total antioxidant capacity of the serum because it is evidencing the amount of reducing substance that has neutralized ROS. The level of DH was decreased in the sera of our patients compared with normal individuals. We think that this reduction is due to the decrease in the protective capacity ensured by antioxidants. Increased oxidative stress and lower antioxidant capacity was found in lichen sclerosis vulvae tissue specimens (27), in serum of patients with OLP and in OSCC saliva, sera and tissue samples (4).

SH groups are very susceptible to oxidation. When the organism is exposed to oxidative stress they are the first antioxidants to be sacrificed (10,11). In our study the level of –SH was lower in the test group than in controls. This decrease illustrates the intensity of the oxidative stress in OLP. The results are in concordance with other studies that have recorded lower levels of serum –SH and higher levels of serum MDA in patients with OLP versus healthy controls (33).

Glutathione is the most important non-enzymatic antioxidant. GSH can act as a nucleophile substrate as well as a reducing agent interacting with various free radicals (19. It plays an important role in detoxification process (32). We found higher levels of serum GSH in tested group compared with controls. Raised serum levels of GSH has also been reported in OSF, leukoplakia, and oral cancer (5,15,18,25). We think that probably this is due to increased resistance to the cytotoxic effects of xenobiotics of altered cells who can selectively grow in a toxic environment relative to the normal cells.

CONCLUSIONS

In conclusion in patients with oral lichen planus there is evidence of the disturbance in the pro-oxidants-antioxidants balance characterized by high levels of pro-oxidants and decrease in the protective capacity ensured by antioxidants which proves the implication of oxidative stress in the pathogenesis of this disorder.

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BALANTA OXIDANTI-ANTIOXIDANTI IN LICHENUL PLAN ORAL

REZUMAT

Lichenul plan este o inflamatie cronica de etiologie necunoscuta. Afectiunea intereseaza mai frecvent mucoasa orala. Cea mai periculoasa complicatie a lichenului plan oral (LPO) este carcinomul spinocelular oral. Recent stresul oxidativ a fost implicat in patogeneza LPO. Scopul aceste lucrari este de evaluare a stresului oxidativ si a apararii antioxidante la pacientii cu LPO. Am determinat si am comparat nivelul mai multor markari ai stresului oxidativ la pacientii cu LPO fata de martori sanatosi. Au fost dozate nivelele serice ale malondialdehidei, proteinelor carbonilate, gruparilor sulfhidril libere, donorilor de hydrogen si a glutationului. Valorile malondialdehidei (MDA), a proteinele carbonilate (PC) si glutationului au fost crescute in timp ce gruparile sulfhidril libere (-SH) si donorii de hIdrogen (DH) au fost scazute la lotul testat comparativ cu lotul martor. In concluzie rezultatele noastre indica o dereglare a balantei oxidanti-antioxidanti in LPO si demonstreaza implicarea stresului oxidativ in patogeneza acestei afectiuni.

Cuvinte cheie: balanta oxidanti-antioxidanti, stres oxidativ, lichen plan oral

EXHALED NO AND RISK FOR ASTHMA IN PATIENTS WITH ALLERGIC RHINITIS

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ABSTRACT

Background: The relationship between the upper and lower airways is well known. Allergic rhinitis is considered a risk factor in asthma development, and is also co-morbidity in asthma. Family history of asthma is known as a risk factor in asthma developing.
The aim of the study was aimed to evaluate the risk of asthma in patients with allergic rhinitis using exhaled nitric oxide (FENO).
Methods: 34 pts with allergic rhinitis were evaluated with Nioxmino for measuring FENO. The medical history of each patient and also the five nasal symptoms score were evaluated. Skin pick test were performed for inhaled allergens (house dust mites, pollens, cat dander, dog dander, moulds).
Results: 15 pts (44.11%) were women (sex ratio f:m = 1:1.26). Mean age of the pts is 28.77 ± 10.38 years. All patients were allergic, 52.9% polysensitized more than one allergen. It was found a significant statistical correlation between family history of asthma an increased values of FENO (p = 0.001). But between polysensitized patients and monosensitized patient there was no correlation with FENO (p = 0.19), and also no correlation of FENO and rhinitis symptoms score (p = 0.08).
Conclusion: Increased values of FENO could be evaluated as a risk factor for developing asthma in patients with allergic rhinitis. In allergic rhinitis severities of symptoms score was not a predictive factor in developing asthma. There was no correlation between FENO and polysensitization.
Key words: exhaled nitric oxide, asthma, rhinitis, family history

INTRODUCTION

During the atopic march the evolution from allergic rhinitis to asthma was evidenced. This two diseases co-exists often, could be named "one allergic disease" (1,2). Allergic rhinitis is the most common IgE mediated disease and is highly increased as prevalence during the last years (3). Allergic rhinitis is a risk factor for asthma (4), and can appear before or after asthma. Allergic inflammation is the key to understand this disease, and also the evolution to co-morbidities, especially asthma.

Allergen inhalation leads to mast cell degranulation in nasal mucosa, and release of mediators, mainly histamine and leukotriens. Th2 cytokines are responsible for the recruitment of inflammatory cells in the tissues, including eosinophils, neutrophils and Th2 lymphocytes (5). The link between the upper and lower airways is not completely understood, it is possible to explain this concept by the fact that inflammatory cells and mediators from nasal secretions enter in the lower airways through inhalation and aspiration, and here they can act as a trigger factor in lower airways inflammation (6).

The question "Which patients with allergic rhinitis will develop asthma?" in the "united airways concept" is important for every practitioner when we have a patient with allergic rhinitis in front of us.

There is a hypothesis that eosinophils recruited in allergic inflammation via expression of adhesion molecules (7), may induce NO-syntetase in bronchial epithelial cells. Exhaled nitric oxide (FENO) is known as a marker of airways eosinophilic inflammation in lower airways (8). Several studies showed that patients with atopic asthma have increased values of FENO than non-atopic asthma. IgE- mediated inflammation leads to elevated values of FENO. Also studies have demonstrated that patients with hay fever during pollen season have an increased values on FENO, even if they had not or had mild asthma symptoms (9).Other study demonstrate that in patients with allergic rhinitis there are increased values of exhaled NO and adenosine compared with healthy subjects, those data suggests that in allergic rhinitis there is an subclinical inflammation in the lower airways (10). In allergic rhinitis an increased in FENO on allergen exposure, particularly in hyperresponsive subjects may be suggestive of airway inflammation and an increased risk for developing asthma (11,12). FENO measurements using Nioxmino are less expensive than other measurements for asthma and very easy to use (13).

Genetic studies in allergies shown that there is a number of susceptibility genes could contribute to the allergic process, and is possible that the "allergic disease genes" and the environment lead to a phenotype which can be asthma and/or allergic rhinitis and/or atopic dermatitis (14).

Clinical aspect of asthma can vary a lot from classical bronchospasm to chronic cough or only effort dyspnoea (2). There are patients with allergic rhinitis with rare and mild asthma symptoms which are not remark due to a severe rhinitis. The difference between rihinitis patients with or without asthma symptoms seems to be a question of perception of dyspnoea. FENO measurements indicate that dyspnoea could be associated with increased inflammation in peripheral airways (15).

The aim of this study was to investigate the risk of asthma in patients with allergic rhinitis using exhaled nitric oxide (FENO).

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MATERIALS AND METHODS

Subjects

A total of 34 patients with allergic rhinitis were evaluated with Nioxmino® for measuring FENO. Doctor's asthma diagnoses according to Global Initiative for Asthma (GINA) in any patient evaluated was not an inclusion criteria. Smokers and patients with respiratory infections within 4 weeks were not included in the study. Positive skin prick tests were mandatory for inclusion. An accurate family history also was obtaining from patients and theirs family. Patients who cannot provide an accurate family history of diseases were excluded. Each patient had to complete the five nasal symptoms score before any measurements.

All patients were attending in Allergy Department of "Octavian Fodor" Emergency Hospital from Cluj Napoca, and they have given the inform consent to participate in the study.

None of the patients use medication 2 weeks before evaluation. Were excluded patient that could not perform FENO measurements

Methods

Allergy screening was made by skin prick tests (Hal Allergy, The Netherlands) (16). A panel of common inhaled allergens was used which included: house dust mites (*Dermatophagoides pteronyssinus, Dermatophagoides farinae*), grasses pollens mix, mug worth, birch, cat dander, dog dander, mould (*Alternaria alternata*).

Five nasal symptoms score

All patients participating in the study complete the five nasal symptoms score. Rhinorrhea, nose itching, nasal stuffiness, sneeze, eyes itching were evaluated retrospectively in 12 hours. They had to interpret the symptoms on a scale from 0 to 3 (0-absence, 1-mild, 2-moderate and 3-severe). The classification of severity score was according to ARIA guide[1]. Symptoms score over 6 included the patient as moderate-severe allergic rhinitis.

Fractional exhaled nitric oxide

All measurements were performed in accordance to recommendations of American Toracic Society, with Nioxmino® (Aerocrine, Sweden) and defined in parts per billion (p.p.b.). No maneuver of spirometry was taken before the test, and all subjects were in seated position when measurements were made. Participants inhaled NO-free air through the mouth piece to total lung capacity, and the exhaled constantly (flow rate was assure as 50ml/min) with a visual feedback display. For patients with pollen allergy measurements were made during pollen season.

Statistical analysis

It was a small group of patients in which we used Microsoft Excel for dates, and Student Test for obtaining *p* value. A *p* value under 0.01 was considered significant.

RESULTS

Patient's characteristics

Thirty-four patients with allergic rhinitis were investigated. 15 pts (44.11%) were women (sex ratio f : m=1:1.26). The age range from 11 to 48 years, patients mean age were 28.77±10.38 years. All patients have skin prick test positive to at least one allergen, a positive skin prick test was considered at least 3 mm diameter with histamine at least 5 mm diameter. Of those 52.9% were polisensitizied to more than one allergen (Table I).

Table I. Sensitization at skin prick tests

Allergen	House dust mites	Pollens	Cat dander	Mould
Positive SPT per allergen	25	24	4	3

FENO values

Between polysensitizied patients and monosensitizied patient there was no statistical correlation with FENO values, p = 0.19 (Figure 1).

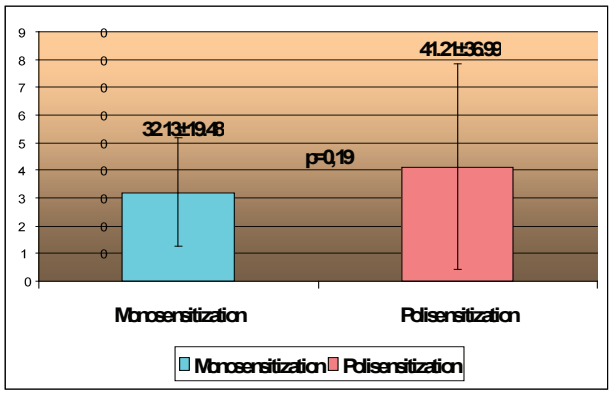

Fig. 1. FENO values correlation with sensitization

As severity of rhinitis patients who have five nasal symptoms score over 6 were included as moderate severe allergic rhinitis patients. Medium FENO values were higher in moderate severe allergic rhinitis group (42.33 ± 35.29 vs. 28.27 ± 18.49), but results showed a non significant statistical correlation, p = 0.08 (Figure 2).

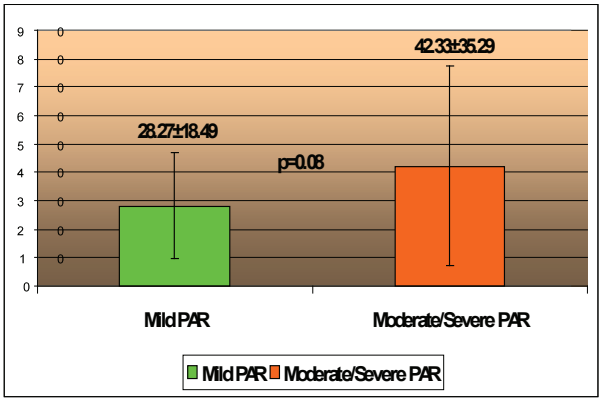

Fig. 2. FENO values correlation with severity of allergic rhinitis

Genetic predisposition for asthma was evaluated with an

accurate family history of the patients. Patients with family history of asthma had higher FENO values than patients with no asthma history (49.44 ± 32.91 vs.22.67 ± 18.95), and the results were statistical significant with p = 0.002 value (Figure 3).

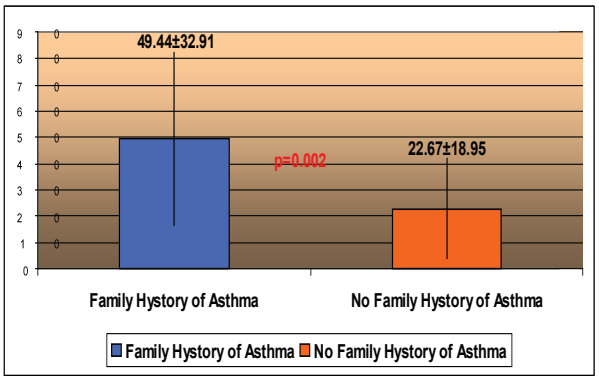

Fig. 3. FENO values correlation with family history of asthma

DISCUSSION

Allergic rhinitis and asthma are considered a single respiratory disease, a single respiratory syndrome involving two parts. Bronchial hyperreactivity is frequently present in patients with moderate-severe allergic rhinitis and should be suspected in the presence of risk factors (17).

In the present study we looked for the presence of lower airways inflammation, a potential subclinical inflammation in patients with allergic rhinitis using FENO, a marker of eosinophilic inflammation. FENO is an accepted marker which increased in lower airway inflammation (18).

The present study investigate the presence of lower airways inflammation in patients with allergic rhinitis

We obtained a correlation of FENO with sensitization, with severity of rhinitis and family history of asthma. Our data not suggested any association with monosensitization and polisensitization in our patient. Although in other studies it was shown, in children an increased of lower airway inflammation when house dust mites sensitization is present (19,20).

Asthma risk factors should be evaluated in every patient with allergic rhinitis. Parental history of atopy or asthma is positively associated with an increased risk for asthma (21,22). In our study we found o statistical significant correlation of FENO and the presence of asthma in family history. Patients with allergic rhinitis and family history of asthma should be closely and more often examine for asthma symptoms.

Although moderate-severe allergic rhinitis was seen often associated with asthma than mild allergic rhinitis (17), in our study the severity of allergic rhinitis was not correlated with FENO values. More studies should be done in order to evaluate the progression from rhinitis to asthma.

Therefore, from daily practice point of view is important to evaluate for asthma every patient with allergic rhinitis (1).Patients with allergic rhinitis presenting other risk factors for asthma should be closely evaluated for subclinical lower airways inflammation which is the substrate for bronchial hyperresponsiveness. Patients' quality of life is better influence when allergic inflammation is controlled using ARIA guideline reccomandations (23).

In conclusion FENO is an important tool to evaluate subclinical allergic inflamationin lower airways in patient with allergic rhinitis and is correlated with other risk factors for asthma develops in allergic patients.

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OXIDUL NITRIC IN AERUL EXPIRAT SI RISCUL DEZVOLTARII ASTMULUI LA PACIENTII CU RINITA ALERGICA

REZUMAT

Introducere: Relaţia dintre căile respiratorii superioare şi inferioare este la ora actuală extreme de bine cunoscută. Rinita alergică este considerată ca factor de risc pentru dezvoltarea astmului şi deasemenea este o comorbiditate în astm. Istoricul familial de astm bronşic este cunoscut ca factor de risc în dezvoltarea astmului bronşic.
Scopul acestui studiu este de a evalua riscul de a dezvolta astm la pacienţii cu rinită alergică folosind determinarea oxidului nitric în aerul expirat (Fe-NO).
Metodă: 34 pacienti cu rinită alergică au fost evaluaţi folosind Nioxmino pentru determinarea Fe-NO. Antecedentele heredocolaterale au fost cu atenţieanalizate, evaluandu-se şi scorul celor cinci simptome nazale. Testele cutanate alergologice au fost efectuate fiecărui pacient pentru alergeni inhalatori (acarieni, polen, epitelii de pisică, epitelii de câine, mucegaiuri).
Rezultate: 15 pacienti (44,11%) din lotul de studiu au fost de sex feminin (sex ratio f:m=1:1.26). Media vârstei acestor pacienţi este 28,77 ± 10,38 ani. Toţi pacienţii au fost alergici, 52,9% polisensibilizaţi la mai mult de un alergen. Am găsit o corelatie semnificativa statistic între istoricul medical de astm broşic şi valorile crescute ale Fe-NO (p = 0,001). Dar, între pacienţii polisensibilizaţi şi monosensibilizaţi nu s-a găsit corelatie cu Fe-NO (p = 0,19), şi deasemenea nu s-a constatat o corelatie semnificativa statistic cu scorul simptomelor rinitei (p = 0,08).
Concluzii: Valorile crescute ale Fe-No ar putea fi considerate ca factor de risc pentru dezvoltarea astmului la pacienţii cu rinită alergică. În rinita alergică, severitatea scorului simptomelor nu poate fi considerat un factor predictiv al dezvoltarii astmului. Nu s-a constatat nici o corelaţie a valorilor crescute ale Fe-NO şi polisensibilizare.
Cuvinte cheie: oxid nitric expirat, astm, rinita, antecedente familiale

DRB1 ALLELE FREQUENCY IN A POPULATION GROUP FROM THE WESTERN PART OF ROMANIA

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ABSTRACT

The present study was conducted between 2008 and 2010 and it aimed to investigate HLA-DRB1 gene polymorphism in population group from the Western part of Romania. PCR-sequence-specific oligonucleotide probes (PCR-SSO) technique was used to type HLA-DRB1 genes. The frequencies of the alleles for male and female population were calculated and compared.
Keywords: HLA-DRB1, alleles, PCR with sequence-specific oligonucleotides (PCR-SSO) technique

INTRODUCTION

The major histocompatibility complex (MHC) encodes class I and class II HLA molecules, and some complement components (1,2).

The HLA complex includes almost four million base pairs of DNA, encoding the HLA-A, B, C (class I), and HLA-DR, DQ, and DP (class II) antigens. To date, numerous studies have been performed to study the HLA polymorphism in different populations and ethnic groups (3).

For example, the HLA-DRB1 with its more than 315 known alleles represents the most polymorphic protein-encoding regions of the human genome (4,5). In addition to their role in conferring immune identity, genetic distances and correspondence analysis demonstrated that patterns of allele and haplotype distribution of class I and class II loci are racially and geographically restricted, thereby allowing their use for population and evolution studies (6).

The aim of our work was to investigate the frequencies of the HLA-DRB1 alleles in Romanian population. The samples were analyzed by the polymerase chain reaction sequence-specific oligonucleotides (PCR-SSO) method.

In this paper, the typing results for the HLA class II loci (DRB1) in a group of 501 individuals from the Western part of Romania are presented. The data were examined at the allele genotype level.

MATERIALS AND METHODS

Samples

The whole blood samples were collected from 501 individuals living in the Western part of Romania between 2008 and 2010. The studied samples were from the Emergency Children Hospital "Louis Turcanu" in Timisoara, Department of Transplant, and also from the Blood Center of Timisoara. Samples were genotyped in the Laboratory of Immunology of Transplant of the Emergency County Hospital Timisoara, Romania. Informed consent was obtained from all subjects and the experiments performed for this investigation complied with current guidelines and ethics.

HLA typing

HLA typing was performed using 2 milliliter peripheral blood collected in ethylenediaminetetraacetic acid (EDTA) tubes for every sample. Blood sample collection and HLA molecular typing were carried out with the national and local ethical consent (7). Blood was collected in order to establish the HLA genotype for medullar transplant. DNA was extracted using an automatic DNA extractor MagNA Pure LC DNA isolation with a specific kit. The kit contained washing buffers, lysis solutions, proteine kinase,

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suspensions with magnetic beads, elution buffers.

The DNA was resuspended in deionised water or the buffer provided with the extraction kit, at a concentration of 13-15ng/µL and A260/A280 ratio of 1.65-1.80.

The Dynal RELI SSO HLA-*DRB1* test was based on three processes: (i) PCR-target amplification (8,9), (ii) hybridisation of the amplified products to an array of immobilized sequence-specific oligonucleotide probes, and (iii) detection of the probe-bound amplified product by colour formation (10). The AutoRELI 48 Instrument automated the hybridisation and the detection reaction. HLA–DRB1 typing was carried out with polymerase chain reaction, using sequence-specific oligonucleotides (PCR-SSO) of HLA–DRB1 allele.

In order to perform the amplification of the samples, it was necessary to have both a positive control, and a negative control DNA. The following reagents were necessary: 7.5 µL (6.0 mM) $MgCl_2$ solution, 15 µL Mastermix, 7.5 µL DNA (concentration of 13-15 ng/µL to give total of approximately 100 ng/reaction).

The plate with samples was introduced in the PCR and underwent a thermal cycler program: a cycle program (35 cycles) at 95ºC for 15 sec., at 60ºC for 45 sec., at 72ºC for 15 sec., hold program at 72ºC for 5 min., hold program 15ºC forever.

It was necessary to pipette 30 µL of denaturizing solution into each reaction, which was then followed by 10 min. incubation at the room temperature, to allow a complete denaturizing.

The biotin-labeled amplicons hybridized to those SSO probes that contained a complementary target sequence and thus were "captured" onto the membrane strip.

The amplicon probe complex was visualized using a colorimetric reaction. Streptavidin-horseradish peroxidase (SA-HRP) conjugate was added to the membrane and bound to the biotin-labelled amplicons captured by the SSO probe. Addition of hydrogen peroxide (H_2O_2) and tetramethylbenzidine (TMB) substrate, results in the formation of a blue color complex in the presence of SA-HRP.

The AutoRELI 48 Instrument automateed both the hybridisation and the detection reactions.

The data were interpreted with the software Dynal Biotech Pattern Matching Program 5.42.

Statistical analysis

Statistical analysis of the data was performed using SPSS v. 15.0. The results for the categorical data were presented as percentages. The Chi-square test was applied to investigate the statistical significance of the observed differences in frequency between the two sexes.

RESULTS AND DISCUSSIONS

The HLA-DRB1 allele frequencies in the Romanian population group (271 males and 230 females) were summarized in Table 1. There were 501 individuals in total, with 14 types of allele. In the locus DRB1, DRB1*11 was the most frequent allele in the present study with a frequency of 43.91 %, followed by DRB1*07 with a frequency of 21.76 %.

Table 1. Allele frequencies of the HLA-DRB1 loci in a Romanian population group of Western part of Romania

HLA-DRB1 allele	*Total N=501*		*Males N=271*		*Females N=230*	
	n	%	n	%	n	%
1	47	9.38	27	9.96	20	8.70
3	52	10.38	26	9.59	26	11.30
4	40	7.98	21	7.75	19	8.26
5	1	0.20	1	0.37	0	0.00
6	1	0.20	1	0.37	0	0.00
7	55	10.98	31	11.44	24	10.43
8	8	1.60	5	1.85	3	1.30
9	2	0.40	1	0.37	1	0.43
10	4	0.80	2	0.74	2	0.87
11	111	22.16	64	23.62	47	20.43
12	7	1.40	4	1.48	3	1.30
13	51	10.18	25	9.23	26	11.30
14	25	4.99	13	4.80	12	5.22
15	49	9.78	26	9.59	23	10.00
16	46	9.18	23	8.49	23	10.00
17	1	0.20	1	0.37	0	0.00
18	1	0.20	0	0.00	1	0.43

HLA DRB1*11 was the most frequent observed allele in both the male individuals and the female individuals, with a percent of 23.62%, and 20.43%, respectively. The difference between male and female population was not significant (Chi-square test, p=0.39). The allele HLA DRB1*07 had also a high percent in male population with 11.44%, while in the female population HLA DRB1*07 had an equivalent occurrence with DRB1*15 and DRB1*16. In female population a high percent of DRB1 allele was observed for DRB1*03 and DRB1*13with a percent of 11.30% each (none were statistically significant). Was also observed that in the male population there were no DRB1*18 allele, while in the female population there were no DRB1*05, DRB1*06, and DRB1*17 allele.

CONCLUSIONS

This study found that the most frequent allele in a population group of 501 individuals was HLA DRB1*11 with no significant difference between the male and female population in the Western part of Romania.

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FRECVENTA ALELEI DRB1 IN GRUPUL POPULATIONAL DIN PARTEA DE VEST A ROMANIEI

REZUMAT

Studiul prezent a fost efectuat intre anii 2008-2010 si si-a propus investigarea polimorfismului genei HLA-DRB1 in grupul populational din partea de vest a Romaniei. In acest scop, a fost utilizata tehnica PCR-SSO pentru tipizarea genei HLA-DRB1. Au fost calculate si comparate frecventele alelelor in cadrul populatiilor de sex masculin si feminin.
Cuvinte cheie: HLA-DRB1, alele, tehnica PCR-SSO

LETTER TO THE EDITOR

RADIOPROTECTIVE PROPERTIES OF WATER WITH LOW CONTENT OF STABLE ISOTOPES: CRITICAL EVALUATION

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ABSTRACT

Deuterium oxide (D_2O) concentration in natural water is about 0,02 %. First symptoms appear in experimental animals when D_2O concentration in body fluids reaches 20-25 %. The stable oxygen isotope ^{18}O is harmless even at highest enrichments. D_2O and ^{18}O concentrations are permanent environmental factors, and all living organisms are adapted to them. The concluding point is that decreased contents of microcomponents such as D_2O and ^{18}O, which are biologically indifferent also at much higher concentrations, cannot impart useful qualities to drinking water.

Key words: deuterium, stable isotopes, radiation safety, spleen, mouse

In 1999 in the Romanian Journal of Physiology was published an article (1), where radioprotective and immunostimulating effects of deuterium-depleted water in mice were reported. During last years, this theme has become popular among some Russian researchers: radio-protective properties of the „light water" were demonstrated in experimental animals predominantly on the basis of histopathological analysis of the spleen. Considerable differences in murine spleen morphology were shown between the radiation-exposed animals receiving usual water on one side, and deuterium-depleted (2) or deuterium- and ^{18}O-depleted water (3) on the other side. The study (3) was funded by the Federal agency for science and innovations of Russian Federation.

Furthermore, protective effect of the deuterium- and ^{18}O-depleted water against radiation-induced lenticular opacity in mice was reported (4). Cataracts are a common pathological abnormality of the lens characterized by the loss of lens transparency. They are the leading cause of blindness worldwide. Because of its high prevalence, this disease has substantial public health implications (5). The lens of the eye is recognized as one of the most radiosensitive tissues in the human body. Ultraviolet rays (5) and ionizing radiation (6) can induce formation of the cataracts. Therefore, a proposition about protective effects of the 'light water' against the cataract is of great potential importance for public health. However, reliability of such statements is doubtful because of the following reasons.

In the nature, the ratio of deuterium to hydrogen (D/H) is about 1:6600, which means that concentration of deuterium in natural water is about 150 parts per million (ppm) or 0.015 atom% (7). First symptoms appear in experimental animals at D_2O concentrations in corporal fluids of more than 20-25 %, while death follows after a concentration increase up to 30-35 % or higher (8). High concentrations (usually more than 20% of body weight) can be toxic to human and animal cells (9). In other words, natural D_2O concentration is more than 1000 times below the threshold level. Should the "light water" have a radioprotective effect, it would mean that D_2O in concentration of 0.015 atom% enhances radiation-induced damage, which has never been observed even at much higher concentrations, exactly as other adverse effects were not. This issue is rather well studied because stable isotopes are used in clinical and experimental research (10,11), and heavy water is used in nuclear reactors. The stable oxygen isotope ^{18}O is harmless even at maximally possible enrichments (10). On the contrary, there is information on the radio-protective and other beneficial effects of water with enhanced deuterium concentration (7,9,12,13), which is not mentioned in the articles (2-4).

D_2O and ^{18}O concentrations in water are constant environmental factors, and all biological species have been adapted to their natural concentrations in the course of the evolution. Besides, the technically complicated process of water clearance from the stable isotopes (rectification with re-mineralisation) (3) can negatively influence the qualities of drinking water. Investigations of the «light water» can be compared with a study of water devoid of some natural microelements: prolonged consumption of such water can cause deficiency of a microelement, but a positive effect from a lowered concentration of a normal environmental micro-component is very improbable. More probable appears adjustment of the morphological description of murine spleen, which provided the basis for the conclusions about the radioprotective effects of the "light water", to a preconceived idea (14,15). For example, it is stated with regard to the spleen morphology in radiation-exposed mice, receiving deuterium-de-

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pleted water (verbatim from Russian): "Morphological parameters of peri-arterial lymphoid mantles are elevated… irregularly enhanced cell destruction in lymphoid zones of the spleen was observed… considerable (2-6 times) elevated contents of granular leukocytes… The quantity of cells with mitoses in germinal centres was 6.9 times elevated" (2). Decreased concentration of biologically indifferent micro-components, such as deuterium and ^{18}O, could not have caused significant morphological differences from the control.

Considering the above, conclusions and recommendations on the basis of the experiments with deuterium- and ^{18}O-depleted water should be regarded as unfounded. The concluding point is that decreased contents of microcomponents such as D_2O and ^{18}O, which are biologically indifferent also at much higher concentrations, cannot impart useful qualities to drinking water.

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PROPRIETATILE RADIOPROTECTOARE ALE APEI CU CONTINUT SCAZUT DE IZOTOPI STABILI: EVALUARE CRITICA

REZUMAT

Concentratia oxidului de deuteriu (D_2O) in apa naturala este de 0,02 %. Primele simptome apa la modelul experimental animal atunci cand concentratia D_2O in fluidele organismului atinge valoarea de 20-25 %. Izotopul stabil al oxigenului ^{18}O este inofensiv chiar si la concentratii crescute. Concentratiile D_2O si ^{18}O sunt factori de mediu permanenti si toate organismele vii se adapteaza la acestea. Concluzia acestui studiu este ca scaderea continutului microcomponentelor de tipul D_2O si ^{18}O, care sunt biologic indiferente, chiar si la concentratii mult mai mari, nu poate afecta calitatile benefice ale apei potabile.

Cuvinte cheie: deuteriu, izotopi stabili, siguranta radiatiei, splina, soarece