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EDITORIAL

CRISPR and precision medicine

Copotoiu Sanda-Maria*

Editor-in-Chief

The deceiving outcome of Jerry Gelsinger's volunteer enrollment in a genetic study threatened to put the brakes on genetic research. Instead, despite the hidden risks, unanticipated and obviously unwanted, knowledge continued to evolve. The tragic death of a naïve volunteer on the altar of genetics ended in four lessons written by the leading personality and at that time, culprit for the obviously surprising collateral loss [1]. These were perceived at the time as a lecture behind the firewall the Penn University managed to build between James M.Wilson (the geneticist in cause) and the prosecutors [2]. Nine years have passed between J. Gelsinger's lethal outcome and Wilson's mea culpa. His death was preceded by seven years of intense research in genetics at the Penn University in the USA.

Today we are confronted with unacceptable mortality in sepsis and septic shock despite large and intense initiatives to oppose it. Antibiotics are either under optimally used, stewardship is reduced sometimes to a matter of perception. These tools are improperly used or inefficient at the end of the day.

Towards the conclusion of his mandate, president Obama launched and supported the initiative of "precision medicine". Precision medicine was defined as an "emerging approach for disease prevention and treatment that takes into account people's individual variations in genes, environment and lifestyle" [3]. The aim of this initiative was to generate the scientific evidence to be used for moving the concept of precision need into clinical practice. This would offer be the best tools to practice individualized medicine and thus become more efficient and make a change to the best in patients' lives. The longer term goals comprised the recruitment of over 1 million American volunteers, the research cohort, who would share genetic data, biologic samples and diet/lifestyle, information, all emerging from their electronic health records [3]. Put this way, it would seem to offer the humanity one exceptional chance to contribute to groundbreaking evidence to support further initiatives and disease management. Naturally, new and ancillary ethic engrams were promoted at this point: engaged participants, responsible data sharing and privacy protection.

Meanwhile, researchers published papers on antibacterial autophagy. Bacteria penetrating the cells are sensed and tagged (the microscopic paint ball war) with molecules called ubiquitin which mark the bacteria for destruction. This process is jeopardized by genetic mutations in the human cells. Invading bacteria are marked by E3 ligases, proteins that decorate the invaders with ubiquitin early in autophagy. According to the Broad Institute, one of the most prestigious North American Institute of Research in bioengineering, there are 617 known ligases [4].

Moreover, it appears that cellular miscommunication plays a crucial role in inflammation. There are cytokines that do not act like switchers to be turned on and off, but rather tuned. Therefor they are considered to behave like biased agonists for triggering some G protein-coupled receptors – GPCRs involved in tunable cytokine activity. It was stated that "variations in a single cytokine can lead to biased downstream signaling and can thereby cause human disease" [5].

The Broad Institute launched recently major research initiatives to study among others, why cancers become drug resistant.

This first 2017 issue of the Acta Medica Marisiensis publishes a welcome review on CRISPR (Clustered Regularly Interspaced Short Pahindromic Repeats) [6]. Genome editing exists since 2012, but in 2013 the TALE Nucleases (Transcription Activator-Like Effector Nucleases) were shadowed by the engineered CRISPR-Cas9 system first harnessed for mammalian genome editing by Fang Zhang of the Board Institute and MIT. The description of the CRISPER-Cas9 system used the words: "efficiency, effectiveness and precision" [4].

The reactions did not wait for long. The United Kingdom expressed its point of view through the Nuffield Council on Bioethics, an organism founded jointly by the Medical Research Council, the Nuffield Foundation and the Welcome Trust. Thus they published their analysis and conclusions on a generous document on genome editing [7].

A quick search last evening on cccDNA clearance revealed largely over 92000 results for cccDNA. Obviously the magnitude of the issue and the potential for groundbreaking results of using bioengineering at this stage is impressive to understate the facts.

This editorial was triggered by the seduction of precision medicine mirrored by the review written by Crauciuc et al and hopefully is deprived of any conflict of interest.

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REVIEW

Development, Applications, Benefits, Challenges and Limitations of the New Genome Engineering Technique. An Update Study

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We assume that the CRISPR Cas9 theory must be delimited by applicability, because the consequences of long term DNA manipulation remain unknown. Moreover, the irreversibility of this procedure should instigate researchers to reserved opinions.
Usefulness as well as benefits of CRISPR Cas9 made it one of the most popular and used genome editing technique. But with its huge potential, ethical and safety concerns emerge. Therefore, before continuing research in this direction we should have a well organized system that is able to make that differentiation between research and reproduction. However we truly believe in the future of genetic engineering and with the CRISPR-Cas9 system we expect that the opportunity of treating now so called incurable diseases arises. Time is all we need.

Keywords: CRISPR-Cas9, Cpf1, Class 2 CRISPR effector, RNA guided

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The development of genome engineering

Genome engineering

An important step for genomic engineering was achieved in 1990 by C. Srinivasana a chemist at Johns Hopkins University, who tried to manipulate bacterial enzymes which could cut DNA [1].

The first step in the genetic engineering process was the division of the chromosomal DNA using genome editing techniques such as zinc finger nucleases (ZFNs) and transcription activator-like effector nucleases (TALENs). The second step was to increase its specificity by using clustered regularly interspaced short palindromic repeats (CRISPR) associated with the Cas9 protein system (CRISPR-Cas9) which has RNA-guided (g-RNA) nucleases that precisely interrupts targeted sequences at the desired locus in the genome of any living organisms [2]. Moreover, there is a possibility to insert specific sequences into the DNA.

Genome editing tools play an important role in genetic engineering, and that is the reason why the CRISPR-Cas9 system has screen platforms which can be operated in various formats, such as the knockout, knockdown and activation screens. Their purpose is to enable high-throughput interrogation of gene functions in healthy and compromised organisms, in order to target coding and non-coding regions throughout the genome, and last but not least, to induce less off-target effects [3].

The CRISPR-Cas9 system

The CRISPR system was used for the first time in 2002 and the theory sustaining it was based on the adaptive immune system of bacteria. Initially, the mechanism had a Cas9 enzyme which used RNA to guide itself on the complementary sequences of DNA [4,5].

Several types of Cas enzymes have been developed, but the most well-known is called Cas9, originally discovered in *Streptococcus pyogenes* [7, 8, 9]. Three major types of the CRISPR–Cas system have been delineated, further divided into several subtypes and a few chimeric variants, [5] of which type II has been studied the most [6].

Scientists' have focused on the immune system possessed by bacteria that are constantly adapting to foreign invaders such as viruses and plasmids. Further analysis detected clustered regularly interspaced short palindromic repeats (CRISPRs), meaning that there are short repetitons separated by nonrepetitive sequences, called "spacer DNA," acquired from an early encounter with foreign DNA.

The nuclease Cas (CRISPR associated proteins) uses spacer DNA to identify and crop the DNA of the invaders. Moreover, in association with a guide RNA, Cas is directed towards a specific target which needs to be removed leading to a resistance to the attack of the virus.

This process could be applied to the nucleus of any living cell. The system would attach a DNA sequence followed by the Cas9 which would unzip the genome, pair it to the target RNA, and two molecules would perform the cut. Afterwards, a repairing process would be initiated, however there would be a high risk of random mutations resulting in a disabled gene. Researchers have the option to replace the mutant sequence with a healthy new version in stem cells or fertilised eggs and to target more than one gene at once, making the CRISPR Cas 9 system an im-

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portant tool for studying complex diseases and improving different biotechnologies [10,11]. Figure 1 illustrates the process of CRISPR-Cas9 associated system.

New proteins discovered (class 2 CRISPR effector)

In the last quarter of 2015, B. Zetche and F. Zhang reported another class of CRISPR effector namely Cpf1 proteins. The purpose of their discovery was to find a safer approach in this technique and to overcome its current limitations. From the total of 16 Cpf1 enzymes discovered, two are compatible with human cells. The CRISPR-Cpf1 technique has the following advantages: a Cpf1 small size enzyme, a single g-RNA molecule compared to Cas9 which needs two molecules and the DNA lysis being produced in several places offering multiple choices. Moreover, the lysis has a different approach by creating "sticky end" which is easier to control compared to Cas9 which creates "blunt ends". Another positive aspect of the "sticky end" is the fact that the insertion of DNA can be directed via the information this procedure carries, making it more manageable [13, 14, 15]. The Cpf1 enzyme is safe to use during the procedure because it is easy to handle [14]. Research institutes have recognised the importance of these new findings and have made the legal preparations in order for it to be beneficial to other researchers and to incorporate the enzyme into their CRISPR system for superior scientific findings [16]. As F. Zhang explained "We are committed to making the CRISPR-Cpf1 technology widely accessible. Our goal is to develop tools that can accelerate research and eventually lead to new therapeutic applications. We see much more to come, even beyond Cpf1 and Cas9, with other enzymes that may be repurposed for further genome editing advances [14, 17]."

Applications and Benefits

The CRISPR-Cas9 system is applied in various scientific fields, such as: medicine and biology, pharmacology and biotechnology engineering.

Medicine

An area where the CRISPR-Cas9 system has provided its advantages was that of genetic recessive disorders. To investigate the possibility of gene correction in adult stem cells using CRISPR-Cas9, the focus was on cystic fibrosis (CF), where adult intestinal stem cells were isolated and expanded in culture, then the mutant F508del allele was corrected using the CRISPR-Cas9 and the functionality of the targeted allele was demonstrated [18, 19].

Another application of this system is in sickle-cell anaemia and Duchene muscular dystrophy. Additionally, Cas9 can be used to correct the causative mutation in dominant negative disorders such as transthyretin-related hereditary amyloidosis or different forms of retinitis pigmentosa [20].

Huntington's is an autosomal dominant neurodegenerative disease caused by trinucleotide repetition (CAG) in the HTT gene after 40 CAG repeats, the majority of patients develop the signs and symptoms of the disease [21,22]. The CRISPR-Cas9 system could treat this illness by excluding the trinucleotide repetition from the patient's DNA. By bringing together the CRISPR-Cas9 system and induced pluripotent stem cell (IPSC) the editing process could be improved [24], and there are several strategies for this procedure [25]. Besides Huntington's disease, this technique could be performed in Rett syndrome [26] and schizophrenia [27].

An enormous milestone is about to be attained in cancer research by providing new methods to configure can-

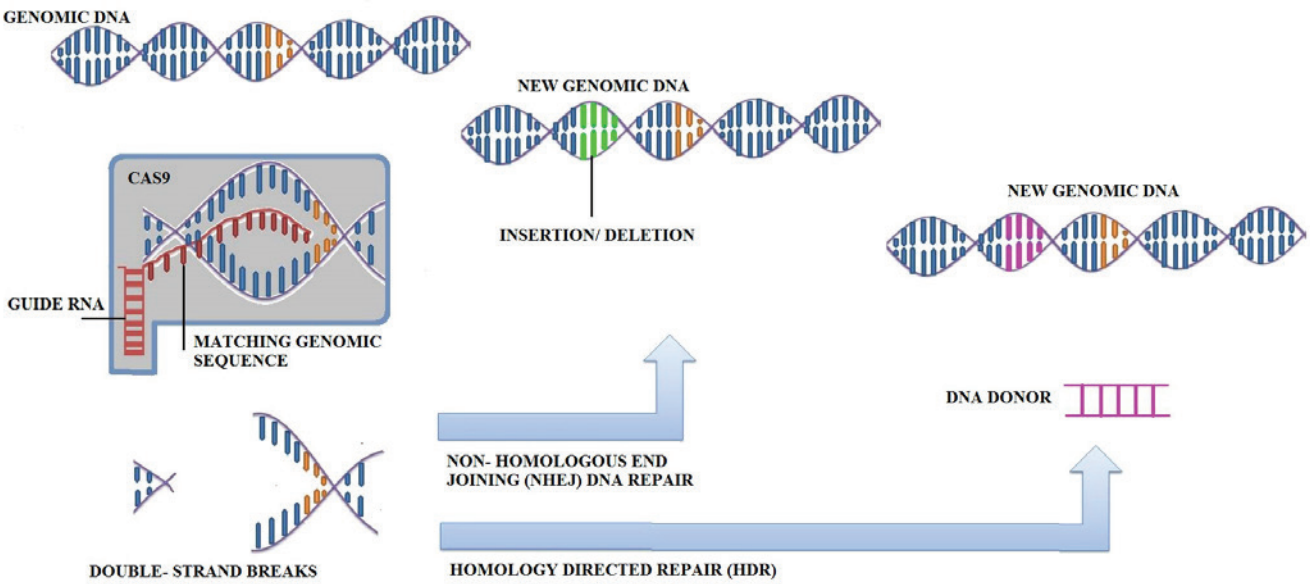

Fig. 1. The process of the CRISPR-Cas9 system. The g-RNA and Cas9 enzyme induce the bicatenar deletion of a targeted DNA fragment, followed by DNA repair methods which either restore the DNA sequence or fail to do so. As a consequence, the genome could become unstable due to chromosomal aberrations such as translocations or deletions if the double strand breaks (DSBs) remain unrepaired or misrepaired. Apoptosis could be induced, and so could senescence, which leads to the loss of heterozygozity [12]. The NHEJ and homology directed repair (HDR), DNA repair methods for DSBs are complex and we elaborated on them in a short description here. Other molecules which take part in the process are: Ku70, Ku80, DNA PK-activation, Ligase IV, XRRC4, XLF, MRN, ATM, Rad 51.

cer progression in vivo. The main focus is on editing and manipulating the genome of somatic cells in mammals by using basic, flexible and cost-effective elements [28]. Progressive genetic and epigenetic alterations will eventually develop cancer in a living organism and the CRISPR-Cas9 system has variable components with applications in treating and modelling this illness. Currently, there are trials focused on modelling oncogenic mutation in cell lines [29], adult animals [30] disabling oncogenic viruses [31] or manipulating the cancer genome [32].

The epigenome and transcriptome is an important cause of cancer genesis, and their consistent exploitation is beneficial in improving the therapy and manipulation of cancer [33].

Concerning prostate cancer cells, a flexible aptamer-liposome CRISPR-Cas9 chimera which integrated an RNA aptamer was used for specific attachment to the cells membrane, while the liposome distributed the CRISPR/Cas9 system that targeted the polo-like kinase 1 in the cells. The results observed in vitro showed a gene inhibition effect, and in vivo, tumor regression [34]. Moreover, the CRISPR- Cas9 editing tool revealed targets for Ibrutinib therapy intended to deactivate the pre B cell receptor signal in acute lymphoblastic leukemia, such as B lymphocyte kinase and Bruton's tyrosine kinase [35].

In addition, a previously unimaginable perspective for CRISPR-Cas9 have been reported in personalised medicine (Figure 2). Regarding lung cancer, biopsy samples were taken from patients' tumours and EGFR mutant genes were identified. With a virus-delivered CRISPR-Cas 9 editing tool the mutation could be detached or even destroyed meaning that in the future „molecular surgery" could be performed on DNA specifically targeting the disease. Nevertheless, a combination of therapies could be used for the patients' benefit, such as: radiation and chemotherapy, and even traditional surgery [36].

Biotechnology engineering and applications in Pharmacology

In the pharmaceutical industry, the CRISPR and Cre/Lox system played an important role when the development

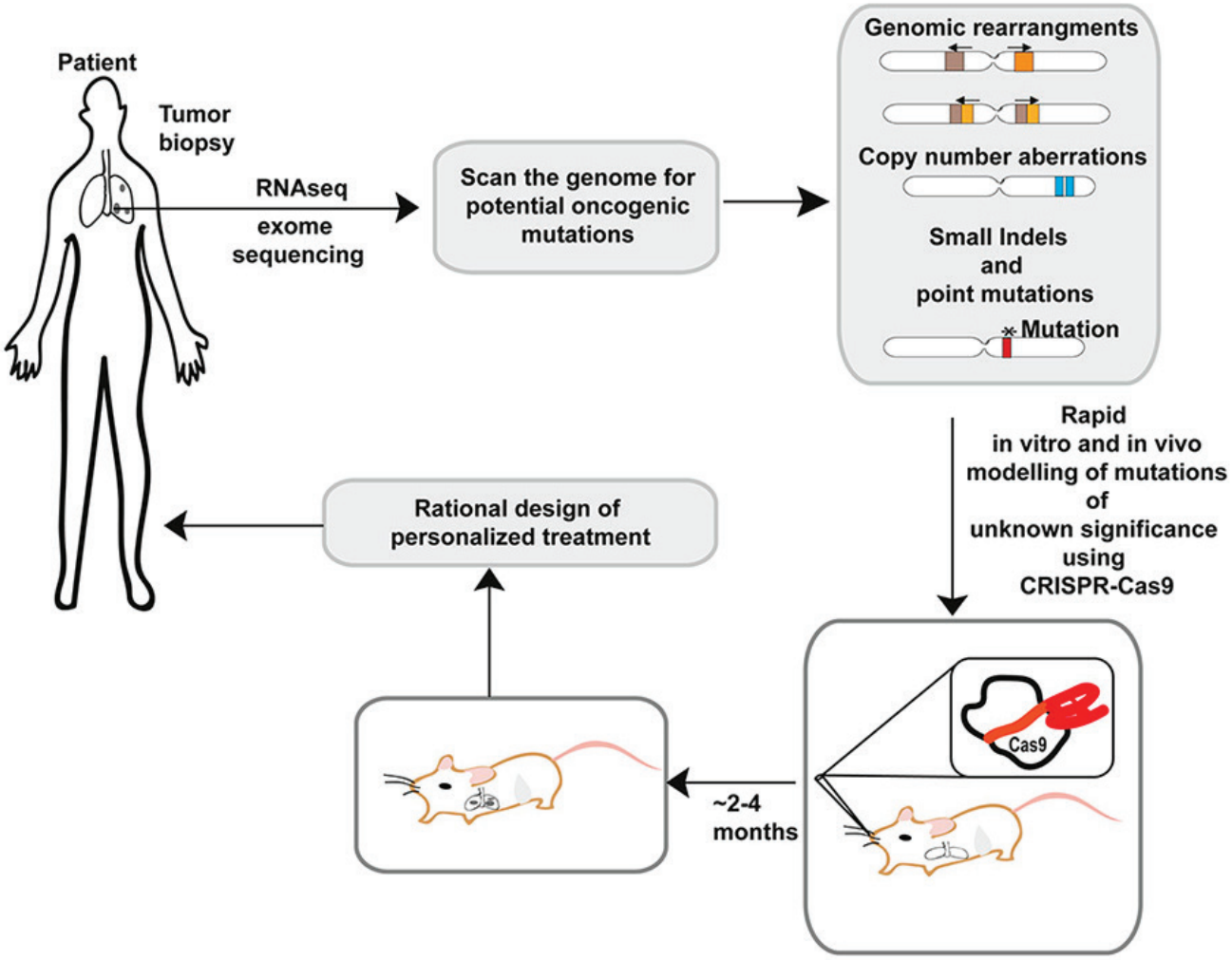

Fig. 2. Applications of CRISPR-Cas9 in personalized medicine (republished with the permissions of Andreea Ventura and Swiss Med Wkly editor-in-chief. Reference no. 28: https://smw.ch/index.php?id=75&tx_ezmjournal_articledetail[identifier]=smw.2015.14230). The importance of this procedure in personalised medicine is the fact that it allows the discovery of functional consequences given by DNA sequences or mutations with unknown pathogenesis and repercussions. The first step consists of the discovery of these mutations in the patient's biopsied tumour tissue. The second step is the creation of these mutations in vitro and in vivo and via the documentation of the functional repercussions (in this example, the case of a mouse). Such information can be used to design an optimised personalised treatment [28].

of a vaccine for the Pseudorabies virus took place with the help of gene editing technology [37]. This technology provided new insights when post-vaccine immunity was inefficient and the evolution of the virus needed to be stopped.

Companies have started to invest in this revolutionary technique. There may be a financial purpose for private companies, as they have invested in the majority of the projects involving the CRISPR system.

„Interest in the genes editing forces of CRISPR-Cas9 by pharmaceutical companies literally exploded in the last year", said Bill Lundberg [38].

Their aim was to engineer immune cells and blood stem cells, discover drugs, new targets in preclinical models for a variety of disease areas and to breed plants for agricultural uses.

Nowadays there are therapies for the suppression of HIV-1 replication, yet there are none for the deactivation of the virus in the latent reservoirs. Using the CRISPR-Cas9 system in combination with two antiviral gRNAs the blocking of viral replication could be attained [39]. Another disease to which this system brings therapeutic value is hepatitis B; knowing that the mini-chromosomal cccDNA generates a new viral antigen, the editing tool could be used to remove cccDNA which would lead to lower HBsAg levels [40].

There is a large number of scientific papers published revealing the successful application of the CRISPR-Cas9 technique, and we would like to mention some of these: the dicot and monocot plant genome [41], plant suspension cells with applications in pharmacology [42], bacteria, zebra fish, non-viable human embryos, mammalian cells, insect-resistant and herbicide-tolerant plant varieties, vaccine development and others. „In the short term, the development of new techniques or the improvement of existing ones will provide further instruments to counter the appearance of resistant weeds and insects and to reduce the use of agrochemicals" [43].

Challenges and Limitations

The delivery of g-RNA and the Cas9 protein has been a constant challenge, but the researchers used plasmids, viruses, and ribonucleoproteins (RNPs) as method for optimizing their gene-editing procedures, even though these three delivery methods had limitations [44,45].

One of the most significant limitations of the CRISPR-Cas9 system was the appearance of certain off-target effects, although these represented a small percentage compared to previous techniques of genetic engineering. Various strategies had been reported to reduce off-target effects [46, 47, 48]. It is very important to select an optimizing RNA guide, Cas9 enzyme and unique target sequences [23]. Moreover, CRISPR-Cpf1 has recently shown promises in diminishing these effects. Additionally, F Zhung maintains that "Cpf1 may overcome one of CRISPR-Cas9's few limitations. The system works well for disabling genes, it is often difficult to truly edit them by replacing one DNA sequence with another" [13].

A limitation and a constant provocation at the same time was the protospacer sequence in the g-RNA represented by the 18-20-nt spacer region, and which the fundamental reason for off-target and on-target effects in CRISPR experiments [45]. Twenty DNA bases pairs (bp) are the maximum target for the CRISPR-single RNA guide (sgRNA) [23]. Thus, no more than 20bp deletions and insertions can be done.

The precision of Cas9 was found to be enormously lower than in the theoretical approach [45]. As a result, new concepts have gone into development, such as: a more accurate Cas9 system, more effective deliveries and more powerful sgRNA [49, 50].

Other researchers found a large number of unexpected mutations, described as off-target effects, which means that the CRISPR-Cas9 system interfered with other regions. Furthermore, there were excessive and unwanted errors made on mice embryos or adult human cells compared to other studies, although we must mention that they only analysed the exons. If they would have had analysed the whole genome, the number of mutations would probably have increased [50].

The consequences were not described in the literature due to the fact that the subjects and their descendants were not monitored for a long period of time. Thus, the consequences were unknown mainly because only a few studies had the objective of following the off-target effects.

Even though this is a controversial subject, there have been studies which reported a minimal percentage of inducing tumours as adverse reactions [51,52,53].

Therapeutic applications have encountered challenges and limitations, since the CRISPR-Cas9 must be precisely defined or improved in the case of safe long term usage in human disease treatments [56]. Depending on DNA's complexity, CRISPR-Cas9 specificity decreases. Therefore, new challenges arise in adapting these techniques arise due to a low proportion of off-target effects seen in bacteria, zebra fish and mice compared to human cells.

This evolution in molecular genetics offers many promises, but it also brings an important ethical debate which was emphasized by use of the technique on human embryos.

The international bioethics committee of UNESCO considers that the CRISPR Cas9 system should only be used as a preventative, diagnostic and therapeutic procedure, without modifying the descendants' genome. Until now, this technique has held the possibility of treating certain diseases, such as: sickle cell anemia, cystic fibrosis and several types of cancer [57].

In March 2015, the researchers who worked to develop CRISPR acknowledged the unsafeness of this method and highlighted the topic of eugenics [49].

At the same time, using the CRISPR-Cas9 method, Chinese scientists have tried to edit the human genome by modifying the gene responsible for beta thalassemia (HBB). In order to avoid ethical concerns, they operated on nonviable embryos [50]. The technique involved the

injection of the CRISPR-Cas9 system into 86 embryos, of which 71 survived. Of the surviving embryos, 54 were tested, only 28 embryos dividing successfully, but not all of them had the replaced genetic information. Following this experiment, the scientists ended the trial and declared that the technology was imperfect, and in order to be used on viable embryos it should have a 100% success rate [50].

They also declared that there were no reasons for ethical concerns because the experiments were made on non-viable embryos.

In December 2013 European Union Council and Parliament adopted Horizon 2020 framework which allow and funding human embryo research if all it's clauses and procedures are accomplished, currently human gene editing is permitted in 18 Member States, whilst 3 prohibit it and the rest have no specific legislation.

Recently in Great Britain, researchers have received the approval to genetically edit viable human embryos, which are to be donated by patients taking part in in vitro fertilization procedures. The scientific study would end in 7 days, and after this time the embryos would be eradicated due to ethical concerns [55] because according to current legislation can't be used human embryos in the blastocyst stage.

Their opponents were worried about the consequences these interventions would have on the human genome, especially in future generations. Likewise, the risk encountered in clandestine exploitation and parents being tempted with a genetically modified embryos in order to gain physical and intellectual traits for their offspring after birth [54].

Conclusion

Without any doubt, the CRISPR system holds many perspectives in the near future, leading to new enzymes being discovered and used as editing tools. For instance, Cpf1 enzyme can be easily and precisely manipulated compared to Cas9 with the purpose of obtaining aimed results without off-target effects. Despite the greater benefits and potential of this technique there is the need for well defined rules and legislations for a consistent worldwide implementation.

Conflict of interest

None to declare.

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RESEARCH ARTICLE

Comparative Analysis of Hepcidin-25 and Inflammatory Markers in Patients with Chronic Kidney Disease with and without Anemia

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Introduction: Hepcidin is a regulatory protein in iron metabolism; we do not know the role in chronic kidney disease anemia. **Methods**: 22 patients with CKD anemia and 15 patients with CKD without anemia were investigated. CKD anemia-inclusion criteria: over 18 years, hemoglobin ≤12 g/dl for women and ≤13 g/dl for men, no treatment for anemia 6 months before enrollment, glomerular filtration rate (eGFR) <60 ml/min/1.73m^2 and stable creatinine three months before enrollment. Exclusion criteria: infection, bleeding, malignancy, systemic or liver disease, immunosuppression, renal replacement therapy. CKD without anemia-inclusion criteria: over 18 years, no anemia or treatment for anemia, CKD with stable creatinine values three months before enrollment. Exclusion criteria: medical conditions known to have a role in the development of polycythemia. Hepcidin-25 and ferritin were measured by ELISA method. Erythropoietin (EPO), tumor necrosis factor (TNF)-α, interleukin (IL)-6 were evaluated using chemiluminescent enzyme immunometric assays. Unpaired T test, Pearson correlation and multiple regression were used for statistical analysis. **Results**: Hemoglobin values were significantly lower in anemia group. There were no differences in terms of eGFR, age, body mass index, serum hepcidin, erythropoietin, fibrinogen, IL-6, and TNF-α between CKD patients with and without anemia. Serum hepcidin correlated positively with ferritin (r=0.45 p<0.05), TNF-α (r=0.54, p<0.05) and negatively with erythropoietin (r=-0.51, p<0.05). Multiple linear regression analysis demonstrated that TNF-α is an independent predictor of serum hepcidin in our patients (p=0.003, R=0.71). **Conclusion**: We found no differences in serum hepcidin, erythropoietin and inflammatory markers in non-dialysis CKD patients with and without anemia.

Keywords: hepcidin-25, TNF-α, IL-6, erythropoietin, anemia, non-dialysis chronic kidney disease

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Introduction

Anemia is a common problem in chronic kidney disease (CKD) patient. Previous studies demonstrated prevalence rates of anemia in CKD up to 47.7% and that glomerular filtration rate, age, gender, race, comorbidities are predictors of CKD anemia. [1,2,3]

Most authors postulated that the prevalence of anemia increases with decreasing glomerular filtration rate in both genders. [4,5]

CKD anemia is a multifactorial process. Erythropoietin deficiency, iron metabolism disorders, uremic toxins, shortened red cell survival, blood loss on hemodialysis sessions or due to repeated blood sampling, lack of essential nutrients like folic acid and vitamin B12, inflammation are involved in CKD anemia. [6,7,8]

Considering the large number of possible etiological conditions, treatment of anemia is sometimes a difficult task for the practitioner.

Recent discovery of hepcidin, considered to be the most important regulator of circulating iron absorption was followed by research to prove her role in the etiology of anemia in chronic kidney disease. [9,10]

Moreover, serum hepcidin concentrations are influenced by inflammation and anemia of chronic kidney disease is often explained by the marked inflammatory status of these patients. [11,12]

In this setting, knowledge of the interplay between hepcidin, serum erythropoietin, inflammatory markers and glomerular filtration rate is a goal for nephrologists. Understanding the relationship between parameters would help us both to understand which categories of patients frequently develop anemia but also to answer an old question about different response to therapy of patients with chronic kidney disease anemia.

The purpose of our study was to compare hepcidin and inflammation markers in CKD patients with and without anemia and to assess the relationship with glomerular filtration rate, serum erythropoietin and inflammatory markers as fibrinogen, hsCRP, IL-6 and TNF-α.

Methods

The study was approved by local ethical board and was conducted according to the Declaration of Helsinki. All

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patients signed an informed consent for plasma sample collection and for the participation in the research.

Study population

Incident chronic kidney disease (CKD) patients referred for medical evaluation in the Nephrology Department of Mures County Clinical Hospital between March 2015 - June 2016 with the diagnosis of chronic kidney disease.

We considered eligible for the study two types of patients

1. Anemia group. Inclusion criteria: over 18 years, hemoglobin ≤12 g/dl for women and ≤13 g/dl for men, no treatment for anemia at least 6 months before enrolment, estimated glomerular filtration rate (eGFR) < 60 ml/min/1.73 mp and stable creatinine values three months before enrolment, ferritin < 300 ng/ml. Exclusion criteria: infection, bleeding, malignancy, systemic or liver disease, immunosuppression, MCV higher than 100 fl, renal function replacement therapy.
2. No anemia group. Inclusion criteria: over 18 years, hemoglobin >12 g/dl for women and >13 g/dl for men, no treatment for anemia at least 6 months before enrolment, estimated glomerular filtration rate (eGFR) < 60 ml/min/1.73 mp and stable creatinine values three months before enrolment. Exclusion criteria: medical conditions known to have a role in the development of polycythemia: chronic obstructive pulmonary disease, sleep apnea, morbid obesity, cardiovascular diseases with right-to-left shunt, renal carcinomas, adrenal carcinomas, hepatomas, adrenal adenomas.

Laboratory data

Blood samples were collected after an overnight fast, between 8:00 and 9:00 in the morning. Hemoglobin, hematocrit, serum creatinine was performed during routine examinations performed in the clinic.

For hepcidin, ferritin, erythropoietin and inflammatory markers assay, serum samples were stored at -80°C. Sample analysis was performed in the same session by the same examiner. Hepcidin-25 was measured by a immunoenzymatically colorimetric method (DRG® Hepcidin 25 bioactive ELISA, DRG International, Inc., USA) according to manufacturer´s indication, with an analytical sensitivity equal to 0.135 ng/ml.

For erythropoietin (EPO), tumor necrosis factor (TNF)-α, interleukin (IL)-6 assay we used a chemiluminescent enzyme immunometric assay (on a Siemens, Germany, Immulite1000 analyzer). Analytical sensitivity was 2 pg/ml for IL-6 assay, 1,7 pg/ml for TNF-alpha, 0.24 mIU/ml for EPO.

Ferritin concentrations were measured with an immunoenzymatically colorimetric method (ELISA Novatec Immundiagnostica GmbH, Dietzenbach, Germany), with an analytical sensitivity of 0.04 ng/ml.

Estimated glomerular filtration rate (eGFR) was calculated using 4-variable Modification of Diet in Renal Disease equation.

Statistical analysis

Statistical analysis was realized using GraphPad Prism 6 Software. Data were presented as mean ± standard deviation.

Differences between groups were evaluated with unpaired Student's t test.

Pearson test was used to verify the existence of correlations between parameters for data with Gaussian distribution and Spearman test was used for variables with non-Gaussian distribution.

Agostino- Pearson normality test was used to verify if values come from a Gaussian distribution. For regression models variables with non- normal distribution were logarithmically transformed.

Results

22 patients with chronic kidney disease anemia (14 females and 8 males) and 15 patients with CKD without anemia (6 females and 9 males) were investigated in the study.

In anemia group, hemoglobin values were significantly lower compared with patients without anemia. Mean age (standard deviation) was 59.55 ± 3.091 for the group with anemia and 62.87 ± 3.205 for the non-anemia group, with no significant differences between age groups. (Table 1)

There were no differences in terms of the mean glomerular filtration rate and body mass index between the two groups (Table 1).

We found no differences in serum hepcidin, erythropoietin, fibrinogen, IL-6, and TNF-α between CKD patients with and without anemia. Serum iron, TSAT, ferritin and (mean corpuscular volume) MCV showed no differences between the two groups. (Table I)

Table I. Clinical and laboratory parameters in patients with CKD

Parameter	Anemia group	Non-anemia group	P
eRFG (ml/min/1.73 mp)	23.75 ± 2.789	27.82 ± 2.882	NS
Hepcidin 25 (ng/ml)	30.48 ± 5.959	27.52 ± 4.405	NS
Body mass index (kg/mp)	27.93 ± 0.9404	30.15 ± 1.105	NS
Fibrinogen (mg/dl)	370.2 ± 28.25	377.7 ± 29.80	NS
Erythropoietin (mIU/mL)	11.12 ± 1.444	10.29 ± 1.084	NS
Age (years)	59.55 ± 3.091	62.87 ± 3.205	NS
TSAT (%)	21.88 ± 1.656	20.65 ± 2.183	NS
TNF-α (pg/mL)	11.45 ± 1.087	11.36 ± 1.101	NS
Ferritin (ng/mL)	86.07 ± 14.32	51.98 ± 9.612	NS
Il-6 (pg/mL)	5.14 ± 1.16	13.87 ± 7.95	NS
Hemoglobin (g/dl)	10.41 ± 0.267	13.84 ± 0.298	p<0.0001
MCV (fl)	85.93 ± 1.606	86.21 ± 1.411	NS
hsCRP (mg/L)	5.980 ± 1.063	8.920 ± 3.235	NS
Serum iron (μg/dL)	59.18 ± 5.125	59.50 ± 5.578	NS

Correlation analyses for all patients showed that serum hepcidin correlated positively with ferritin (r=0.45 p<0.05) and TNF-α (r=0.54, p<0.05). Hepcidin correlated negatively with erythropoietin (r=-0.51, p<0.05). We found no correlation between serum hepcidin and hemoglobin, iron, hsCRP, Il-6, fibrinogen and eGFR in our patients.

Multiple linear regression analysis for the whole population studied, with hepcidin as dependent and ferritin, erythropoietin and TNF-α as independent variables demonstrated that TNF-α is an independent predictor of serum hepcidin in our patients (p=0.003, R=0.71).

Discussion

Chronic kidney disease is a serious medical condition that evolves with numerous complications that reduces lifespan significantly. As filtration rate decreases, the prevalence of complications like cardiovascular disease, hyperparathyroidism or anemia increases significantly. [3] These big issues and spread in the population makes chronic kidney disease to be extensively studied in order to decrease associated comorbidities.

One important aspect of the disease is anemia. It is known that its prevalence increases with decreased glomerular filtration rate, charging the health care systems with huge costs. [13]

Chronic kidney disease anemia is a multifactorial process. Uremic milieu, low folate and B12 vitamin, malnutrition and inflammation are important causes of anemia. To these we can add frequent blood sampling in hospital setting or loss of blood in the hemodialysis circuit. Low erythropoietin production by the diseased kidney and disorders of iron metabolism are recognized as the main causes of anemia in chronic disease. [14,15]

Hepcidin is a relatively recently discovered molecule. The main function of hepcidin is to regulates iron absorption by binding to ferroportin, the only element involved in cellular iron export. [16]

The main site of production is the liver; hepcidin expression depends on iron stores but also by endogenous and exogenous EPO, infection, inflammation and hypoxia. [17]

Nephrologists community consider that hepcidin is an important issue because understanding its role in anemia of chronic kidney disease could provide important details on therapy.

This study focuses on characterizing of serum hepcidin profile in CKD patients with and without anemia and also on characterizing the relationship between hepcidin, glomerular filtration rate, endogenous EPO and inflammatory markers as fibrinogen, hsCRP, IL-6 and TNF-α.

We studied patients in whom history, clinical and paraclinical examination, has ruled out active infections or obvious causes of inflammation (see inclusion and exclusion criteria). Also, there were no significant differences in terms of age and glomerular filtration rate between the two groups of patients, to eliminate the possible impact of this parameters on serum hepcidin values.

Some authors demonstrated a negative correlation between serum hepcidin and glomerular filtration rate in CKD patients. [18,10]

Although this claim is not accepted by all nephrologists, in CKD serum hepcidin values are raised compared to the general population; it is also demonstrated a significant increase in hepcidin in hemodialysis patients. [19-22]

Previous studies demonstrated that patients with chronic kidney disease have increased plasma hepcidin levels and that patients with absolute iron deficiency have lower levels of hepcidin, the latter being considered a marker that can differentiate the anemia of chronic iron deficiency anemia. [23,24]

In our study, hemoglobin levels were different (p<0.0001) but hepcidin values were not different in anemia compared with non-anemia patients.

One possible explanation is lack of significant differences in terms of serum iron. Also, iron deficiency has not been a feature of the two groups studied and this may have influenced our results. Hepcidin expression is dictated by the body iron load [25]; and at comparable iron concentrations serum hepcidin values can also be comparable.

In these conditions, why some patients experience low hemoglobin values and others do not?

The question is even more present given that we demonstrated in the two groups of patients amounts of erythropoietin, IL-6 and TNF-α who do not show significant differences.

CKD anemia is a condition is a condition thought to be caused primarily by a lack of erythropoietin. Conversely, we would expect that patients without anemia present elevated serum erythropoietin.

Fehr et al found negative correlation between EPO and hemoglobin for a creatinine clearance above 40 mL / min. The author did not show a significant correlation below this clearance (despite a high rate of anemia) suggesting a lower set point for erythropoietin regulation. [26]

In a recent analysis of determinants of EPO in CKD, Mercadel et al postulated that CKD is characterized by early relative EPO deficiency, but several factors besides hemoglobin may persistently stimulate EPO synthesis ensuring normal hemoglobin levels in patients with measured GFR above 30 ml/min/ 1.73 mp. [27]

For both our groups mean glomerular filtration was under 30 ml/min/ 1.73 mp but with the specification that we used estimated and not measured glomerular filtration rate.

In our study, hepcidin correlated positively with ferritin. This correlation is the only one supported by all authors and it seems logical because the main regulator of hepcidin is the body's iron reserves. [28,29]

The fact that chronic kidney disease is a proinflammatory status that worsens as declining glomerular filtration is now universally accepted. [30,31]

Hepcidin production is directly regulated by proinflammatory cytokines. Nemeth et al demonstrated that hepci-

din is induced by IL-6 in human hepatocyte culture, and this response is rapidly followed by hypoferremia. [32]

Even if hepcidin expression is up-regulated by IL-6, CKD clinical studies are not consistent in supporting the correlation between hepcidin and IL-6. [33,34]

The negative correlation between EPO levels and hepcidin was an expected result; increased erythropoiesis requires a low hepcidin concentration that will allow absorption of iron needed to restore erythrocyte. [35]

Similar evidence was obtained recently regarding anemia in acute and chronic inflammatory processes where hepcidin- independent effects of inflammation on the suppression of erythropoiesis were demonstrated. [36]

In our study on non-dialysis chronic kidney disease, multiple regression analysis showed that TNF-α was the main predictor of hepcidin levels.

Inflammatory cytokines have active roles in influencing hepcidin expression. It is known that IL-6 has an important role in stimulating hepcidin but little are known about the direct influence of TNF-α.

Studies in rheumatoid arthritis patients showed that administration of TNF-α inhibitors reduced serum hepcidin-25 but the mechanisms are largely unknown. [37]

Our study limits are: limited number of patients, estimation of the glomerular filtration rate, that patients were not divided depending on the stage of chronic kidney disease.

In conclusion, our study has demonstrated lack of significant differences in terms of hepcidin profile and inflammatory markers in patients with CKD anemia compared to patients with CKD without anemia.

Hepcidin-25 does not correlate with glomerular filtration in non-dialysis chronic kidney disease and TNF-α was the main predictor of hepcidin-25 levels.

We conclude that hepcidin, iron markers, erythropoietin and inflammatory markers cannot fully explain chronic kidney disease anemia.

Chronic kidney disease anemia might be a more complex matter than we previously thought and further studies are necessary for provide the whole mechanism and to understand the role of hepcidin in iron metabolism in patients with CKD.

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Conflict of interest

None to declare.

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RESEARCH ARTICLE

The Prevalence of Dysphotopsia in Patients with Recent Cataract Surgery

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Pseudophakic dysphotopsia are becoming increasingly important as unwanted side effect after cataract surgery. **Objective**: The purpose of this study is to compare the photic symptoms experienced by patients after cataract surgery. **Material and method**: This is a prospective study that included 105 eyes from 99 patients, which underwent uncomplicated phacoemulsification and IOL implantation, between June 2015 and June 2016, performed at Ophthalmology Clinic Tg Mureș. Patients without visually consequential ocular co-morbidity completed a questionnaire, designed to assess subjectively perceived visual functioning and identify symptoms of dysphotopsia. **Results**: From the total number of patient, hydrophobic lenses were implanted in 95 patients and 10 patients received hydrophilic lenses. Photic effects were reported in 18% of treated eyes. Although the percentage of dysphotopsia is higher in the hydrophobic lenses category, there was no significant statistical difference between the two categories. **Conclusion**: The incidence and significance should not be overlooked, thus visual acuity is not enough for evaluating postoperative visual function.

Keywords: dysphotopsia, glare, survey, cataract, intraocular lens

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Introduction

The major concerns, nowadays in cataract surgery, are enhancing vision and retina protection against light toxicity. [1-5]

With all the advances in the intraocular lens design, that have improved visual outcome, patient often complain of optical side effects known as dysphotopsia. [4-12]

Negative dysphotopsia represent the absence of vision in the temporal region, located at 20^0 from the center, whilst positive dysphotopsia, the presence of glare, halo, or streaks of light. [5,8,-16] Negative dysphotopsia is a more enigmatic symptom.

Glare is caused by the interaction between the stray light and refractive media leading to a reduced contrast sensibility. [12,17,18] Halos (discs of light) are usually perceived in bright light, or foggy weather. [9,16] Thus patient can experience an interference with their mundane activities.

These unpleasant ocular symptoms can appear as early as one week postoperative, and lest up to several months. [8,11,13,14]

The mechanism in which this phenomenon is produced is still a matter of much controversy; factors pertaining to lens design are currently debated.

On the one hand these symptoms can often be caused by the optic shapes and diameters of the intraocular lens, current studies shown higher incidence of dysphotopsia correlated with sharp-edge optical design. [5,12,14,17-19] Thus negative photopsia is produced by parallel rays of light that pass thru the lenses edge resulting into two divergent rays that project onto the retina, creating a temporal shadow.[8,10,13,14]

Another possible explanation of dysphotopsia is the interaction between the optical pathway of the eye and the intraocular lens, the mechanism is the reflection of light rays between the optic edge and the anterior capsulorhexis; irrespective of intraocular lens material, nevertheless increased anterior chamber is also know for inducing negative dysphotopsia [4,6,7,9,14]

The use of acrylic copolymers as lens material, can also impact the incidence of dysphotopsia.

Hydrophilic acrylic lenses have higher water content then hydrophobic ones, which lead to a higher incident of capsular opacification and a lower incident of dysphotopsia. The higher water content compared of hydrophilic lenses lead to a lower refractive index. A high refractive index is known to cause dysphotopsia.

Aspheric intraocular lenses improve vision by reducing the optical aberration, especially spherical aberration at the retina. These lenses generate a degree of negative spherical aberration thus compensating the positive spherical aberration induced by the cornea. [6,19-22]

Studies show that the blue light-filtering yellow chromophore in some hydrophilic lenses can reduce the effect of positive dysphotopsia, without affecting color vision. [16-19] The blue light filtering lenses can improve vision by enhancing contrast, where initially designed to protect the retina form phototoxicity. [16,18,21]

Majority of unpleasant symptoms are transient, thus the "watchful waiting" is the best solution in many cases. Although the majority of symptoms fade in time due to neuroadaptation, if symptoms persist, some measures should

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be taken: first of all eliminating any refractive error with spectacles, then proper diagnosis of dry eye, thus leading to treatment, the use of thick framed glasses may be of use, so is the intermittent use o pupillary dilatation. [3,5,8,13,14]

There is a percentage of 1-2% of patients who present with debilitating, prolonged symptoms, for whom additional surgery may be the solution. Treatment options cited by the current literature are: anterior laser capsulotomy, reverse optic capture, piggy-back intraocular round edge lens in sulcus (reducing the space behind the iris), or iris suture fixation of the capsular bag and lens complex, thus covering or eliminating the anterior capsule. In extreme cases, with debilitating symptoms, lens exchange is discussed. [3-5,8,12-14,18]

The purpose of our study is to determine the incidence of dysphotopsia compared to the type of intraocular lens and examine if there is a correlation between patient satisfaction and the presence of dysphotopsia,

Material and Method

This is a prospective, observational study that included 105 eyes from 99 patients that underwent uncomplicated phacoemulsification and IOL implantation, between June 2015 and June 2016, performed at Ophthalmology Clinic Tîrgu Mureş.

There is no objective test to diagnose the presence of dysphotopsia

Surgery

The surgery was performed by two surgeons using the same method, with similar visual outcome. Uneventful standard phacoemulsification was performed; all eyes received an acrylic single-piece intraocular lens in the capsular bag.

Participants

The patients were divided into categories, based on the IOL type implanted, hydrophilic lenses (Medicontur 640AB and 640ABY) and hydrophobic ones (AcrySof SN60WF and SA60AT). Due to the fact that aspheric lenses have a reduction in optical aberration compared to spherical ones, we only included in the questionnaire phase aspheric acrylic lenses.

The exclusion criteria applied were:

– preoperative condition: retinal or optic nerve damage (macular degeneration, glaucoma neuropathy), corneal or vitreous opacities, amblyopia, corneal astigmatism over 1,25 diopter, dry eye, traumatic cataract, other pathologies that can influence the comprehension of the questionnaire (Alzheimer's disease)
– intraoperative complications(capsular rupture, IOL out of the capsular bag),
– postoperative complications: corneal oedema, significant inflammation, tilted intraocular lens, best corrected vision under 0.1 – unexpected vision loss, cystoid macular oedema, refractive errors (>1,00 spherical diopter, or astigmatism). [4,7,19,23]

Questionnaire

This study was designed to evaluate the photic phenomena from the perspective of subjective multiple question questionnaire, participants were surveyed at the two week postoperative examination. The questionnaire used was a validated questionnaire described in previous studies. [17,23] A single investigator performed all the questionnaires

Our primary outcome measured was regarding patient satisfaction, followed by uncorrected/corrected visual acuity (measured using Sneller chart) and glass independence.

Subjects that complain about dysphotopsia were asked about the nature of these symptoms, and about the way these affect their life. (Table I.)

Results

The study contained a number of 95 hydrophobic acrylic lenses and 10 hydrophilic lenses.

86% of the respondents were satisfied with the postoperative vision.

The mean age of patients in the study is 70.33, without a significant difference between the two groups, 63% women and 36% men.

Dysphotopsias reported at the two week follow-up were present in 24.3% of cases, 52.63% of these patients reported the presence of halos and 26.31% complained of temporal shadow.

Patients were asked to rank the severity of their symptoms, as shown in Fig 1. Sever symptoms were not reported.

The mean for best corrected visual acuity, situated between 0.8-1.0 for both groups was 70.14 % (Fig 2.)

In the study 38.29% were within the range of +/- 0.50DSph refraction, and 28.08% within +/- 1,00DSph refraction.

The presence of dysphotopsia was correlated with the degree of postoperative satisfaction as shown in Fig 3.

Discussion

The photic phenomenon is a well known consequence of cataract surgery and pseudophakia. [3,4,8,10,11,13] The

Table I. Questionnaire

Postoperative satisfaction grading	spectacle independence	ease of performing mundan activities (grading)	presence/degree of dysphotopsia	willingness congener ocular surgery
1 dissatisfied	near vision yes/no	driving	0 absent	yes
2/3moderat (neutral)	distance vision yes/no	watching TV/phone/computer	1 minimal (barely notice)	no
4 satisfied		reading book/drug prospectus	2-4 annoying	
5 highly satisfied			5 debilitating	

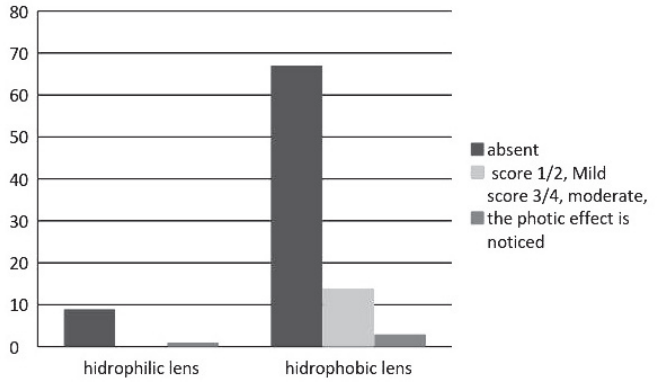

Fig. 1. Grading scale of dysphotopsia

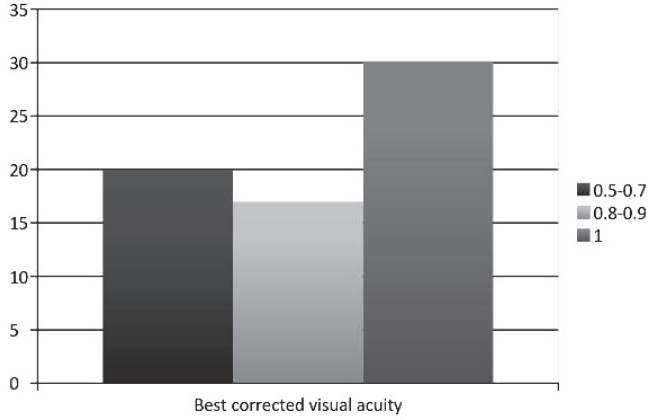

Fig. 2. Postoperative best corrected vision

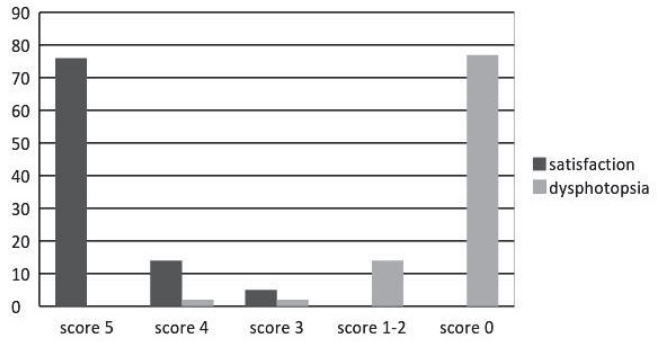

Fig. 3. Patient satisfaction and dysphotopsia presence correlation

simplest way to describe dysphotopsia is that it represent an unwanted patterns on the retina.[5]

The lens visual side effects are reported in the current literature as being between 2 and 18%, with a slight increase for multifocal lenses, were as known, the patients have higher demand. [8,11,13,14,22] The incidence of dysphotopsias in our study is 24.03%, we think that this may be due to our sample size and the phrasing of our question; some questing may have implied an certain answer.

Due to the prevalence of posterior capsule opacification, the study contained more hydrophobic square edge lenses then hydrophilic ones, thus the possible explanation for the high rate of dysphotopsia.

High demand in term of visual outcome, and postoperative satisfaction, make the need of additional chair time. [8,18,22] Thus the postoperative visual acuity is not enough for evaluating the visual function. [5]

Finding these complaints is quite surprising, given that often during the postoperative ocular exam the surgeon finds a good visual acuity. [9,11]

Photic phenomena are produced by two major factor lens geometry and ocular physiology.[5,7,9-14,22]

Theoretically all the lenses are subjected to some degree of dysphotopsia, as long as they are placed in the capsular bag, nevertheless it is known that hydrophilic lenses are the most commonly used, due to the low incidence of dysphotopsia, although the lens material makes it prone for the development posterior capsular opacification. [4,7,20-22]

When conducting a subjective questionnaire, minor changes in the way questions are phrased can change the outcome. We observed when asking broad questions the patients tend not to mention dysphotopsia symptoms, when the questions were explained the number of patients with visual complaints drastically changed. As a result we preferred the direct inquiry. This can also explain the high number of patients with dysphotopsia compared with the high degree of satisfaction.

While we did not find a correlation between the type of intraocular lens implanted, nor the presence of sharp-edge lens, and the presence and severity of dysphopsia, it is currently known that there is a link, thus additional studies should be conducted.

Due to the careful selection of the patients, one of the weak points of our study is the lack of reaching the statistical significant, we think that this can be solved by enlarging the sample size, and including lenses that are not exact replicas of one another.

What we found interesting is that we did not determine a correlation between the objectified outcome of the patient (presence of dysphotopsia) and patient dissatisfaction. One answer can be that the vision before the surgery was very low, thus making any increase in vision a definite increase in patient satisfaction.

Although many patients experienced some degree of dysphotopsia, only a few reported them as being bothersome, or even mentioned them before the deliberate questioning, thus leading to the conclusion that additional time for postoperative consultation is needed to fully understand the extent of these symptoms. Case by case treatment is required. [9]

After extensive literature review what we found is that with all the studies conducted in this department, there isn't a way to predict who will experience dysphotopsia. [10]

In order to minimize the prevalence of dysphotopsia surgeons should opt for unfinished or frosted lens edge, or materials with low index of reflection.[6,9,12]

In the current state, the surgeon has a wide range of IOL to chose from, as regarding the materials used, refractive and additional profiles, nevertheless, we would like to point out that due to the enigmatic mechanism, patient related factors play an equally important role (idiosyncratic predisposition, prominent globe, shallow orbit) [5,22]

Conclusion

The optical side effects gradually gained ground, being the major postoperative concern.

When choosing the right intraocular lens for the patient it is important to inquire about his/hers daily activities, which can be an indicator about the tolerance of possible dysphotopsia.

Treatment should be adequately timed because the symptoms usually fade in time due to capsular fibrosis, cortical adaptation, or the patient final compromise.

Photic phenomena are produced by two major factor lens geometry and ocular physiology.

Placing the lens haptics horizontally appears to reduce the presence of negative dysphotopsia.

Although hydrophilic lenses have a lower incidence of dysphotopsia than hydrophobic ones, when choosing the right intraocular lens it should be known that hydrophilic lenses have a higher incidence of posterior capsule opacification.

Conflict of interest

None to declare.

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RESEARCH ARTICLE

Characteristics of Sleep Apnea Assessed Before Discharge in Patients Hospitalized with Acute Heart Failure

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Objectives. Evaluation of the characteristics of sleep apnea (SA) in patients hospitalized with acute heart failure, considering that undiagnosed SA could contribute to early rehospitalization. **Methods**. 56 consecutive patients (13 women, 43 men, mean age 63.12 years) with acute heart failure, in stable condition, underwent nocturnal polygraphy before hospital discharge. The type and severity of SA was determined. Besides descriptive statistics, correlations between the severity of SA and clinical and paraclinical characteristics were also analyzed (t-test, chi-square test, significancy at alpha < 0.05). **Results**. 12 (21.4%) subjects were free of SA (AHI - apnea-hipopnea index <5/h), 15 (26.7%) had mild SA (AHI=5-14/h), 17 (30.3%) had moderate SA (AHI 15-30/h), and 12 (21.4 %) had severe SA (AHI>30/h). The apnea was predominantly obstructive (32 cases vs. 12 with central SA). Comparing the patients with mild or no SA with those with severe SA, we did not find statistically significant correlations (p>0.05) between the severity of SA and the majority of main clinical and paraclinical characteristics – age, sex, BMI, cardiac substrates of heart failure, comorbidities. Paradoxically, arterial hypertension (p=0.028) and atrial fibrillation (p=0.041) were significantly more prevalent in the group with mild or no SA. **Conclusions**. Before discharge, in the majority of patients hospitalized with acute heart failure moderate and severe SA is present, and is not related to the majority of patient related factors. Finding of significant SA in this setting is important, because its therapy could play an important role in preventing readmissions and improving prognosis.

Keywords: acute heart failure, obstructive sleep apnea, central sleep apnea, prognosis

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Introduction

The relationship between sleep disordered breathing (SDB), represented by obstructive and central sleep apnea (SA), and cardiovascular diseases has been demonstrated in numerous studies. SDB confers an increased risk for cardiovascular complications and events, playing role in the pathophysiology of resistant hypertension, arrhythmias (from those benign to sudden cardiac death), myocardial ischemia, pulmonary hypertension and heart failure [1-4].

The prevalence of symptomatic obstructive SA in the general population is about 4% in middle-aged men, and 2% in middle-aged women. SDB is common in heart failure (HF), being present in nearly one half of the patients, impairing both the quality of life and prognosis. The specific manifestation is central SA, which is directly related to the level of congestion and functional status [5-7].

Acute HF is a common cause of hospitalizations in the course of HF, moreover, readmissions represent the most predictive factor for poor prognosis and increased mortality. Rehospitalizations within 30 days post-discharge are high, almost one third of patients being involved. The precipitating factors for these admissions are multiple, including cardiac and noncardiac conditions, like myocardial ischemia, arrhythmias, infections, treatment non-compliance, improper medications, etc., and, also, undiagnosed and/or untreated SDB [8-10] .

Thus, identifying potential factors related to early readmissions is mandatory. In line with this, the aim of our study was to evaluate the characteristics of SDB in patients hospitalized with acute HF, when already compensated, before hospital discharge. Also, the feasibility of this approach was tested: the yield of routine nocturnal poligraphy for identification of those patients who could benefit from specific treatment of SDB, as a possible tool for preventing rehospitalizations.

Methods

Patient population

We enrolled in the study 56 consecutive patients (13 women, 43 men, mean age 63.12 years) hospitalized with acute HF in the Department of Cardiology of Clinical County Hospital Mureș, between 01.01.2015- 01.06.2016. At the admission, all the patients signed the general consent form used in our institution, agreeing with anonymous data collection and usage for scientific purposes. Approval of local ethical committee (3865/01.03.2016) was obtained for confidential data processing and publication.

Patients who had a severe clinical/hemodynamical instability during the admission, those with marked insomnia and those already on treatment with continuous positive airway pressure (CPAP) therapy for obstructive SA were excluded from data collection.

During the hospital stay, all the patients received an usual care, involving common diagnostic (clinical follow-

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up, ECG, chest X-ray, echocardiography, laboratory tests) and therapeutic (oxigen, oral and parenteral medication) procedures. Clinical and paraclinical data were recorded in a custom designed database for further statistical analysis.

In-hospital sleep-study

When in stable clinical condition, as a rule, during the night before discharge, all the patients underwent nocturnal polygraphy (Stardust II®, Phillips Respironics) for assessing the presence and severity of SDB. The device was applied at bedtime by a member of the medical staff, while a trained night shift nurse controlled intermittantly the course of registration during nighttime.

Poligraphy involved continuous registration of the following six parameters: nasal airflow, arterial oxygen saturation, heart rate, thoracic movements, body position and snoring. The recordings were edited and corrected manually. The episodes of apnea and hypopnea were defined according to the criteria of American Academy of Sleep Medicine [11, 12]. The overall SDB of the patient was labelled as central or obstructive depending on the predominance (more than 50 % of all episodes) of the central or obstructive events. The level of severity was expressed using the apnea-hipopnea index (AHI) – number of events/hour. Central events also included Cheyne-Stokes respiration, a specific pattern of SA in HF, which was diagnosed when three consecutive cycles of respiration had a cresscendo-descrescendo pattern in association with central hipopnea or apnea.

Statistics

We applied descriptive statistics for the characterization of the prevalence, type and severity of SA, while the correlations between SDB severity and diverse clinical and paraclinical parameters were analysed using t-test and chi-square test - statistical significance set at alpha < 0.05 (GraphPad InStat 3.0 software).

Results

The occurrence, severity and type of SA found in our patients are presented in Table I. SDB was present on nocturnal poligraphy performed before hospital discharge at the majority of patients. More than a half of the patients presented moderate or severe forms of SA, the obstructive form being more prevalent (about 2/3 of cases).

To study the correlations between the severity of SA and diverse clinical and paraclinical characteristics of the patients, we compared the parameters of two groups – (1) patients with AHI <15/h (mild or no SA), and (2) patients with AHI >30/h (severe SA). The data and results of comparisons are presented in Table II, III and IV.

The two groups were comparable regarding the general characteristics, like age, sex and body mass index (BMI). Also, we did not find statistically significant differences regarding the majority of cardiac substrates, non-cardiac comorbidities and echocardiographic parameters. Paradoxically, two characteristics, the presence of arterial hypertension and atrial fibrillation, proved to be significantly more prevalent in patients with AHI<15/hour. Also, a tendency ($p<0.1$) was observed for older age and higher values of pulmonary artery pressure in the AHI>30/hour group.

Table I The prevalence, severity and type of sleep apnea in the patient population

	AHI <5 nr. of patients (%)	AHI = 5-14 nr. of patients (%)	AHI = 15-30 nr. of patients (%)	AHI>30 nr. of patients (%)
Apnea (total)	12 (21.4%)	15 (26.7%)	17 (30.3%)	12 (21.4%)
Central	-	4 (26.6%)	4 (23.5%)	4 (33.3%)
Obstructiv	-	11 (73.3%)	13 (76.4%)	8 (66.6%)

Table II Comparison of general parameters in the two groups

Parameter	AHI>30 (12 patients)	AHI<15 (27 patients)	p
Age	*60.67 ±2.0	63.22 ±1.9	0.442
Age>65 years	**3 (25%)	15 (18.5%)	0.095
Body mass index	27.87 ± 1.2	28.27 ± 1.2	0.849
Body mass index >30 kg/m2	4 (33.3%)	8 (29.6%)	1.00
Sex (nr. women/men, %)	1/ 11 (8.3%/91.7%)	7/20 (25.9%/74.1%)	0.393

*for all values: mean ± standard deviation, ** for all values: nr. of patients (%)

Table II Comparison of general parameters in the two groups

Parameter	AHI>30 (12 patients)	AHI<15 (27 patients)	p
Age	*60.67 ±2.0	63.22 ±1.9	0.442
Age>65 years	**3 (25%)	15 (18.5%)	0.095
Body mass index	27.87 ± 1.2	28.27 ± 1.2	0.849
Body mass index >30 kg/m2	4 (33.3%)	8 (29.6%)	1.00
Sex (nr. women/men, %)	1/ 11 (8.3%/91.7%)	7/20 (25.9%/74.1%)	0.393

*for all values: mean ± standard deviation, ** for all values: nr. of patients (%)

Table III Comparison of clinical characteristics in the two groups

Charactersitic (cardiac substrates, comorbidities)	AHI>30 (12 patients)	AHI<15 (27 patients)	p
Dilated cardiomyopathy	*5 (41.6%)	14 (51.8%)	0.731
Hypertension	1 (8.3%)	13 (48.1%)	**0.028
Ischemic heart disease	2 (16.6%)	6 (22.2%)	1.00
Atrial fibrillation	0	8 (29.6%)	**0.041
Significant mitral valve regurgitation	6 (50%)	13 (48.1%)	1.00
Severe aortic regurgitation	2 (16.6%)	7 (25.9%)	0.692
Severe aortic stenosis	3 (25%)	3 (11.1%)	0.348
Severe tricuspid regurgitation	4 (33.3%)	11 (40.7%)	0.734
Severe pulmonary hypertension (>60 mmHg)	7 (58.3%)	7 (25.9%)	0.074
Chronic obstructive pulmonary disease	2 (16.6%)	1 (3.7%)	0.219
Diabetes	2 (16.6%)	6 (22.2%)	1.00
Renal dysfunction during admission (creatinine>1,5 mg%)	4 (33.3%)	8 (29.6%)	1.00

*for all values: nr. of patients (%), **statistically significant

Table IV Comparison of echocardiographic parameters in the two groups

Parameter	AHI>30 (12 patients)	AHI<15 (27 patients)	P
Left ventricular (LV) end-diastolic diameter (mm)	*61.17 ± 2.7	58.00 ± 1.65	0.316
Left ventricle (diastole) >60 mm	**7 (58.3%)	12 (44.4%)	0.487
Left atrial PA diameter (mm)	48.17 ± 1.5	47.83 ± 1.31	0.874
Left atrial PA diameter > 50 mm	6 (50%)	9 (33.3%)	0.4
LV ejection fraction (%)	41.46 ± 4.33	42.79 ± 3.26	0.815
LV ejection fraction <40 %	6 (50%)	14 (51.8%)	1.00
Pulmonary artery systolic pressure (mmHg)	69.09 ± 5.55	55.63 ± 3.95	0.0672

*for all values: mean ± standard deviation, ** for all values: nr. of patients (%)

Discussion

The pathophysiology of harmful effects of SA in HF is complex and involves haemodynamic, mechanical, humoral, neural, metabolic and inflammatory factors. Obstructive SA induces marked intrathoracic pressure changes, which lead to a higher left ventricular transmural pressure-gradient. SA (both central and obstructive) is characterized by repetitive episodes of hypoxemia/hypercapnia (followed by reoxygenation), which induce oxidative stress and hypoxic pulmonary vasoconstriction. The increase of left ventricular afterload combined with hypoxemia, together with an increase in heart rate and blood pressure (both consequences of sympathetic nervous system activation during arousals) could lead to myocardial ischemia and/or arrhythmias and could trigger acute cardiac decompensations too. The high level of inflammatory mediators (e.g., C-reactive protein) and the oxidative stress determines endothelial dysfunction and metabolic disturbances, which have an important role in atherogenesis [1, 3, 10].

Several studies demonstrated that up to 40-50% of patients with HF (both with preserved and reduced ejection fraction) suffer from clinically relevant SA, with AHI greater than 15/hour [6, 10, 13]. This fact was confirmed also by our study, we observed a prevalence of moderate/severe SA in about 50% of the patients, the obstructive form being more frequent.

Many observational studies demonstrated, that the occurrence and severity of SA in HF is correlated with several general and specific factors - e.g., age, gender, body mass index, level of congestion, NYHA class, left ventricular ejection fraction, cardiac and non-cardiac comorbidities [1-3, 10]. Comparing the group of patients with no or mild SA with that with severe SA – with two exceptions - we did not find significant differences between the two groups in this regard. The increased prevalence of hypertension and atrial fibrillation in the mild/no SA group could be explained by chance, by the relatively small number of cases. On the other hand, the tendency of increased pulmonary artery pressure in the severe SA group is in line with the literature [1-3].

The feasibility of our approach is supported by the studies of Khayat et al., which demonstrate the value of in-hospital SA testing in the setting of acute heart failure. These data support, that both central and obstructive SA diagnosed during hospital stay are independently related to postdischarge mortality (multivariable hazard ratio 1.61 and 1,53) and hospital readmissions (adjusted rate ratio 1,53 and 1,49) [14-16].

Finding a marked SA at hospital discharge could have multiple significances: (1) persisting congestion (including subclinical) as a contributing factor, (2) poor prognosis regarding mortality and readmissions, and (3) the need to start specific therapy for improving symptoms, quality of life and prognosis. Regarding the latter aspect, presently there exist supporting data only for treatment with CPAP in HF patients with significant obstructive SA [10, 17]. Suppressing central SA by adaptive servo-ventilation is still debated after the worrying results of SERVE-HF trial – increased mortality in the patients on nocturnal non-invasive ventilation [10, 18].

Conclusion

Our data supports the value of predischarge testing for SA in patients hospitalized for acute heart failure. Initiation of specific therapy in the case of finding moderate or severe forms of SA (obstructive) can be an important part of management, having a definite role in preventing readmissions and improving prognosis.

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Conflict of interest

None to declare.

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RESEARCH ARTICLE

Kinetics and Mechanism of Drug Release from Loratadine Orodispersible Tablets Developed without Lactose

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Objective: The aim of this study is to develop lactose-free orodispersible tablets with loratadine for patients with lactose intolerance. **Materials and methods:** Seven compositions (F1-F7) of 10 mg loratadine were prepared in form of orally disintegrating tablets, by direct compression, using croscarmellose sodium and pre-gelatinized starch in various concentrations as superdisintegrants, diluted with microcrystalline cellulose and combined with mannitol and maltodextrin as binder agents. The tablets had been studied in terms of their pharmacotechnical characteristics, by determining: the weight uniformity of the tablets, their friability, breaking strength and disintegration time, drug content and the dissolution profile of loratadine. The statistical analyses were performed with GraphPad Prism Software Inc. As dependent variables, both the hardness of the tablets and their disintegration ability differ between batches due to their compositional differences (as independent variables). DDSolver were used for modeling the kinetic of the dissolution processes by fitting the dissolution profiles with time-dependent equations (Zero-order, First-order, Higuchi, Korsmeyer-Peppas, Peppas-Sahlin). **Results:** All proposed formulas shows rapid disintegration, in less than 15 seconds, and the dissolution loratadine spans a period of about 10 minutes. Akaike index as well as R2 adjusted parameter have demonstrated that the studied dissolution profiles are the best fitted by Zero-order kinetic. **Conclusion:** In conclusion, association of croscarmellose sodium (7.5%) with pre-gelatinized starch (6%) as superdisintegrants and mannitol as the binder agent (35%), positively influences the dissolution properties of loratadine from orally fast dispersible tablets.

Keywords: loratadine, orodispersible, dissolution kinetic, lactose-free, superdisintegrant

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Introduction

Orodispersible tablets are characterized by enhanced dissolution and release of the active substance compared to conventional pharmaceutical forms. Considering their easy administration, this pharmaceutical form is preferred for children *[1,2,3]*, elder *[4]* and psychiatry ill *[5]*. The orally disintegration is attributed to the rapid water penetration into the tablets matrix, which creates a porous structure, and results in a fast disintegration. Considering this, the formulation and development of orodispersible tablets includes the maximization of porous structure of the matrix by incorporating a proper disintegrant and by using highly water soluble excipients *[6]*.

In order to formulate antihistaminic (loratadine) containing tablets for patients with lactose intolerance the following excipients were proposed: Manitol SD200, maltodextrin and microcrystalline cellulose as diluents, pre-gelatinized starch and croscarmellose sodium as superdisintegrants. In this study, the influence of formulation factors on the pharmacotechnical properties of tablets and the kinetic of the dissolution process were evaluated.

Methods

Materials

Loratadine, Mannitol SD 200, Maltodextrin (*kindly* supplied by Arena Group SA, Romania); Microcrystalline Cellulose NF 101 – Unitab®, Pre-gelatinized Starch – Lycatab®, Croscarmellose Sodium – Vivasol® (JRS Pharma GmbH & Co. KG, Germany); Silicon Dioxide – Aerosil® 200 (Evonik-*Degussa* GmbH, Germany); Magnesium Stearate, Aspartame, Cloves Aroma (*kindly* supplied by Gedeon Richter Romania SA); Reagents (analysis grade purity).

Apparatus and equipment

Eccentric Tablet Machine, punches of 7 mm diameters (Æ); Electronic Balance, precision of 0.01/0.1 mg (Kern&Sohn GmbH , Germany); Friability tester (Erweka, Germany); Tablet Hardness Tester (Erweka, Germany); Spectrometer Spektromom 195 D (MOM, Hungary); Dissolution tester type 1 (Erweka, Germany), rotating basket (#60 *mesh*).

-Preparation of Loratadine- orodispersible tablets: The ingredients were weighed, mixed for 10 minutes, sifted and mixed again in mortar with the help of pastel, without rubbing. The tablet machine was adjusted to achieve tablets of 200 mg weight. The homogenously mix of the ingredient was then directly compressed using a pair of punches of 7 mm diameters. The prepared tablets were kept in tightly closed containers, protected from light and sudden movements, at room temperature (15-25 °C).

-Mass uniformity test: 20 tablets had been accurately weighted and the average mass of a tablet was calculated (M). Same tablets were then individually accurately weighted (M_i). The M_i-M difference was expressed as percentage of M [7].

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-*Tablet friability:* 20 tablets of knowing mass (M_{in}) were stirred in the friabilator drum for 4 min (corresponding to 100 rpm), de-dusted and then accurately weighted again (M_{fn}). The difference M_{in}-M_{fn} was expressed as *percentage of M_{in} [7]*.

-*Tablet hardness:* 20 tablets were individually crashed in the harness tester, determining for each of them the diametrically pressing force, in newton (N), which crushed the tablet. The tablets hardness was then expressed as the calculated mean force *[7]*.

-*In vitro disintegration test:* 20 tablets were individually kept (not more than 3 min) in 50 mL distilled water, unstirred and maintained at 37 °C, without stirring. There has been recorded the time (in seconds/ minutes) passed till the tablet was turned into soft mater. The disintegration time was then expressed as the calculated mean time *[7]*.

-*Uniformity of loratadine content test*: 20 tablets were weighed and powdered in mortar rubbing vigorously with the pestle. The powder equivalent to 10 mg of loratadine (»200 mg) was weighed, then extracted by vigorously stirring for 10 minutes in 50 mL of hydrochloric acid 1N and diluted to 100 mL with the same solvent, in volumetric flask. After discarding the first filtered portion, 10.0 mL of filtrate was diluted with hydrochloric acid 0.1N to 25 mL, in a volumetric flask (sample). The absorbance of sample was then determined by spectrophotometry at 280 nm, using the hydrochloric acid 1N as blank. *The calibration curve* was prepared in same conditions, using 10 mg of loratadine (reference substance) dissolved in 1-2 mL of ethanol and diluted to 100 of hydrochloric acid 1N, in volumetric flask (stock solution: 0.1mg loratadine/1mL). The series of etalon solutions was prepared by diluting the stock solution as it follows: 5 mL, 7.5 mL, 10 mL, 15 mL, 20 mL and 25 mL of stock solution were diluted to 25 mL, by adding hydrochloric acid 0.1N, in volumetric flasks. The absorbance of each etalon solution was determined at 280 nm, using the 1N hydrochloric acid as blank *[7,8]*.

-*In vitro dissolution study:* The release rate of loratadine from tablets formulated for orally fast disintegration was determined by the United States Pharmacopeia (USP) method, using a tester similar to apparatus 1 (the rotating basket method) *[8]*. The test was performed on 6 tablets collected from each batches. Each tablet was individually introduced in a rotating basket (#60 *mesh*) and then immersed for 10 minutes in 900 ml hydrochloric acid 1N (media), at 37±0.5 °C and 50 rpm. The sample (5 ml) was withdrawn from the dissolution apparatus at every minute, each time replacing the subtracted solution with fresh dissolution media. The collected samples were filtered through filter paper and then the absorbance of the clear solution was measured by spectrophotometry (at 280 nm), using the hydrochloric acid 1N as blank. *The calibration curve* was prepared in same conditions as it is described above. Loratadine determined as released in media was expressed as percentage of 10 mg (the initial dose of Loratadine in tablet) and then plotted against time to determine the dissolution (release) profile.

Statistical analysis

GraphPad Prism software (GraphPad Software, Inc., v.5 trial demo version [9]) were used for performing: Anova test, Bonferroni multiple comparison, linearization of the curves by regression, calculation of and the descriptive statistical indicators. DDSolver -an Excel Ad in Program [10]- were used for modeling the kinetic of the dissolution processes by fitting the dissolution profiles with time-dependent equations (Zero-order, First-order, Higuchi, Korsmeyer-Peppas, Peppas-Sahlin) and for calculating the goodness of fit parameters (R^2 adjusted coefficient, Akaike index).

Results

Seven compositions (F1-F7) of 10 mg loratadine were prepared in form of orally disintegrating tablets, by direct compression, using croscarmellose sodium and pre-gelatinized starch in various concentrations as superdisintegrants diluted with microcrystalline cellulose and combined with mannitol and maltodextrin as binder agents, as it is shown in Table I. All tablets were in form of small white discs (Æ 7 mm), with flat faces and intact edges. After the organoleptic examination, the tablets had been studied in terms of their pharmacotechnical characteristics, by determining: the weight uniformity of the tablets, their friability, breaking strength and disintegration time, and the dissolution profile of loratadine previously assessed as tablet active dose, respectively.

Table I. Composition of the orodispersible tablets

Ingredient (agent type)	Formula / Composition (%)						
	F1	F2	F3	F4	F5	F6	F7
Loratadine (API)	5	5	5	5	5	5	5
Mannitol SD 200 (binder agent)	34.38	34.38	34.38	-	-	20.5	20.5
Maltodextrin (binder agent)	-	-	-	32.5	32.5	12	12
Microcrystalline Cellulose NF 101 (diluent)	46.87	44.37	41.87	48.45	52.45	48.45	52.45
Pre-gelatinized Starch – Lycatab® (superdisintegrant)	6	6	6	4	-	4	-
Croscarmellose Sodium - Vivasol® (superdisintegrant)	5	7.5	10	7	7	7	7
Aerosil® 200 (glidant)	1	1	1	1	1	1	1
Magnesium Stearate (lubricant)	0.25	0.25	0.25	0.25	0.25	0.25	0.25
Aspartame (sweetener)	1.5	1.5	1.5	1.5	1.5	1.5	1.5
Clove Aroma	-	-	-	0.3	0.3	0.3	0.3
Weight = 200 mg ± SD (n=20):	± 0.1%	± 1.0%	± 0.2%	± 0.1%	± 0.8%	± 0.2%	± 0.1%
Loratadine = 10 mg ± SD (n=20):	± 0.7%	± 1.4%	± 2.3%	± 1.0%	± 2.1%	± 0.5%	± 2.7%

Compared to the average mass (experimentally determined) and to the declared active content (established by formulation), the individual weight of tablets had the percentage deviations less than ±1% and the assessed active content less than ±3%, ±7.5% being the maximum allowed in both of the cases [7]. In terms of friability, two of the formulations (F4 and F6) do not meet the imposed requirements [7], their friability being greater than 1% (Figure 1).

As dependent variables, both the hardness of the tablets (expressed by the crushing force) and their disintegration ability (expressed by the period of time in which the tablet is transformed in very fine particles) differ between batches due to their compositional differences (as independent variables). The interactions of the two dependent variables can be considered extremely significant (P < 0.0001), so that the P values that follow for the row and column effects are difficult to interpret (excepting F4 for which the value of P is > 0.05, that means an insignificant effect of the interaction). In the same time, both of the dependent variables are directly affected by the ingredients of the tables, as these effects are also considered extremely significant (P < 0.0001). However, all tablets batches disintegrate without stirring in less than 3 minutes (the maximum admissible limit for this kind of tablets [7])

Although it is supported by a fast disintegration (in less than 15 seconds), the dissolution of loratadine lies on a period of about 10 minutes for all batches, but showing significantly different dissolution profiles that all are perfectly linearized by the simple regression (Figure 2).

In order to prove their linearity, the dissolution curves were supplementary fitted with usual time-dependent kinetic equations (Zero-order, First-order, Higuchi, Korsmeyer-Peppas, Peppas-Sahlin) and in all cases, the Akaike index as well as R^2adjusted parameter have demonstrated that the studied dissolution profiles are the best fitted by Zero-order kinetic. This means that in all seven cases, the dissolution of loratadine from tablet occurs at a constant rate which is numeric quantified by the value of k_0 - the constant of the implied dissolution process (Figure 3).

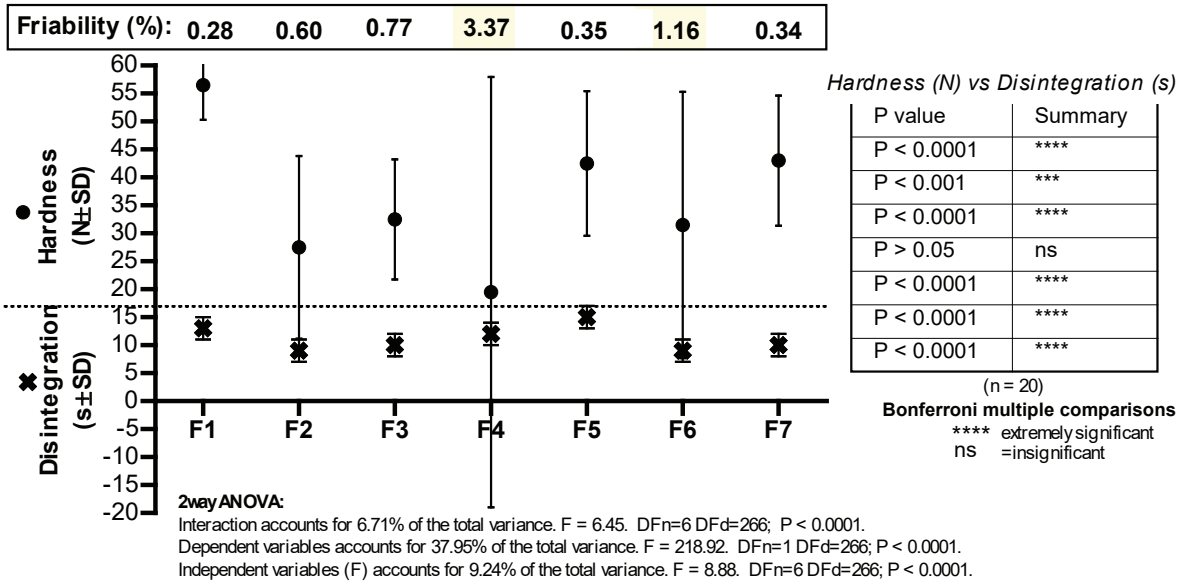

Fig. 1. The mechanical resistance vs. the disintegration ability of the orodispersible tablets

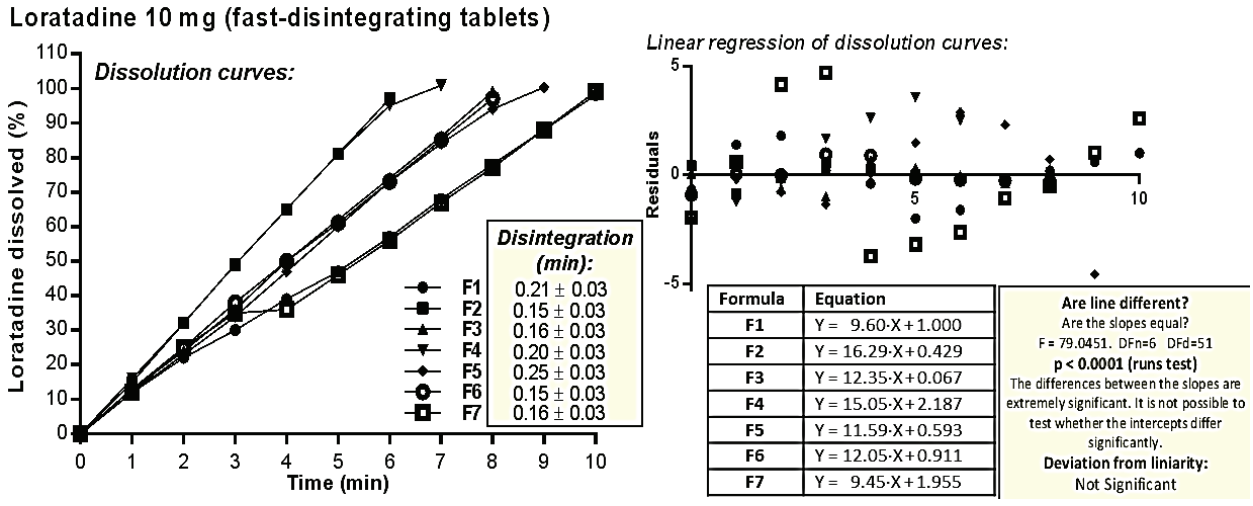

Fig. 2. The dissolution profiles of Loratadine vs. the disintegration time of tablets

The lower value of R^2adj coefficients indicates that the dissolution process of loratadine from the tablets of F3, F6, F5 and especially F1 and F7 batches, is also accompanied by other kind of processes, possibly due to the associated ingredients which interact into the matrix of the tablet. F2 and F4 batches stand out by their faster dissolution rates, significantly higher than the others (k_0 constants showing the highest values). However, the very poor resistance of F4 tablets (both hardness and friability) determines its exclusion from the consideration. It follows that F2 batch shows the most suitable characteristics for orally administration of tablet and its dispersion: the loratadine dissolution is running steadily from the start (Figure 4) and over all period of at about 6 minutes (when at least 97% of active dose is dissolved), after few seconds of latency (Figure 5), but the kinetic process does not appear to be influenced by the interactions between its associated ingredients.

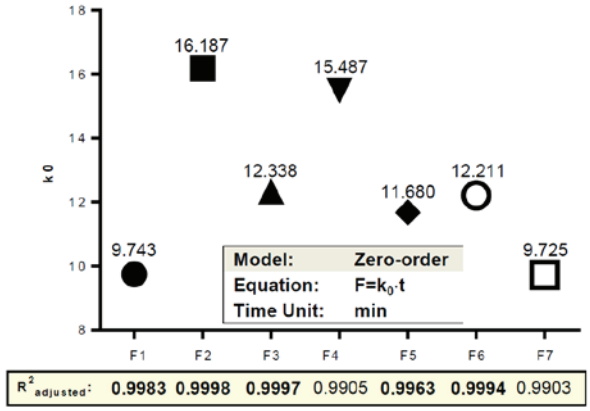

Fig. 3. The degree of curve fitting with Zero-order kinetic (R^2 adjusted) vs. the dissolution rate (k_0)

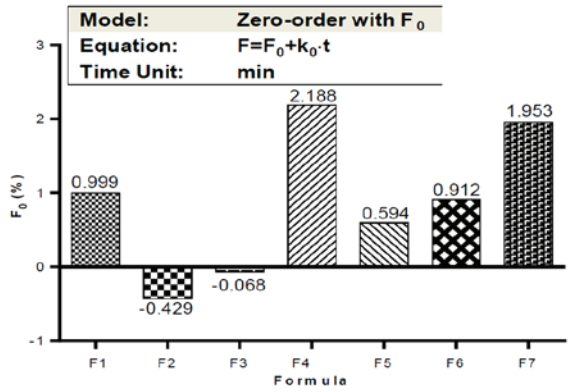

Fig. 4. The burst fraction (F_0) released previous to the dissolution process

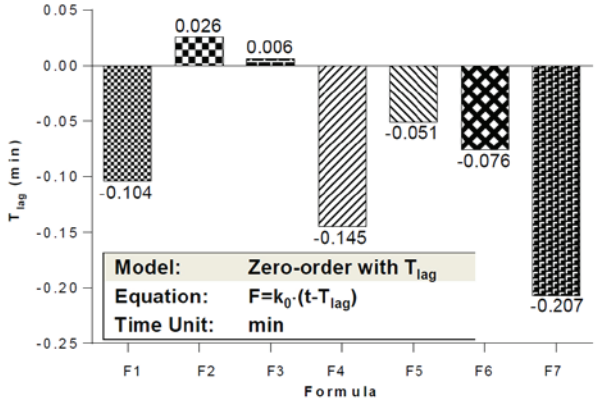

Fig. 5. The latency (T_{lag}) with which the dissolution process occurs

Conclusions

Association of croscarmellose sodium (7.5%) with pregelatinized starch (6%) as superdisintegrants, positively influences the dissolution properties of loratadine (5%) from orally fast dispersable tablets if mannitol is added as the binder agent (35%). The use of maltodextrin instead of mannitol determines a dramatic decrease, below the level of acceptability, in the hardness of tablets.

Pre-gelatinized starch (4%) associated to maltodextrin (12%) favors the dissolution processes, but only in the presence of mannitol (20%), otherwise the dissolution processes being slowed down. Approximately the same effect is determined if mannitol (35%) is associated to a combination of croscarmellose sodium (5%) and pre-gelatinized starch (6%).

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Conflict of interest

None to declare.

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RESEARCH ARTICLE

Precursor Synthesis of Some New Macrocyclic Compounds

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Objective: Development of new electronic devices with applications in computer science as well as new medical devices pushed the researcher to find new technologies. Based on those new techniques we have designed and synthesized compounds with possible application in the field of advanced materials. **Material and method:** Compounds were analyzed by TLC and NMR. Routine 1H NMR (250 MHz) spectra were recorded at room temperature in deuterated acetone, unless stated otherwise. Thin-layer chromatography (TLC) was carried out on aluminum sheets coated with silicagel 60 F254 Merck TLC plates. **Results:** Starting from commercial available compounds intermediates were obtained in a good yield. 4,4'-(2,4,8,10-tetraoxaspiro[5.5]undecane-3,9-diyl)diphenol was obtained starting from pentaerythritol and p-hydroxy-benzaldehyde in the presence of catalytic amounts of APTS (p-toluensulfonic acid). The product was purified by recrystallization and characterized by NMR spectroscopy. The structure exhibit 2 different signals for equatorial and axial position. Furthermore di, tri and tetra ethylene glycol were obtained by microwave assisted synthesis in a matter of minutes. Compounds were separated by recrystallization. **Conclusions:** In conclusion, several intermediates were synthesized and characterized from spectroscopic point of view. Further analyses should be carried out and the compounds should be tested as advanced materials.

Keywords: spiran, 1,3-dioxan, polyethylene glycol, inside&outside methylene group

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Introduction

Macrocyclic compound played a major role in the field of supramolecular chemistry because they can form organized supramolecular assemblies. Usually these macrocyclic have cavity of different size and can accommodate small or large molecules by host-guest interactions [1].

The design and synthesis of new macrocyclic compounds remain of great interest due to wide applications such as catalysis [2-4], advanced materials [5-7] and medicine [8-11].

One narrow domain of supramolecular chemistry is the field of molecular machine. Such as cars are driven by the conversion of piston action into a rotary motion, there are molecules that can undergo programmed motions in response to different stimuli. Molecular analogues of a variety of mechanical devices such as molecular rocking chair [12], rudder, wringer [13], shuttles [14], molecular elevator [15], unidirectional rotors [16], and tweezers have been created. But these "molecular machines" have been controlled by structural transition between two or more stable states in a reversible manner by one single moiety using external stimuli such as light, temperature or pH.

The aim of our work was the design and synthesis of some precursors of some macrocyclic compounds which incorporate 2 different switchable units and can be valuable compounds for synthesis of new photochemically and conformational controlled molecular devices. Converting light-energy in "molecular motion" by *cis-trans* isomerization is one of the most studied and applied system by researchers. Usually azobenzene [17] or stilbene [18] derivatives are used due to the possibility of controlling the isomerization by both UV light and thermal relaxation [19]. Photoisomerization of azobenzene derivatives have been used to control electronic properties of more complex compounds [19] or to drive the folding/unfolding of peptide chains [20]. Therefore, we have considered of great interest the synthesis of some intermediates as building blocks for new macrocycles which can incorporated two or more switchable units.

Material and methods

All reagents were acquired from Sigma Aldrich, Merck or Alfa Aesar and were used without any further purification. Routine ^{1}H NMR (250 MHz) were recorded at room temperature (r.t.) in CD_3COCD_3, unless stated otherwise on a Bruker 250 MHz spectrometer, using the solvent line as reference. Chemical shifts (δ) are reported in parts per million (ppm) values using residual solvent peak as internal reference and the coupling constants (J) are in Hertz (Hz). Multiplicities are abbreviated as: *s*-singlet; *d*-doublet; *dd*-doublet of doublet; *t*-triplet; *q*-quadruplet and *m*-multiplet. Compounds were analyzed by TLC and NMR. Thin-layer chromatography (TLC) was carried out on aluminum sheets coated with silicagel 60 F_{254} Merck TLC plates.

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Melting points were determined using a Boetius microscope and represent uncorrected values.

The irradiation was carried out in a domestic microwave oven (Samsung, model MS23F301EAK).

Synthesis of 3,3'-(2,4,8,10-tetraoxaspiro[5.5]undecane-3,9-diyl)diphenol (3)

A mixture of 3-hydroxybenzaldehyde (1.22 g, 1 mmol), pentaerythritol (680 mg, 0.5 mmol) and paratoluensulfonic acid (38 mg, 0.2 mmol) were stirred under reflux in a two-necked glassware flask connected to a Dear-Stark apparatus in about 100 mL toluene. The reaction was monitored by thin layer chromatography on silica gel coated plates Merck 30-F_{254} using pentane:ethylacetate mixture (9:2 v/v). After completion of the reaction the mixture was washed three times with water, dried over Na_2SO_4, and filtered. Evaporation of the solvent in vacuo gave the desired crude product. The product was purified furthermore by recrystallization from ethanol (yield: 23%, mp 243-247 °C). ^{1}H NMR (250 MHz, CD_3COCD_3) δ/ppm 2.62 (dd, *J*=11.7 Hz, *J'*=1.8 Hz, 2H, H_{eq}); 3.01 (m, 2H, H_{ax}); 3.33 (d, *J*=11.7 Hz, 2H, H_{ax}); 4.15 (dd, *J*=11.7 Hz, *J'*=1.8 Hz, 2H, H_{eq}); 5.23 (s, 2H, Ar-C*H*); 6.80 (overlapped, 2H, H-6', H-6"); 6.94-7.07 (m, 4H, H-2', H-2" and protons H-4', H-4"); 7.18 (overlapped dd, 2H, H-5', H-5"); 8.70 (s, 1H, O*H*).

General procedure for synthesis of di-, tri- and tetraethylene glycol ditosylate

All compounds were obtained using a modified procedure described by Kazemi and coworkers [21]. 10.13 g K_2CO_3 (73.42 mmol), 50 mmol alcohol and 13.24 g (69.5mmol) p-toluenesulfonyl chloride were charged into a mortar and grinded vigorously for about 15 minutes. A small amount of the mixture was dissolved in dichloromethane and the reaction progress was monitored by thin layer chromatography on silica gel coated plates Merck 30-F254) using dichloromethane:pentane mixture (1:2 v/v). The excess of tosyl chloride was removed by addition of about 2 mL of *t*-BuOH and irradiated (600 W) in a domestic microwave for about 3 minutes (*caution: a smoke was generated during the irradiation; therefore, the entire process was carried out under a very good ventilated chemical hood*). The reaction mixture was stirred with ethylic ether washed twice with small portion of brine and the combined organic layers were dried over Na_2SO_4 and the solvent was removed using a rotary evaporator. The crude product either purified by recrystallization or by flash-chromatography (using a mixture of pentane:dichloromethane as eluent) affording pure tosylated compound.

Diethyleneglycol ditosylate (6a): Colorless crystals: m.p. 86-89°C. Yield: 87%. ^{1}H NMR (250 MHz, $CDCl_3$) δ/ppm 2.46 (s, 6H, CH_3); 3.63 (t, 4H, OCH_2); 4.11 (t, 4H, OCH_2); 7.35 (d, 4H, H-3', H-3"); 7.76 (d, 4H, H-2', H-2");

Triethyleneglycol ditosylate (6b): Colorless crystals: m.p. 76-80°C. Yield: 88%. ^{1}H NMR (250 MHz, $CDCl_3$) δ/ppm 2.31 (s, 6H, CH_3); 3.45 (s, 4H); 3.56 (t, 4H, OCH_2); 4.02 (t, 4H, OCH_2);7.26 (d, 4H, H-3', H-3"); 7.69 (d, 4H, H-2', H-2");

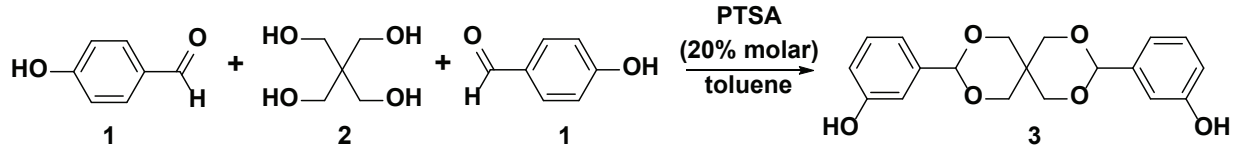
Tetraethyleneglycol ditosylate (6c): Tick oil. Yield: 84%. ^{1}H NMR (250 MHz, $CDCl_3$) δ/ppm 2.22 (s, 6H, CH_3); 3.38 (m, 8H); 3.45 (t, 4H, OCH_2); 3.96 (t, 4H, OCH_2);7.16 (d, 4H, H-3', H-3"); 7.58 (d, 4H, H-2', H-2");

Results

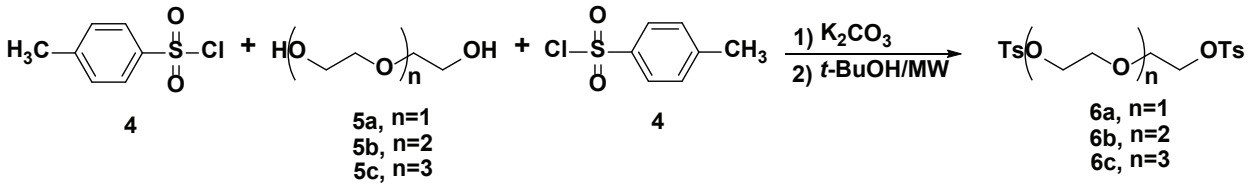
Since molecular devices are nowadays mostly controlled by mean of UV light we have thought that also conformational changes can be a way of controlling nanoscale devices. Therefore, we have designed a macrocyclic compound that bears both a spiran unit and a azobenzene moiety linked by polyethylene glycol linkage. In the first step, we have synthesized the spiran unit starting from commercially available reagents (scheme 1) in the presence of para-toluene sulfonic acid (PTSA) as catalyst.

Spiran 3 was characterized by NMR spectroscopy and melting point was determined using a Boetius microscope. Both melting point [22] and NMR are in accordance with literature data.

Also, we have synthesized 3 polyethylene glycol ditosylate starting from corresponding alcohol and tosyl chloride in a manner of minutes using a solvent-free modified method described previously in the literature by Kazemi and coworkers [21] (Scheme 2).



Scheme 1.

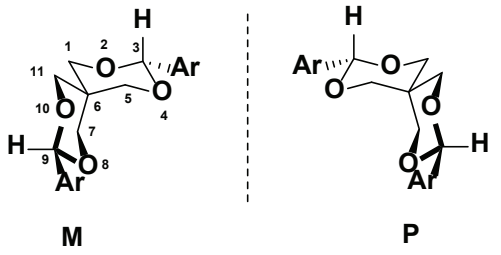


Scheme 2.

Discussions

Since saturated six membered ring are one of the most studied class of compounds regarding the conformational changes we had in mind the design and synthesis of some macrocyclic compounds bearing this moiety. Since we intended to have a friendly green synthesis we have avoid using toxic solvents in the synthesis and we have tried to obtain the compound 3 following a method described in the literature [23] using a domestic microwave. Unfortunately, after irradiation in a domestic microwave an insoluble black tar was obtained. Therefore, we have followed classical method of synthesis of diacetals using acidic conditions (scheme 1).

Aryl derivatives of 2,4,8,10-tetraoxaspiro[5.5]undecane have specific helical and axial chirality [24]. On the other hand, 1,3-dioxane rings have an anancomeric structure where the conformational equilibrium is strongly shifted towards the conformer where aryl groups are in equatorial position. Due to the spiro skeleton which exhibit helical chirality, under reaction condition, the product is a racemic mixture of both M and P helix configuration [24] (Scheme 3).



Scheme 3. (reprinted from Grosu et al., 2003 [22], with permission from Wiley)

Because of this conformational arrangement, NMR spectrum of compound 3 exhibit different signals for axial and equatorial protons from positions 5 and 7, as well as protons from positions 1 and 11. Methylene from positions 5 and 7 are oriented towards the other 1,3-dioxane ring, being called methylene inside. The other two positions (1 and 11) which are oriented in the opposite direction are called methylene outside [25]. All the protons from spiran skeleton exhibit two AB (AX) systems. Also, a long-range coupling split supplementary the equatorial protons. This was happened because of a "W (M)" arrangement of the bonds in a way of H_{eq}– $C^{1(11)}$–C^{6}–$C^{5(7)}$–H_{eq} between equatorial positions, therefore the signals for protons form equatorial positions are seen as doublets of doublets (figure 1).

Also, the equatorial protons of the methylene inside positions are more deshielded than those of outside position.

Furthermore, three ditosylated polyethylene glycol were synthesized using a very convenient solvent-free method,

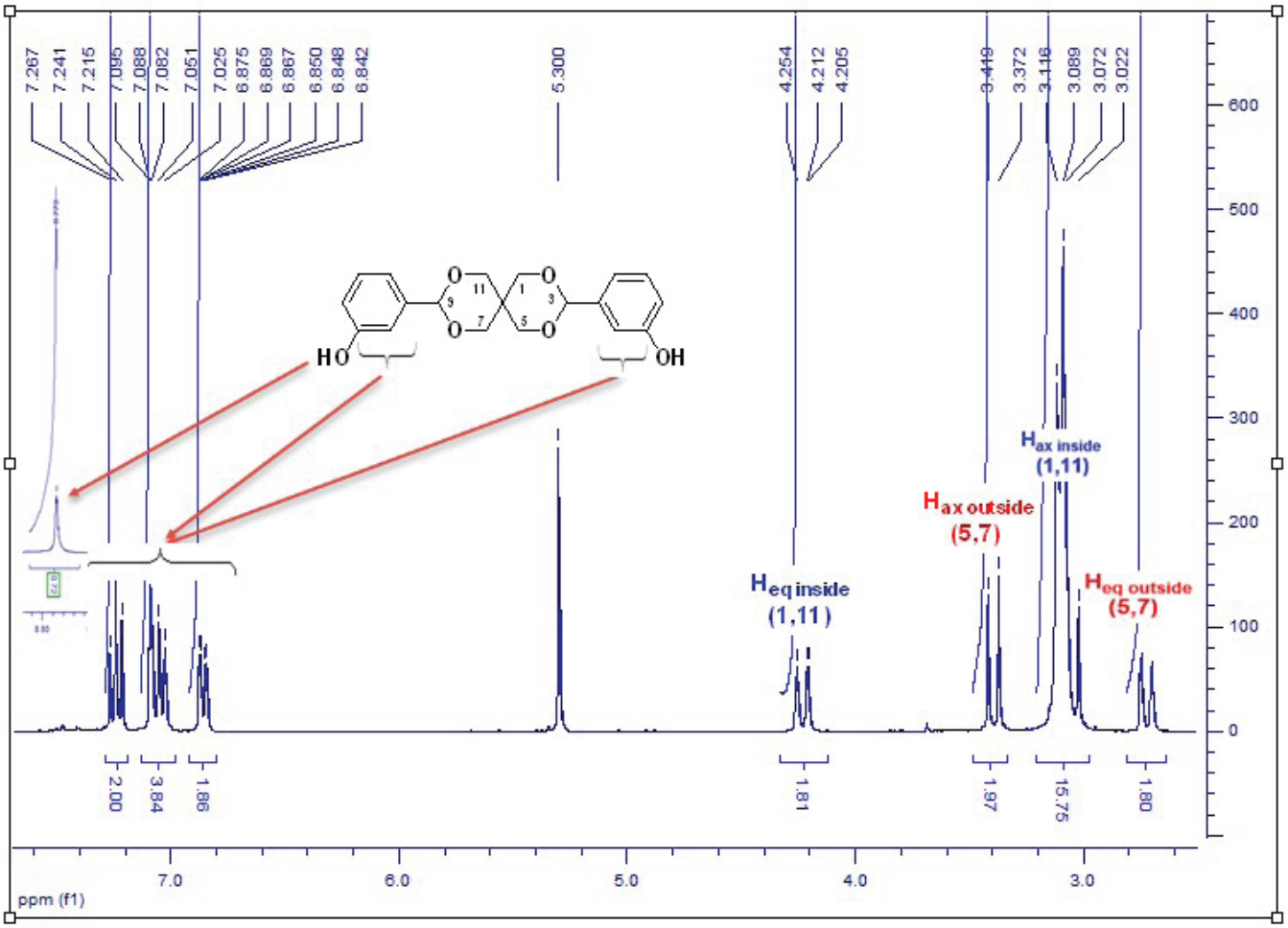


Figure 1 ^{1}HNMR spectrum (Acetone-D_6, rt) of compound 3

starting from alcohol and tosyl chloride in basic media. Reaction is very quick and occur in very good yield.

Conclusions

In conclusion, we have synthesized and characterized one spiran showing different signals in NMR spectra for equatorial and axial positions. Also, due to spatial conformation of the spiran skeleton differences occur between inside and outside methylene protons in ^{1}H-NMR. Di-, tri- and tetra glycol ditosylated were obtained in good yield in a solvent-free condition.

Acknowledgements

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Conflict of interest

None to declare.

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RESEARCH ARTICLE

Relationship Between High Levels of Salivary Cotinine Test and Demographic Characteristics of Pregnant Smokers from Mures County

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Objectives: To evaluate the relationship between the frequency of self-declared status regarding smoking in a group of pregnant women from Mures county, Romania and the high levels of Salivary Cotinine (SC) like biomarkers. **Material and methods**: It was conducted a retrospective study among 230 pregnant women presented for prenatal care at 50 General Practitioners cabinets in Mures county, Romania, in 2015. Data were collected with a validated questionnaire which included age, level of education, socioeconomic status and ethnicity, also the self-reported smoking status. The Salivary Cotinine level was evaluated using NicAlert Saliva test kits. **Results**: Using salivary test we identified a high prevalence of involuntary exposure to cigarette smoke among both non-smokers and those who quit smoking before pregnancy. Also we registered pregnant women that although declared smoking cessation before pregnancy their salivary Cotinine levels were high, almost like to an active smoker, probably because of second-hand exposure or because they didn't say the truth about their habit. **Conclusions**: We underline the importance of implementing more efficient community interventions among this vulnerable group in order to reduce the frequency of smoking and sustain quitting.

Keywords: smoking, pregnancy, cessation, cotinine test

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Introduction

Exposure to cigarette smoke in utero, whether it is direct or indirect via secondhand smoking, can be associated with negative consequences and severe health problems for the newborns, children, and adults [1-5]. Studies revealed that maternal smoking during pregnancy was associated with ectopic pregnancy, placental abruption, prematurity [6-8], low birth weight babies, and increased risk of having a baby with stunted neuro-development growth and cognitive problems [9,10,11]. Pregnant women, who are often aware of the risks of smoking, may be hesitant to disclose their true smoking status during a clinical encounter, limiting opportunities for provider-based counseling and support [12].

The most commonly used biomarker of exposure to tobacco smoke is Cotinine, as a main metabolite of nicotine. The measurement of the Cotinine concentration in various biological fluids is directly proportional to the degree of exposure to nicotine [13]. The determination of Cotinine is recommended for the assessment of active tobacco smoking, monitoring of environmental tobacco smoke (ETS) exposure, and impact evaluation of smoking cessation programs [14].

In a study on pregnant women made in Scotland, there was a 25% underestimation of smoking using self-reported data that was validated with Cotinine [15]. A similar survey conducted in Sweden revealed that 6% of self-reported non-smokers were probably smokers and 3% had Cotinine levels suggestive for secondhand smoking using Cotinine validation [16].

There are scientific proofs that smoke-free environment represents the only strategy for protecting the population from second-hand smoking negative effects [17]. For this matter, many countries have implemented legislations requiring all public places, workplaces and all indoor places to be free of secondhand smoking [18]. In 2004, Ireland was the first country which implemented the smoke-free legislation. Since then, other European and non-European countries followed Ireland; Norway, New Zealand, Italy, Uruguay, England and several provinces in Canada, the USA or Australia [19,20] had implemented the smoke-free law.

In Romania the anti-tobacco law was implemented in June 2002, and stipulate smoking only in specially arranged public spaces and in February 2016 it was modified by banning smoking in all public spaces. A study performed after the adoption of smoke free law in Uruguay showed a measurable decrease of passive smoking exposure in indoor public places and workplaces, the level of exposure was assessed by measuring air nicotine concentrations [21].

The goal of our research was to evaluate the relationship between the frequency of self-declared status regard-

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ing smoking in a group of pregnant women from Mures county, Romania and the high levels of Salivary Cotinine (SC) like biomarkers.

Material And Methods

Settings and population

*Population***.** We conducted a convenience study among pregnant women presented for prenatal care at 50 General Practitioners (GP) cabinets out of 90, in Mures county, Romania. From 324 women which were presented for monitoring purposes to GP's, only a group of 230 pregnant women agreed to fill in a questionnaire regarding smoking status and Cotinine testing, group that formed the final sample of our research.

Measurement: Data were collected based on a validated questionnaire which included age, level of education, socioeconomic status and ethnicity. We also measured self-reported smoking status using the following questions (I have never used cigarettes and I was a regular smoker during pregnancy). Responses were categorized into two groups: smokers and non-smokers.

Salivary Cotinine level was evaluated using NicAlert Saliva test kits. The test principle is based on the use of monoclonal antibodies for Cotinine, and was developed for the identification of smoking status in research studies, on individuals that are monitored for smoking cessation, not for medical diagnosis or therapy. If Cotinine is present in the sample, it will bind to the antibody through binding sites. The number of sites is occupied according to the amount of Cotinine present in a sample. Cotinine, one of the major metabolite of Nicotine, is a suitable candidate as a marker, because it has a relatively long half-life of 10-40 hours and was found to be more sensitive and specific than carbon monoxide in the air.

To achieve regression models we have encoded variables of interest and marked it in the below table 1 as legend that follows: the dependent variable was the amount of Cotinine (the N00, N11, N22 were coded 0 for N33, N44, N55, N66 were coded 1), the independent variables were: Q2 (less than 8 years of school. 8 years of school. Vocational school - coded 1. High school. Graduated. University - encoded 0), Q4 (Married. Concubinage - encoded 0. Not married. Divorced. Widow - coded 1), Q5 (Romanian. Hungarian - encoded 0. Roma people - coded 1. Than, the level of smoking was evaluated based on), Q7 (I have never used cigarettes. I stopped smoking before I was pregnant - I do not smoke. I stopped smoking when I found out I'm pregnant - do not smoke - smoke regularly encoded 0 and for the same number of cigarettes as before I was pregnant. I smoke but I reduced the number of cigarettes after I got pregnant. I increased the number of cigarettes consumed when I found out I was pregnant - coded 1). Q9 (1. coded 0; 1. 2. 3. 4 encoded), Q10 (Nobody smokes where I stay. Smokers can smoke only in certain rooms in the house - encoded 0. Smokers can smoke wherever they want - coded 1), Q11 (Did not show any change. People smoke anywhere in the house even if they found out they were pregnant - coded 1. No one smokes in the house when I was pregnant - Smoke outside. People smoke in other rooms when they found out I was pregnant - encoded 0), Q18 (I did not smoke cigarettes during the past 30 days. coded 0; Less than one cigarette per day. One cigarette per day. 2-5 cigarettes a day. 6-10 cigarettes per day. Between 11 to 20 cigarettes a day. Between 21 to 30 cigarettes a day. More than 30 cigarettes a day: coded 1), Q19 (I have not smoked a cigarette in the last 30 days - code 0; In 5 minutes. In 6-30 min. In 31-60 minutes. After 60 minutes - coded 1), and Q25 (Yes. in the next 30 days. Yes. the next 6 months. Yes. but not in the next 6 months - there encoded 0. I'm not going to give up - coded 1).

Samples Collection/Preparation

Saliva Testing: Saliva was collected using a funnel, and the collection tube to fill at least one third of its capacity. In the first 4 hours after collection, eight drops of the sample were extracted and deposited on the end of the tape lined Accutest NicAlert test strip, which previously was placed on a flat surface. After transferring saliva from the red zone into the white, the red color must appear in at least one zone (levels 0-6), otherwise, the test results are not valid [25].

Interpretation: Identifying areas of salivary Cotinine labeled bands in the area ranging from 0 (0-10 ng / ml) to 10. Any concentration greater than or equal to 10 ng / ml (zones 1-6) represent a positive result. Salivary Cotinine concentrations and interpretation are as follows: level 0 (1-10 ng / ml) is a nonsmoker; 11 (10-30 ng / ml) corresponds to involuntary smoking; level 22 (30-100ng / ml) - confirmed smoker with low tobacco consumption; 33 (100-200 ng / ml) is a confirmed smoker with moderate tobacco consumption; and 44 (200-500 ng / ml) – is smoker with a high level of tobacco consumption.

The research protocol was approved by the ethics committee at the University of Medicine and Pharmacy Tirgu-Mures, as part of a larger study on Building Capacity for Tobacco Research in Romania.

Statistical analysis

Statistical analysis was performed using the Statistical Package for Social Sciences (SPSS, version 20, Chicago, IL, USA). The association between qualitative variables was assessed using chi-square test or Fisher's exact test. The relationship between the explanatory variables and other Cotinine were evaluated by logistic regression.

The results were presented by odds ratio (OR) and 95% CIs. For all statistical tests of significance alpha level was set at 0.05.

Results

The bivariate analysis of the relationship between Cotinine levels and socio-demographic showed that the smoking status of pregnant women monitored was significantly influenced by the low level of education ($p = 0.0001$. OR:

5.69 for 95% CI 0.86-11.25) and Roma ethnicity (p = 0.0001, OR: 4.9, 95% CI = 1.90-12.77). No statistically significant data were registered regarding marital status or presence of intervention from a General Practitioner (GP) related to the risk of active or secondhand smoking.

Behavioral parameters data revealed that tobacco use in pregnancy was influenced by smoking status in pre-pregnancy period. Smoking family members also influenced attitudes towards smoking of pregnant women inside the home and those with no changes done during pregnancy about quitting (p = 0.0001, OR: 6.1, 95% CI = 2.88-12.89) or (p = 0.0001, OR: 6.1, 95% CI = 2.67-13.62). The lack of change in smoking behavior by direct family members in the presence of pregnant women and inside the house, had a bad impact on tobacco consumption in subjects investigated (p = 0.0001, OR: 2.26, 95% CI = 1.22-4.20), also the attitude of pregnant women towards smoking in the last 30 days prior to questioning was associated with an increased risk of continued smoking during pregnancy for women who have lit a cigarette at least a day. in the last 30 days (p = 0.0001, OR: 55.5, 95% CI = 22.22-138.61). The short time between the first cigarette in the morning with the lack of any concerns of cessation, had a significant bad influenced upon smoking status of subjects investigated (p = 0.0001, OR: 56.0, 95% CI = 22.43-139.79) (see Table I).

In the group of women informed by the GP's about the smoking consequences during pregnancy, was found high levels of Cotinine especially in those with low education, those living in houses where are smokers with no restrictions and those who said they are not planning to quit smoking in the next 30 days.

High levels of Cotinine were found in pregnant smokers, in those with one or more family members who are smokers, those who smoked in the last 30 days, women who smoked within the first hour after awakening, and those that do not think seriously about quitting as well, all subjects from both groups monitored (trained and untrained by the GP's) (see Table II).

Our data showed that an increased level of Cotinine is associated with active smoking (Q7), and with secondhan smoking as well (Q9) (Table III).

An increased Cotinine levels has been highlighted among Roma pregnant women (68.2%), and in 64.7% of pregnant women with low education (less than high school) also at 33.6% of married women.

The level of Cotinine was dependently influenced by the number of smokers in the pregnant household (p = 0.0004, OR: 5.19, 95% CI = 2.08-12.96), by the number of cigarettes smoked in the last 30 days (p = 0.0001. OR: 73.8, 95% CI = 19.53-279.31), and how long after awaken g the pregnant light up the first cigarette (p = 0.0001, OR: 53.45, 95% CI = 17.13-79.36).

Our data showed that saliva Cotinine cut-off level of 10 ng/ml was found to be the optimum cut-off value that differentiates pregnant smokers from non-smokers or secondhand smoking when all testing conditions were possible to fulfill.

Table I. Bivariate analysis of the relationship between Cotinine levels and socio-demographic parameters

Socio-demographic parameters		Cotinine**		OR	IC	P
Questions	Codes *	1	0			
Education level	1	64.7%	24.4%	5.69	2.86-11.25	0.0001
	0	35.3%	75.6%			
Marital status	1	37.5%	33.7%	1.18	0.54-2.57	0.69
	0	62.5%	66.3%			
Ethnicity	1	68.2%	30.3%	4.9	1.90-12.77	0.0001
	0	31.8%	69.7%			
Intervention	1	51.0%	45.2%	1.26	0.51-3.09	0.64
	0	49.0%	54.8%			
Time	Pretest=1	43.7%	69.1%	0.35	0.19-0.62	0.0004
	Post-test=0	56.3%	30.9%			
Behavioral parameters						
Questions	Codes					
Self-declared smoking status	1	84.8%	10.1%	50.0	20.93-119.41	0.0001
	0	15.2%	89.9%			
Number of smokers in the family	1	47.3%	12.8%	6.1	2.88-12.89	0.0001
	0	52.7%	87.2%			
Exposure to passive smoking	1	69.0%	27.6%	6.1	2.67-13.62	0.0001
	0	30.3%	72.4%			
Avoiding passive smoking in the family	1	47.8%	28.8%	2.26	1.22-4.20	0.0001
	0	52.2%	71.2%			
Smoking status in the last 30 days	1	84.6%	9.0%	55.5	22.22-138.61	0.0001
	0	15.4%	91.0%			
Time duration between -waking and first cigarette in the last 30 days	1	84.8%	9.1%	56.0	22.43-139.79	0.0001
	0	15.2%	90.9%			
Planning to quit smoking	1	32.3%	62.5%	0.286	0.119-0.688	0.0001
	0	67.7%	37.5%			

* Legend explained in Methods section.
** Bivariate analysis of variable Cotinine, taken binary 0-absent and 1-present.

Table II. Cotinine levels correlated with socio-demographic parameters, depending on the intervention of GP's or not

Questions*	Cotinine**	Pregnant women with intervention for cessation			Pregnant women without intervention for cessation		
		1	0	P OR (CI95%)	1	0	P OR (CI95%)
Education level	1	71.4	17.6	0.003 11.6 (2.12-64.1)	34.6	12.0	0.057 2.88 (0.90-16.5)
	0	28.6	82.4		65.4	88.0	
Marital`status	1	28.6	23.5	0.75 1.3 (0.25-6.52)	7.7	0.0	0.15 2.04 (1.53-2.71)
	0	71.4	76.5		92.3	100.0	
Ethnicity	1	42.9	11.8	0.09 5.62 (0.91-34.5)	11.5	0.0	0.08 2 08 (1.55-2.80)
	0	57.1	88.2		88.5	100.0	
Self-declared smoking status	1	85.7	0.0	0.0001 9.5 (2.56-35.2)	88.5	12.0	0.0001 56.2 (10.23-308.8)
	0	14.3	100.0		11.5	88.0	
Number of smokers in the family	1	100.0	64.7	0.02 0.44 (0.28-0.68)	88.5	40.0	0.0001 11.5 (2.71-48.7)
	0	0.0	35.3		11.5	60.0	
Exposure to passive smoking	1	50.0	0.0	0.001 3.42 (1.83-6.39)	15.4	12.0	0.72 1.33 (0.26-6.66)
	0	50.0	100.0		84.6	88.0	
Avoiding passive smoking in the family	1	35.7	17.6	0.25 2.59 (0.49-13.6)	44.0	35.0	0.54 1.45 (0.43-4.90)
	0	64.3	82.4		56.0	65.0	
Smoking status in the last 30 days	1	84.6	0.0	0.001 9.5 (2.56-35.2)	88.5	14.3	0.001 46.0 (8.27-255.6)
	0	15.4	100.0		11.5	85.7	
Time duration between -waking and first cigarette in the last 30 days	1	85.7	0.0	0.001 9.5 (2.56-35.2)	88.5	14.3	0.001 46.0 (8.27-255.6)
	0	14.3	100.0		11.5	85.7	
Planning to quit smoking	1	0.0	43.8	0.005 2.55 (1.53-4.25)	40.9	57.1	0.45 0.52 (0.09-2.9)
	0	100.0	56.2		59.1	42.9	

*Legend explained in Methods section.
**Multivariate analysis of variable Cotinine, taken binary 0-absent and 1-present.

Table III. Correlations between socio-demographic variables and Salivary Cotinine level

Variable	Odds Ratio	95% CI	P
Intervention	1.99	0.20 to 19.53	0.55
Education level	1.03	0.10 to 9.72	0.97
Age	1.09	0.89 to 1.34	0.37
Marital status	16.51	0.86 to 316.55	0.06
Ethnicity	6.27	0.20 to 188.49	0.29
Smoking status	109.12	16.76 to 710.38	<0.0001

Discussions

Starting with estimated rates of daily smoking for 31-37% of the Romanian adult population and 24% among students it is critically needed to develop efficient programs for tobacco cessation. In this context, research studies are important to find the best techniques to be used for a sustainable network dedicated to population support for smoking cessation or even better for avoidance of the onset [13, 17].

Prolonged exposure to tobacco smoke, especially to cigarettes, has been scientifically proven to have harmful effects on organs, systems and processes in the human body, both for active as for the secondhand smokers. Inhalation of more than 4800 different chemicals by smoking, creates fertility problems and an increased risk of health problems during pregnancy, for mother or fetus [17].

The conditions of appearance and maintenance of this habit are influenced by economic status, education and culture, family background, emotional climate and level of addiction.

After analyzing pregnant self-reports on smoking status, our study showed a high frequency of women who smoked before pregnancy (30.04%) of which a high percentage (43.3%) continued to smoke during pregnancy, a high frequency even for Romania [22,23,24].

In another similar study conducted in Romania in 2002 on a sample of 286 people, based on sample self reports about smoking status the initial classification was: 50.56% active smokers, 3.5% occasional smokers and 55.94% nonsmokers [26]. After performing the Cotinine test strips the subjects distribution by their smoking status was represented as: 44.06% active smokers, 39.50% passive smokers and 16.43% non-smokers [26]. These results showed that more than a half of the subjects participating in this study self-declared being nonsmokers, but this percent decreased dramatically after the test performing, and higher frequency of positive Cotinine levels was encountered in women than in men [26,27,28].

Regarding the limitations of our study we can mention that there were situations where testing conditions were not always possible to fulfill. In heavy smokers case, their saliva was more thicker than secondhand smokers or nonsmokers women, which did not allowed Cotinine migration into test strip without a thermostatic period. This has not always been possible because not all health facilities within which they carried out the collection were equipped with a thermostat. Thus, there were cases where the Cotinine test showed negative results while the study participants declared themselves as active smokers (6 out of 31).

The etiological factor for tobacco addiction is nicotine. The recent smoking cessation approaches tend to focus on this chronic, relapsing dependence on tobacco, even by monitoring components of nicotine in the blood, or sa-

liva. The success of such approach involves understanding of the chronic nature of tobacco addiction, the monitoring period (not just interventions in the acute stages of manifestation) associated with behavioral educating both for healthcare professionals and also for pacients [20,23].

We believe that the results of this study will enable a shift in starting up community interventions more effective in smoking cessation outcomes, manifest especially among young women who are pregnant or are preparing to be, from different ethnicities and specific socio-demographic and cultural profiles.

Conclusion

Using salivary Cotinine test we identified a high frequency of involuntary exposure to cigarette smoke among both non-smokers and those who quit smoking before pregnancy. Also we registered pregnant women that although declared smoking cessation before pregnancy their salivary Cotinine levels were high, almost like to an active smoker, probably because of secondhand exposure or because they did not say the truth about their habit. We underline the importance of implementing more efficient community interventions among this vulnerable group in order to reduce the frequency of smoking and sustain quitting.

Acknowledgments

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Conflict of interest

No conflict of interest.

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RESEARCH ARTICLE

The Influence of Some Parameters on Chiral Separation of Ibuprofen by High-Performance Liquid Chromatography and Capillary Electrophoresis

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Objective: The aim of the study was to compare the influence of mobile phase composition and temperature on chiral separation of racemic ibuprofen by capillary electrophoresis and high performance liquid chromatography with UV detection. Materials and methods: Racemic ibuprofen was analysed on a chiral OVM column with an HPLC system 1100 Agilent Technologies, under isocratic elution, by using potassium dihydrogen phosphate 20 mM and ethanol in mobile phase. The flow rate was set at 1 mL/min, UV detector at 220 nm and different column temperatures were tested. For electrophoresis separation an Agilent CE G1600AX Capillary Electrophoresis System system, with UV detection, was used. The electrophoresis analysis was performed at different pH values and temperatures, with phosphate buffer 25 mM and methyl-β-cyclodextrin as chiral selector. Results: The chromatograhic analysis reveals a high influence of mobile phase pH on ibuprofen enantiomers separation. An elution with a mixture of potassium dihydrogen phosphate 20 mM pH=3 and ethanol, at 25ºC, allowed enantiomers separation with good resolution in less than 8 min. Conclusions: The proposed HPLC method proved suitable for the separation of ibuprofen enantiomers with a good resolution, but the capillary electrophoresis tested parameters did not allow chiral discrimination.

Keywords: ibuprofen, enantiomers, HPLC, CE, chirality

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Introduction

Ibuprofen or (*R,S*)-2-(4-isobutylphenyl)propanoic acid is an anti-inflammatory agent with chiral structure (Figure 1). The two enantiomers have different biological behavior, the S(+)-enantiomer being the eutomer, but R(-)-ibuprofen is partially converted to form S(+) in biological environment [1,2]. There were many attempts to isolate the eutomer from racemate or to find a specific chemical synthesis of single enantiomer. The high costs of processes and their difficulties, together with the partial biological conversion of the inactive enantiomer explain why ibuprofen is used as racemase in drugs.

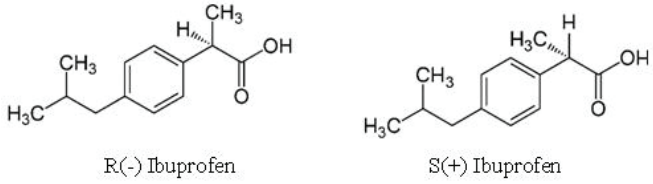

Fig.1. Ibuprofen enantiomers

There are many published methods regarding ibuprofen chiral resolution, mainly made by chromatographic methods [3-11] and few by capillary electrophoresis [12-16]. In general, the methods are designed to analyze enantiomers in biological environment for different purposes such as enantiomers conversion, differences regarding their biological behavior etc.

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Ovomucoid is a chiral chromatographic support suitable for HPLC separation of many active pharmaceutical ingredients from different pharmacological classes [17-19]. Capillary electrophoresis is today a powerful chiral separation technique with many applications in enantiomers separation of drugs.

The aim of the present work is to propose a new application of ovomucoid chiral stationary phase for HPLC separation of ibuprofen enantiomers and to test the ability of methyl-β-cyclodextrin to discriminate the same enantiomers by capillary electrophoresis.

Methods

HPLC analysis

Apparatus: The HPLC analysis was performed on HPLC system 1100 Agilent Technologies, using an Ultron ES OVM column, 150x4.6 mm, 5um (Shinwa Chemical Industries LTD., Agilent Technologies).

Materials and methods: Ibuprofen racemic working standard from Societa Italina Medicinali Scandicci, Italy, was used. For HPLC analysis, the following reagents and solvents were necessary: ethanol and methanol for chromatography, potassium dihydrogen phosphate (Merck products), and purified water obtained from a purifying system Direct Q5 (Millipore).

Methods: The stock racemic ibuprofen solution was prepared in methanol at a concentration of 0.1 % (m/V). The stock solution was further diluted in order to obtain the working solution of 10 μg/mL. The solutions were stored at 8°C to avoid any potential degradation. The mobile phase consisted of 90% potassium dihydrogen phosphate

buffer 20 mM and 10% ethanol, and delivered at a flow rate of 1 mL/min. The samples were injected in volumes of 5 µl and the analytes were detected at 220 nm.

Capillary electrophoresis separation

Apparatus: Capillary electrophoresis separation was performed using a HP AGILENT 3D CE G1600AX system with UV detection, glass capillary with optic window 8 mm, 65 cm x 75µm (PolymicroTehnology, Phoenix, AZ, USA).

Materials: ibuprofen racemic working standard, methanol (Merck for liquid chromatography), ultrapure water (18.2 MΩ·cm) disodium phosphate, monosodium phosphate, sodium borate hydrate, methylbetacyclodextrine (CycloLab).

Methods: The ibuprofen solution of 0.5 mM in methanol:water 1:1 was injected (100 µL), under 50 mbar pressure for 2 seconds, with an applied current of 20 kV. For system preconditioning, the capillary was washed with NaOH 1 M solution for 30 minutes, with water for 15 minutes, then with buffer solution (phosphate buffer 25 mM) for 5 minutes. The cyclodextrine solutions were prepared in phosphate buffer 10 mM.

Results and discussions

HPLC analysis

The ovomucoid chiral column contains a glycoprotein present in eggs with large chiral recognition ability. The recommended pH analysis domain is 3-7.5 with phosphate buffer at concentration of 20 mM. The maximum allowed proportion of organic solvent in mobile phase composition is 50% and the best resolution is provided with the flow rate of 0.8-1.2 ml/min. Acid compounds retention is maximum at isoelectric pH of analytes.

The concentration of the test solution, 10 µg/ml, is correlated with the plasmatic concentration of ibuprofen after its oral administration in low to high doses [20].

Acetonitrile, a powerful elution solvent, was not suitable for ibuprofen retention, even at high composition of aqueous phase. Because the differences between ethanol and methanol in mobile phase were not so significant and considering its lower toxicity, ethanol was used as organic modifier of the mobile phase.

The influence of pH on enantiomers separation was performed using different pH values for mobile phase: pH=3, pH=4.7, pH=6.1, with a proportion of aqueous phase of 90% and the column temperature maintained at 25°C. The best resolution of separation was obtained using a mobile phase pH=4.7, with a resolution of 2.46, but the retention times for ibuprofen enantiomers were high: tR1 = 19.80 min and tR2 = 23.49 min (Figure 2). At higher pH of 6.1, the separation was not possible (Figure 3); the low pH of 3 allowed the enantiomer separation with a very good resolution in less than 8 min (Figure 4, upper image).

As it is well known, the temperature is an important parameter of enantiomer separation, thermodinamic processes of chemical compounds retention and separation being strongly influenced by this parameter. In this case, we carried out the chromatographic separation at the optimum mobile phase pH (pH=3.00) at different column temperatures (20°C, 25°C, 30°C and 35°C). The other chromatographic conditions were the same as previously described (Figure 4).

In accordance to our results, an acid pH of 3 for aqueous mobile phase was an optimum value for a compromise between good separation and adequate retention time.

As it can be seen from Figure 4 and Table I, we can conclude that a higher column temperature did not lead to a better resolution of enantiomers separation and a column temperature similar with the room temperature is an optimum value.

Thus, we can conclude that the mobile phase consisting in phosphate buffer 20 mM, pH 3 and ethanol (90:10),

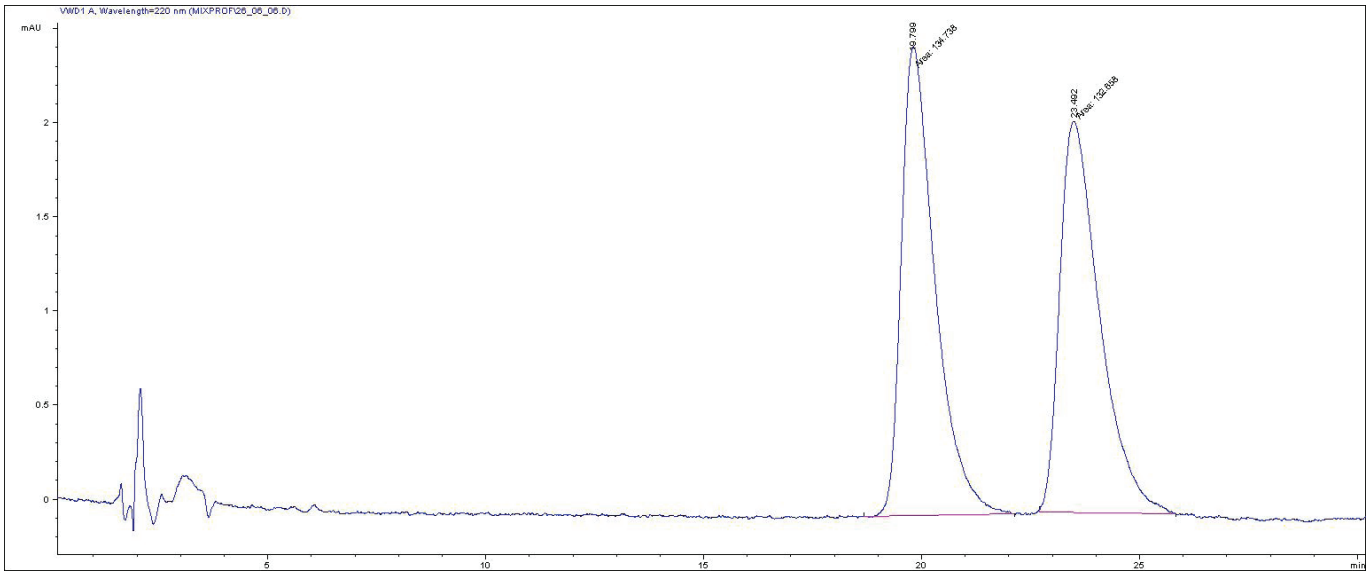

Fig. 2. Ibuprofen enantiomers separation with mobile phase A - KH2PO4 20 mM (pH=4.7), B - ethanol, 90%A:10%B, flow rate 1 ml/min, detection at 220 nm, column

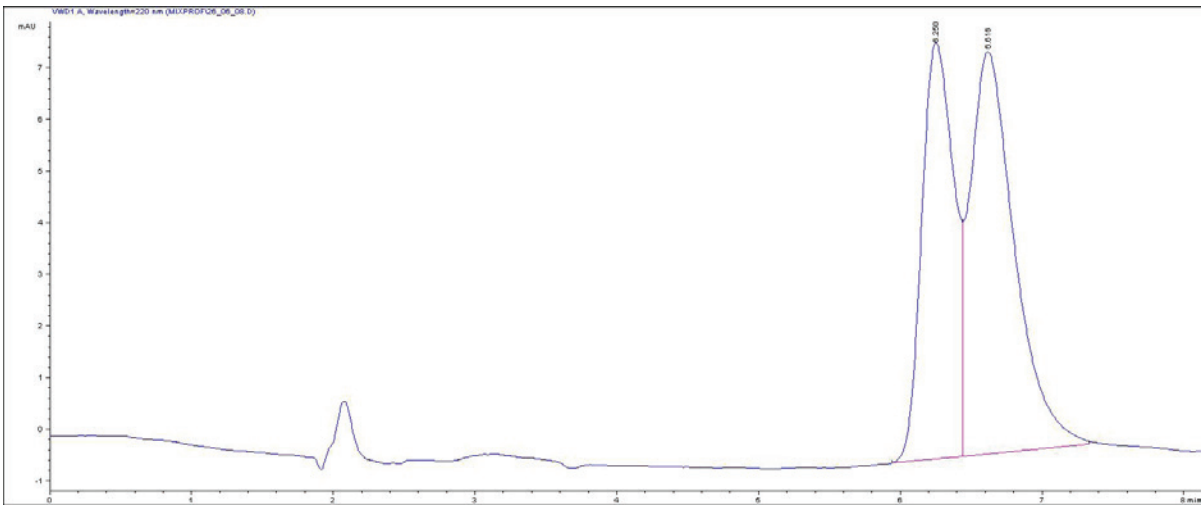

Fig. 3. Ibuprofen enantiomers separation with mobile phase A - KH2PO4 20 mM (pH=6.1), B - ethanol, 90%A:10%B, flow rate 1 ml/min, detection at 220 nm, column

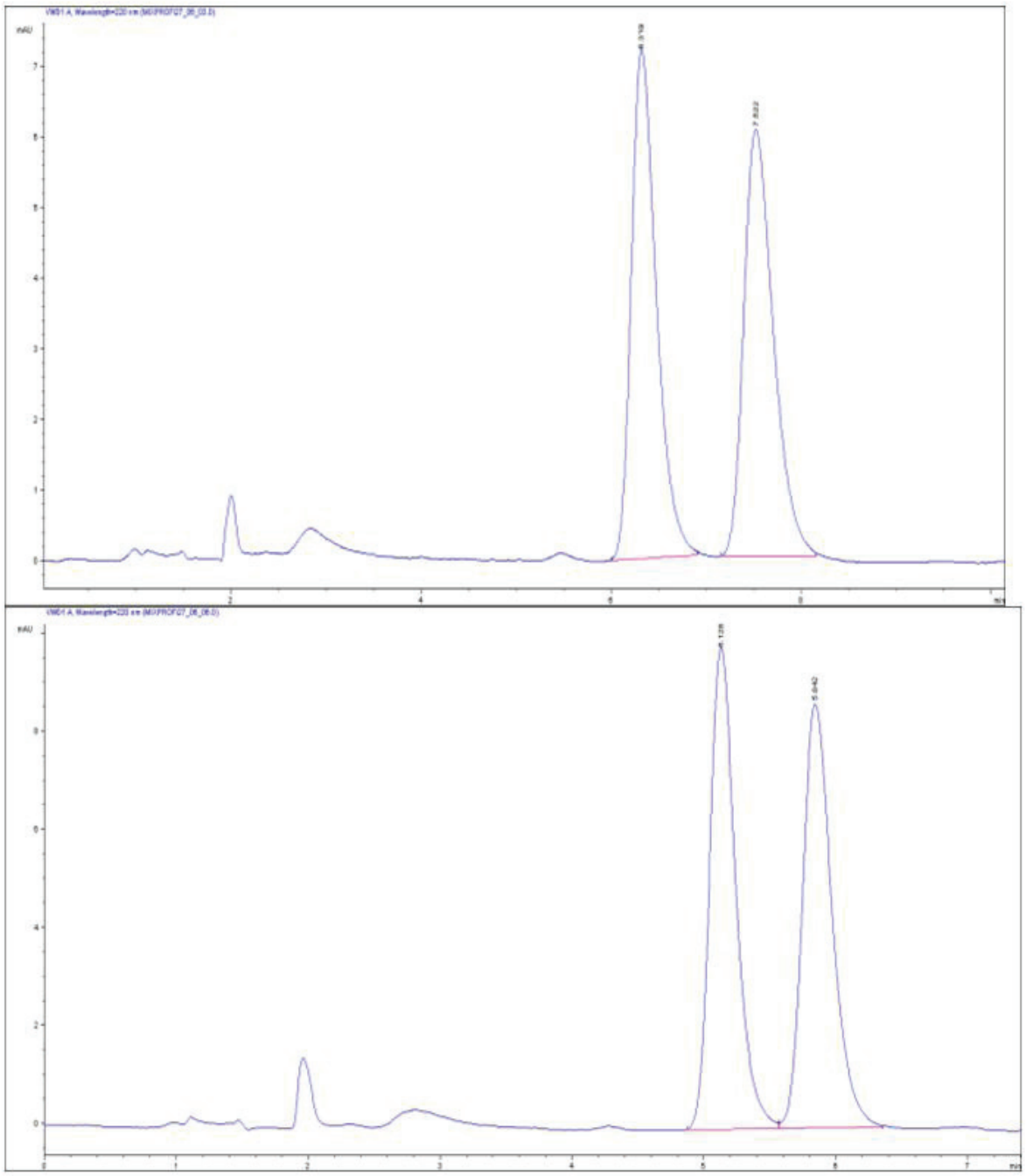

Fig. 4. Ibuprofen enantiomers separation with mobile phase A - KH2PO4 20 mM (pH=3.0), B - ethanol, 90%A:10%B, flow rate 1

Table I. The influence of temperature on chromatographic parameters of ibuprofen enantiomers

Parameters	Temperature			
	20°C	25°C	30°C	35°C
tR1, min	6.81	6.32	5.69	5.13
tR2, min	8.25	7.52	6.64	5.84
Rs	2.45	2.33	2.20	1.93

delivered at a flow rate of 1 ml/min, with a column temperature around room temperature and detection at 220 nm, are the separation conditions required for optimal resolution and detection of ibuprofen enantiomers. The separation process revealed first the R enantiomer and secondly the S enantiomer.

Capillary electrophoresis

The influence of buffer pH, temperature, cyclodextrine concentration and the nature of buffer was tested. Evaluation of different pH values for the buffer solution at room temperature has revealed a peak characteristic for ibuprofen racemate at pH=6.8. In this case, a lower pH of the buffer solution did not improve the separation. Furthermore, different temperatures of separation, 20°C, 25°C, 30°C, were tested. Unfortunately, no tendency of separation was noticed (Figure 5). Other tests were performed using different cyclodextrine concentrations (15 mM, 10 mM and 5 mM), and we observed a minimum of retention at 10 mM without separation of enantiomers. By replacing the phosphate buffer with borate buffer, the separation was not achieved.

Conclusions

The proposed HPLC chiral method is suitable for the separation of ibuprofen enantiomers. Further tests are necessary to conclude that the proposed method is suitable for chiral separation of ibuprofen in biological samples (i.e. plasma samples). None of the attempts regarding chiral capillary electrophoresis separation was successful.

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Conflict of interest

None to declare.

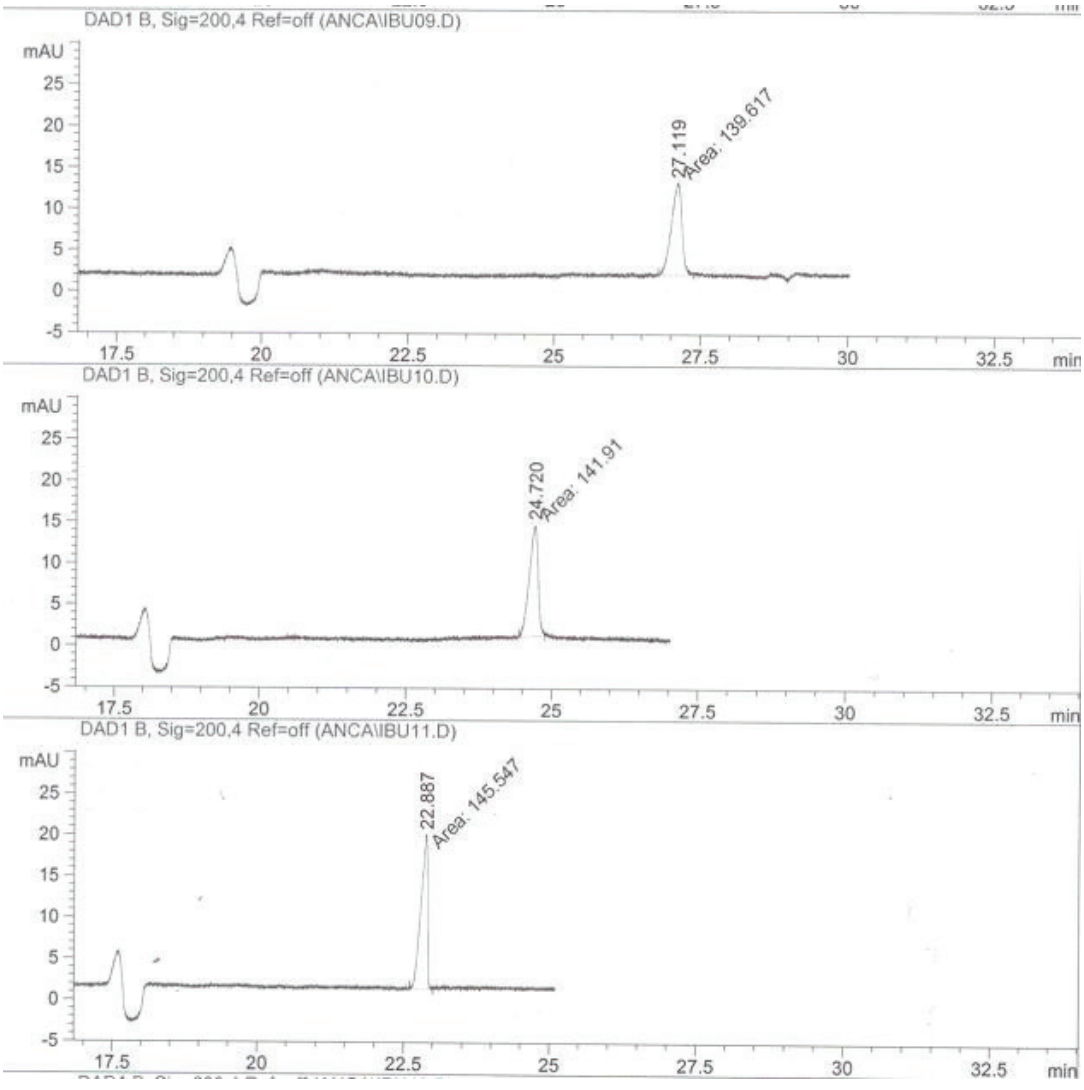

Fig 5. Ibuprofen electropherogram with phosphate buffer pH=6.8, methylbetacyclodextrine 10 mM, at 20C (up), 25C (middle), 30C (down)

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CASE REPORT

Practical Advantages of CBCT in the Surgical Treatment of Impacted Lower Third Molar

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University of Medicine and Pharmacy Tirgu Mures, Romania

Introduction: The imaging method of cone beam is an improved, extremely accurate computed tomography applicable in the whole field of dentistry. Due to its ability to locate the exact position of the impacted teeth, CBCT software has an important role in the management of difficult cases of impacted third molar. In some situations, the lower third molar is quite near to the inferior alveolar nerve that the surgical extraction can present a high risk of post-operative sensitive impairs of the skin and mucosa of the lower lip and chin on the same side. **Presentation of case series**: Our study tried to assess the contribution of CBCT in the pre-operative evaluation and further treatment of patients with impacted third molars in mandibular bone with high risk of inferior alveolar nerve injury. The paper presents three clinical cases showing positive signs on standard OPG, which exhibit indicators of a potential contact between the inferior alveolar nerve and the impacted lower third molars. For an improved exploration Dental CT Scan, DICOM image acquisition program, and 3D reconstruction with a special software were used. **Conclusions**: The study showed that compared with panoramic radiography, CBCT improve the evaluation of the surgical risk and allow a more accurate planning of surgery.

Keywords: impacted third molar, CBCT, inferior alveolar nerve, mandibular canal

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Introduction

In young adults, over 20 years old, the frequency of maxillary third molars impaction is considered to be about 46%, while the mandibular wisdom teeth impaction is approximately 73%. The incidence is equal for men and women. [1] One of the reasons that third molars inclusion can affect the health of the oral cavity is the large number and the frequency of clinical complications associated with it.[1,2]

The most common complications of the third molar eruption are: infections, dental crowding, tooth decay, periodontal disease, receding gums, loosened teeth, root resorption of adjacent teeth, and difficulties in adapting dental prostheses. Other modifications such as mandibular fractures, development of cysts and tumors, pain in head and neck area, trismus, trophic disorders are much more rare.[3]

To choose the right treatment is necessary to know the position and inclination of the tooth's long axis and the relationship to adjacent structures. Such information can be acquired using radiological examination.[4]

Since the first dental radiography performed in 1896 by Otto Walkhoff [5], radiological investigation methods used in dentistry have developed from standard x-ray images to digital radiology, CT scan, and MRI but especially to CBCT. The expense of MRI and high doses of radiation of classic CT limit their use to selected cases in maxillofacial area.[6]

The emergence of CBCT corrected many deficiencies of existing technology, expanding the use of 3D technology to other fields of dentistry. Cone Beam imaging method named CBCT is based on a perfected computed tomography technology applicable throughout the dentistry area.[7]

In maxillo-facial osseous conditions, CBCT provides information on the precise location of various pathological processes developed in the jaws or facial soft tissues and data about the adjacent anatomical structures.[8]

The presence of certain radiological signs on panoramic radiography such as narrowing, darkening or deflection of the root, dark, bifid or island-shaped apex, interruption of the mandibular canal cortical contour, canal deflection or narrowing, are associated with a veritable relationship between the roots of the third molar and the mandibular canal. However, only cross-sectional CT images obtained by conventional CT or CBCT can define the root-canal relationship in a buccal or lingual direction.[9]

Our study tried to assess the role of CBCT in the treatment of patients with impacted mandibular third molars in a difficult position and high risk of inferior alveolar nerve injury. The injury of the inferior alveolar nerve may represent a rare but serious neurologic complication in the surgery of the impacted third molars requiring a careful pre-operative imagistic evaluation of their anatomical relationship with the inferior alveolar nerve.

Presentation of case series

Panoramic radiography is considered to be sufficient in most cases prior removing mandibular third molars. However, CBCT is indicated when one or more signs of close contact between the wisdom tooth and mandibular canal are present in the two-dimensional image.

This paper presents three clinical cases showing positive signs on standard OPG, which exhibit signs of a potential

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contact between the inferior alveolar nerve and the impacted lower third molars. For a better defining the relationship between third molars and the mandibular canal were used Dental CBCT (Cranex 3D provided by Soredex - Tuusula, Finland), DICOM image acquisition program, and 3D reconstruction with a special software.

Case 1

A 25-year-old female patient was referred to maxillo-facial surgery department for moderate pain in the right lower jaw. On oral examination, inferior anterior dental crowding and absence of bilateral inferior third molar were identified.

The panoramic radiography (Figure 1) showed mandibular canal image superimposed over the third molar roots, on both sides. Contour lines of the left mandibular canal were barely visible, indicating a most likely lingual location. On the right side was found interrupted the cortical bone of the upper part of the canal and darkening of the roots of the third molar.

To proceed to a safer surgery, the patient underwent a CBCT examination to better evaluate the connection between the mandibular canal and the roots of the third molars (Figure 2). CBCT examination confirmed the lingual position of the left mandibular canal to the roots of left third molar and right mandibular canal crossing through the roots of the right third molar.

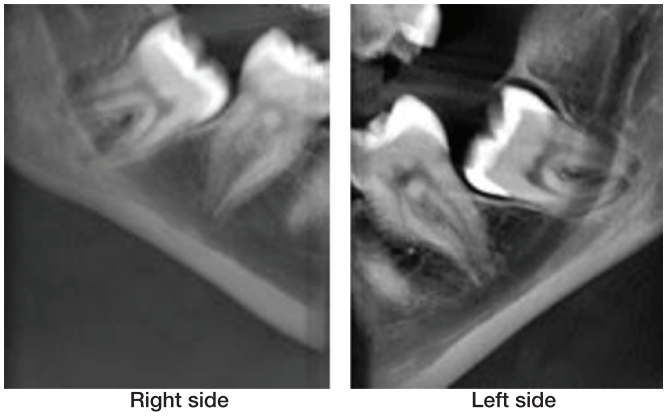
The 3-D reconstrucion confirmed the information provided by CBCT images and offered the possibility to appreciate the arrangement of anatomical details.

After studying CBCT, the surgeon decided to perform odontotomy, roots separation and gentle removing of segments in both third molars in order to avoid inferior alveolar nerve lesion. The patient developed a post-operative sensorial deficiency in left inferior alveolar nerve territory which was recovered almost completely in 8 weeks.

Case 2

A 22-year-old woman was referred to our department for pain, radiating from the left lower jaw to the left ear which appeared approximately one week ago, with increasing intensity, that did not respond to ibuprofen and metamizole. On intraoral examination, was found left inferior third molar in an abnormal position, with extensive caries and dental crowding both in the lower and upper dental arches.

On panoramic radiography (Figure 3.) was observed partial inclusion of both mandibular third molars with complete root formation. The left third molar had a mesial-angulated position, in contact with the second molar, showing a deep carious process and mesial bone resorp-



Right side Left side

Fig. 1. Panoramic radiography

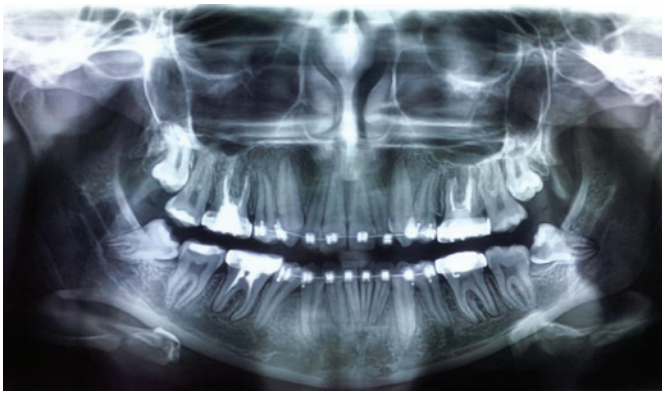
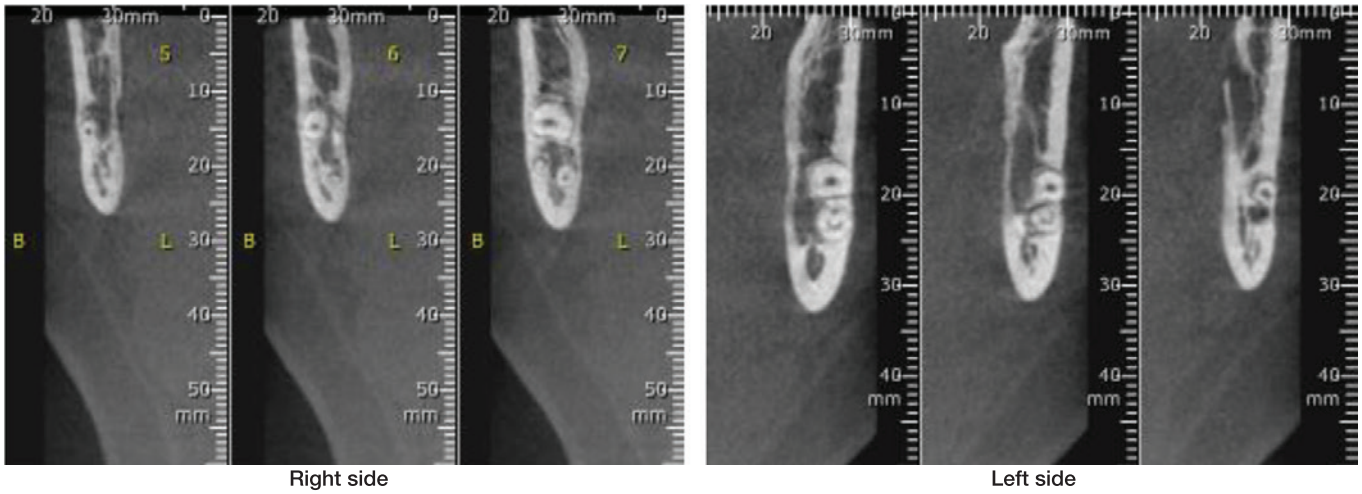


Fig. 3. Panoramic radiography with evident signs of close relationship of both lower third molars with mandibular canal



Right side Left side

Fig. 2. CBCT section showing the intimate relationship between lower third molars roots and mandibular canal

tion. The roots were convergent overlapping on the mandibular canal. The mandibular canal cortical lines appeared well defined showing a slight deviation near the root apex.

On CBCT examination were specified the position of the left mandibular canal near the vestibular cortical and it contacts with the third molar roots (Figure 4.).

CBCT analysis indicated the position of the roots in contact with the mandibular canal and high risk of inferior alveolar nerve harming, the surgical option being modified for careful separate roots removal. The patient presented slight sensorial deficiency at the left lower lip level which has recovered in 2-3 weeks.

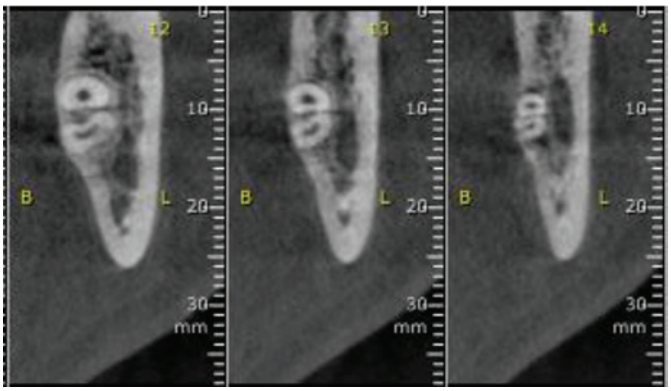


Fig. 4. CBCT sections indicating the left lower third molar roots in contact with mandibular canal

Case 3

A 24-year-old woman was examined in the emergency service for severe, radiating pain at the right lower jaw, which started 24 hours ago, with no obvious response to ibuprofen. Her medical and dental histories were unremarkable.

On panoramic radiography (Figure 5.) were found both mandibular third molars partially impacted with complete root formation. At the right second molar was observed a distal cavity associated with impacted third molar in a mesial incline. The right third molar presented roots overlapping on the mandibular canal whose shape appears as two radiopaque, net lines, which can be easily distinguished, not showing deviations, probably in a vestibular position.

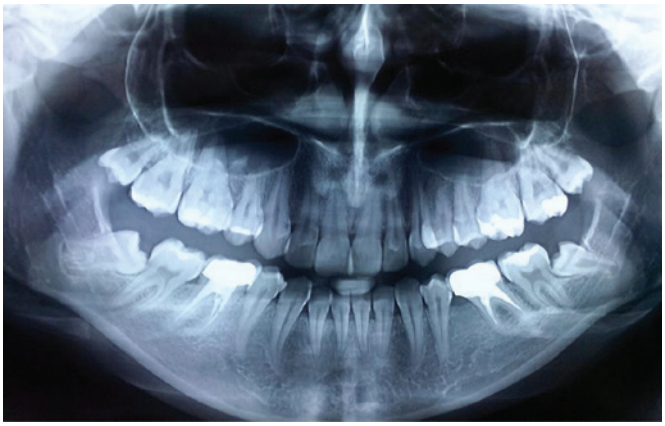


Fig. 5. Panoramic radiography that indicate signs of right trird molar roots in posible contact with mandibular canal

On CBCT examination was identified a vestibular position of the mandibular canal and a punctiform contact with the roots of the right third molar (Figure 6.).

Surgical plan was modified from initial odontectomy to odontotomy with roots separation. The patient presented no post-operative sensory impairment.

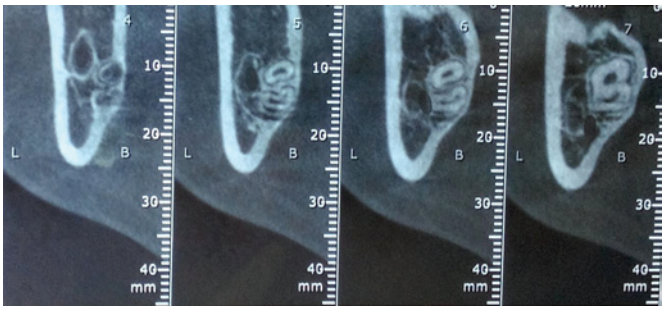


Fig. 6. CBCT sections showing the contact of the right lower third molar roots with mandibular canal

Discussion

In oral and maxillo-facial surgery, panoramic radiography is the first-level imaging of choice in the pre-operative evaluation of the third molar.[10]

Radiographic signs, detectable on the panoramic radiography that indicate the presence of a close relationship between the inferior alveolar nerve and the lower third molar are [11,12]:

– Radiotrasparent band darkening the root of the third molar because of the decreasing of bone density produced by the mandibular canal crossing the area.
– Interruption of the line marking the roof of the canal due to the root of the third molar crossing it.
– Sudden change of direction or narrowing of the mandibular canal at the point in which it is in contact with or superposed on the roots of the third molar.
– Abrupt deviation of the roots of the third molar at the point where they superpose on or come in contact with the mandibular canal.
– Bifid apex with third molar root darkening or root depression at the point where they are crossed by the inferior alveolar nerve.
– Superposition of the roots of the third molar and the mandibular canal.
– The roots of the third molar are in contact with the roof of the mandibular canal

There are studies which report that darkening of the roots, interruption of the cortex of the roof of the canal, and canal deviation detected pre-operatively are the radiographic signs that are most often associated with inferior alveolar nerve exposure and intraoperative injury. [10,13,14,15]

The absence of positive radiographic signs on the panoramic radiography is preferable to their presence for preoperative diagnostic purposes and for a reliable surgical approach. Without positive radiographic signs on pano-

ramic radiography, the risk of inferior alveolar nerve injury is considered to be small, but the presence of one or more signs might indicate a high probability of intra-operative exposure of the vascular-nervous bundle.[16]

Being a bi-dimensional examination, OPT does not provide information on the depth of the anatomical structures studied and locates the mandibular canal only in the vertical and not in the horizontal plane. On the other hand, it provides a distorted magnification by a variable factor greater horizontally than vertical, and anatomical structures are overlapped - air shadows, soft tissues, and phantom images.[16,17]

Some authors compared the diagnostic accuracy of OPT vs. CBCT in detecting the relationship between the root apex of third molars and the mandibular canal. A significant difference was found between the two techniques on the horizontal plane, but the diagnostic information provided by CBCT was far better.[18]

Most authors consent that CBCT and Dental CT Scan are the most effective imaging techniques to identify the localization of the mandibular canal regarding its superior/inferior and buccal/lingual dimensions, and the precise crown-root morphology of third molars.[18]

CT examination should be used only for patients in whom panoramic radiography shows one or more of the radiographic signs indicating an intimate relationship between the mandibular canal and the third molar root, but this relationship cannot be defined sufficiently using conventional radiology. [12,15,19,20] Thereby, it may substantially contribute on the planning of the surgical approach and on the evaluation of consequences and results. [16]

CBCT gives undistorted three-dimensional images with a very good resolution that allows visualization of anatomical structures shape and their real size.[21]

CBCT exam permits to evaluate the buccolingual relationship between the mandibular canal and the roots of the third molar thus avoiding to push the tooth during surgical movements and to hurt the inferior alveolar nerve. In this way can be planned the appropriate interradicular section if it is evident that the inferior alveolar nerve crosses the roots. CBCT exam can also identify the presence or the absence of cortical bone around the inferior alveolar nerve and allows to detect the number of roots of the third molar and the precise their anatomy. Furthermore, CBCT determines the inclination of the tooth and the position of the crown in relation to the buccal or lingual surface of the mandible.[16,22]

The usage of CBCT has reduced the cost for patients, and mostly it has improved the risk-benefit ratio by reducing the dose of radiations for patients compared to standard CT exam.[15] In cases where the roots of the third molars have a complex morphology being located in contact with the mandibular canal, the new 3D reconstruction programs assisting CT images provide sharp visualization in the three spatial planes of the structures from the mandibular canal that have to be respected.[16]

3D images are not mandatory for the pre-operative evaluation of third molars. They only complete anatomical image's details that might influence the surgical approach: single or multiple odontotomy, depth of osteotomy or direction of luxation which can be programmed more accurately.[16]

Conclusions

CBCT is an excellent diagnostic method for selected situations in oral and maxillo-facial surgery, including evaluation of mandibular third molars, but its efficiency has been lesser studied yet. Panoramic radiography may be sufficient in most cases before removal of mandibular third molars, but CBCT may be indicated when one or more signs of close contact between the tooth and the mandibular canal are present in the standard panoramic radiography. In these situations, CBCT might change the surgical approach and patient's outcomes.

The study have shown that CBCT contributes to optimal risk assessment and an adequate surgical planning, compared with panoramic radiography.

The mandibular morphology at the third molar region with impacted teeth and the location of the mandibular canal could be distinctly determined using cross-sectional CBCT images.

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Conflict of interest

None to declare.

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