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CUPRINS

In memoriam
Docent Professor Dr. Gheorghe Arsenescu
1919-1987

July 15th of this year, 2019, marks the 100th anniversary of the birth of Professor Gheorghe Arsenescu, he who headed the department of Physiology at our University for almost a quarter of a century, between 1963 and 1987.

75 years previous, in September 1944, a young medical officer, a recent graduate of the Military-Sanitary Institute of Bucharest, originally from Cocu-Arges, fought with weapon in hand, after all the officers of his subunit were killed, to conquer back the Sângeorz hill near Oarba de Mureș. Many of the 11,000 Romanian soldiers who died in this battle, one of the fiercest fought by the Romanian army for the liberation of northern Transylvania, under Hungarian occupation following the 1940 Vienna Dictate, were killed under his very own eyes.

For these deeds, doctor Gheorghe Arsenescu was awarded with the order of Mihai Viteazul with swords, a medal only ever bestowed to a few, and those few only ever officers distinguished in combat.

The city of Tg. Mureș was liberated by Romanian and Soviet troops on the 28th of September, 1944, while the fighting in Oarba was still raging, to end only on the 6th of October.

Destiny has contrived to make his last and most fruitful years be spent here in Tg.Mureș, not far from the place where he risked his life so that we can be who we are today.

After the war, Gheorghe Arsenescu became a scientist at the Institute of Normal and Pathological Physiology "Daniel Danielopolu" in Bucharest. He returned to Tg. Mureș 19 years later, in 1963, as Associate Professor and then Professor at the Department of Physiology, a position which he held until his death in 1987.

As a teacher, he was always preoccupied with the level of preparation of his students, whom he treated with unrivalled understanding and kindness. His lectures were of the highest level, perhaps harder to understand by some students, but admired and respected by all for the unequalled way in which they were presented.

Both leader and mentor to his doctoral students, among whose number I am honored to count myself, he endeavored to make of them his true friends and collaborators, to teach them to be exigent with themselves, to spare no effort in achieving something worthy of the trust he had granted them. He was sparing with praise, but deep down in his heart he always knew how to hold those he loved and cherished in the high place they deserved.

His greatest passion was research, a passion learned from his mentor Professor Danielopolu.

In the 24 years spent at Tg. Mures he was ever preoccupied with everything novel, and together with the team he led he created new fields of activity. He introduced, for the first time in our country, experimental myocardial microelectrophysiology, the use of radioisotopes in experimental cardiology, clinical use of orthogonal electrocardiography and vectorcardiography, as well as studies on the automatic computer processing of ECG data.

His more than 150 scientific works made him well known, in the country and abroad. In his capacity as a member of the International Council of the Society of Electrocardiology, he participated with presented papers at 15 international congresses of the society.

He achieved everything through hard, often exhausting, work. His was the legacy of a long line of people who knew they had to strive forward with nothing beyond their own wits and strength of arms.

He was a modest, honest and fair man, with tremendous will and strength, with a robustness you would not have guessed in his ascetic body.

He was passionate about history especially that of his country of which he was supremely proud, like many in his area of the Arges sub-Carpathians. A colonel Gheorghe Arsenescu is known today as an anti-communist resistance fighter in the Muscel area, who was executed for his struggles in 1962.

Even as the merciless illness laid him dying on his hospital bed, he continued to work on the manuscript of a lecture, until his final moments. He died in June 1987 and was buried in his hometown, with his forefathers, in the land he had loved so deeply.

Now, 100 years after his birth, we remember our teacher, with gratitude for all that he has done so that we who followed him can take his faith further and farther, to new heights.

Prof. dr. Marius Sabău
UMF Targu Mures

HUMAN INDUCED PLURIPOTENT STEM CELLS-DERIVED CARDIOMYOCYTES FOR CARDIAC APPLICATIONS

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ABSTRACT

Induced pluripotent stem cells (iPSCs) are a type of cells that can be generated directly from adult cells and can be differentiated into many cell lines, including cardiomyocytes. The first reprogramming methods had low efficiency but lately safe lines of iPSCs without insertional mutations have been achieved. The human iPSCs technology offers a unique opportunity to obtain patient-specific hiPSC-derived cardiomyocytes. This platform allows iPSCs to be used for drug testing, disease modeling and cell therapy development. In this review, we discuss the progress and applications regarding human iPSCs, including their potential, limitations and future directions.

Keywords: cardiomyocytes, embryonic stem cells, end-stage heart failure, induced pluripotent stem cells, long QT syndrome, torsade-de-pointes.

1. INDUCED PLURIPOTENT STEM CELLS – FROM PAST TO FUTURE

The discovery that somatic cells could be used to obtain pluripotent stem cells (PSCs) has made revolutionary changes in stem cell research. Induced pluripotent stem cells (iPSCs) from mouse embryonic and adult fibroblast cultures were first reported in 2006 by Yamanaka and Takahashi [1]. After that, the same researchers obtained iPSCs from human fibroblasts by viral overexpression of the same specific transcription factors with mouse iPSCs, OCT3/4, SOX2, Klf4 and c – Myc. The obtained human iPSCs were very similar to human embryonic stem (ES) cells in many aspects. Regarding morphology, the iPSCs had large nuclei and scant cytoplasm, like ES cells. They showed feeder dependency with no attachment to gelatin-coated tissue-culture plates and similar proliferation as ES cells. iPSCs expressed human ES cell-specific surface markers like stage-specific embryonic antigen (SSEA-3, SSEA-4), tumor-related antigen (TRA-1-60, TRA-1-81) and NANOG protein. The studied cells expressed undifferentiated ES cell-marker genes such as: OCT3/4, SOX2, NANOG, growth and differentiation factor 3 (GDF3), fibroblast growth factor 4 (FGF4), reduced expression 1 (REX1) and many others. IPSCs showed high telomerase activity and teratoma formation when injected subcutaneously to immunodeficient mice. Also, human iPSCs showed *in vitro* differentiation to multiple cell lines like neuronal or cardiac [2]. After this important discovery, iPSCs were obtained from many other cell-specific tissues, such as keratinocytes or blood cells [3,4].

Initially, retroviruses and lentiviruses were used to obtain iPSCs from somatic human cells. The main risk using these techniques was the development of insertional mutations in the cells. To avoid it, other methods of reprogramming with nonintegrating viruses were studied. A good expression vehicle for generating human iPSCs was adenovirus being a non-integrating virus. The disadvantage of this method was having a low efficiency [5]. A much safer and efficient method of obtaining human iPSC was using non-viral reprogramming methods like mRNA transfection [6], removable PiggyBac transposons [7] and plasmid vectors [8].

The low efficiency of the reprogramming methods was significantly improved by adding different chemical compounds such as valproic acid [9], sodium butyrate [10] or creating a hypoxic environment [11]. From these, valproic acid and sodium butyrate are the most commonly used in obtaining human iPSCs.

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Human iPSCs give patient-specific derived cells being a faithful genetic copy of the cells taken from a donor. Their use comes with its own challenges and limitations such as difficulty in maintaining fully differentiated cardiomyocytes (CMs) in culture. Reprogramming human adult somatic cells into iPSCs solved the ethical issues of using human ES cells in research.

2. CHARACTERIZATION OF INDUCED PLURIPOTENT STEM CELLS LINES

Since more than a decade ago, when iPSCs have been discovered, a great progress was made in stem cell biology and regenerative medicine. This progress came together with an important concern for their safety and use in clinical applications and disease modeling. Unfortunately, studies showed that iPSCs could sometimes develop genetic abnormalities during reprogramming or cell culture, being unqualified for further use. Researchers advise the genome of iPSCs to be monitored regularly during experiments, otherwise the tumorigenic risk of such cells could increase.

One factor that can produce genomic alterations is a prolonged cell culture. The first reported abnormality in iPSC lines was aneuploidy [12]. In the same time, it is very important that culture conditions to not influence the cell genetic integrity. Jacobs et al. showed in a study that higher-density culture resulted in DNA damage and genome instability [13]. Another study demonstrated that enzymatic single-cell passaging can produce karyotype abnormalities to human iPSCs genome such as trisomy 12 or 20q11.21 amplification [14].

Studies showed that the process of reprogramming can cause genomic alterations when speaking about methods using integrating vectors, such as human retroviruses. To overcome this problem, other methods were preferred such as plasmids or Sendai viruses. Variable levels of aberrant DNA methylation were described in some studies as epigenetic differences between iPSC lines. Differences in DNA methylation was found especially in iPSCs derived from different sources such as CD34+ cells from peripheral blood or adult dermal fibroblasts [15]. Another study had controversial results showing that genomic patterns of different iPSC lines were similar from different cell sources when using the same reprogramming approach [16]. These problems can be partially resolved using different methods to decrease the genomic alterations during cell reprogramming such as supplementation of the medium with nucleosides [17]. However, some studies concluded that these changes in the iPSCs genome during the reprogramming protocol are not so harmful and usually don't stop the usage of the cells for research [18].

A new way of genome editing called clustered regularly interspaced short palindromic repeat (CRISPR)-Cas systems provide adaptive immunity against genomic alterations. This is a simple platform to generate site-specific nucleases (SSN) for targeting genome mutations. It also passes this resistance mechanism vertically to its progeny [19].

Initially, human iPSCs were cultured on mouse embryonic fibroblasts (MEFs) feeder cell lines. This protocol was efficient and fast but could come with the disadvantage of transferring animal pathogens and inducing immune response. Human dermal fibroblasts (HDFs) were also used but they could allow contamination which can be a problem when using the cells in therapeutic approaches. The solution for these problems is to replace the MEFs and HDFs feeder cell lines with something else. For human iPSCs, the field is moving towards feeder free culturing on Matrigel in supplemented media. Xeno-free media makes things more clinical applicable [20].

3. CARDIOMYOCYTES DERIVED FROM HUMAN iPSCs

First attempt in obtaining cardiomyocytes from human iPSCs was by using embryoid bodies but with a poor efficiency, limiting their utility for further applications [21]. The use of monolayer culture methods has been developed as a solution for a better cardiomyocyte production. Exposing the human PSCs to activin A and bone morphogenetic protein 4 (BMP4) in RPMI/B-27 medium was reported to be more efficient generating around 30% cardiomyocytes in the H7 human ESC line [22]. A protocol for obtaining cardiomyocytes upgraded the previous one by using TGF-β super family growth factors, lacking insulin in the differentiation medium and using GSK3 (glycogen synthase kinase 3) inhibitor pretreatment of undifferentiated human PSCs with a result of 30-90% cardiomyocytes across human PSC lines. Regulation of Wnt/ β-catenin using small molecules or shRNA showed to be a reliable method for obtaining cardiomyocytes from human iPSCs in a monolayer culture. Using this technique, cardiomyocytes showed a high degree of purity (85%) and mature electrophysiological properties [23].

When human iPSCs are differentiated into cardiomyocytes they become a heterogeneous mixture of several subtypes, including ventricular cardiomyocytes (VMs), atrial cardiomyocytes (AMs) and CMs from the sino-atrial node (SAN). So, the idea was to create each CM subtype using physiological and molecular markers to help discriminate them. A study conducted by Blazeski showed that most of the protocols are generating ventricular-like cells [24]. Protocols for AMs differentiation are not that efficient than those for VMs. Another study showed that relatively homogeneous VMs and AMs populations can be derived from human ESCs by regulating Noggin and retinoid signals [25]. CMs from the sino-atrial node are still difficult to produce. One of the most efficient protocols of obtaining SAN cells is by inhibition of neregulin1β/ErbB signaling and upregulation of SAN-CMs specific genes [26].

The main problem when obtaining CMs from iPSCs is their immaturity and the fact that they share more similarities with fetal than with adult human cardiomyocytes. Early hPSC-CMs are mostly round and small (like fetal cardiomyocytes), becoming later more elongated but not as much as adult cardiomyocytes [27]. There are four characteristics used to prove cardiomyocyte's maturity: composition and organization of the contractile apparatus, specific genes expression, metabolism and electrophysiological features. Studies showed that membrane composition, specific intracellular structures (such as the T-tubules which give the maturity of CMs are lacking in the *in vitro* adult cardiomyocytes), gap junctions are different in natal CMs when compared with CMs obtained from iPSCs. All of these can slow the conduction leading to arrhythmias [28]. When analyzing the specific gene expression (such as RyR2, SERCA, NCX, Hey2, Mlc2v, etc.), one can see that they are present in both CMs – native and *in vitro* with some differences in their expression levels [29]. Regarding the metabolic changes during CM maturation, embryonic glycolysis is changed to adult fatty acid β-oxidation. Also, a high oxidative redox potential is required in mature CMs [30]. Maybe the most important criteria when analyzing CM maturation is its function. For example, the expression ratio of β1/ β2 adrenergic receptors seems to be extremely important [31]. Human PSC-CMs have a small contraction force. Because of insufficient Ca^{2+} release and uptake, in human PSC-CMs there is a negative force-frequency relationship [32].

As explained above, it is very important that iPSC-CMs to be very similar to native CMs so that can be used for disease modeling and cardiac regeneration.

4. PREDICTION OF DRUG – INDUCED ARRYTHMIA

Before approving drugs for use, the potential to produce Torsade-de-Pointes (TdP) is tested by evaluating their ability to block the human ether-á-go-go related gene (hERG) channel *in vitro* and assessment for QT prolongation risk *in vivo* according to the ICH S7A/B guidelines. TdP is a polymorphic ventricular tachycardia that can lead to sudden cardiac death when it's not treated immediately.

Latest studies in the field tried to evaluate drug-induced cardiotoxicity using human induced pluripotent stem cell-derived cardiomyocytes. This method may offer a better evaluation of cardiac safety and efficacy for tested drugs. One important example in this direction is the Comprehensive in Vitro Proarrhythmic Assay (CiPA) initiative to detect drug-induced Torsade-de-Pointes (TdP). The main purpose of this initiative is to categorize drugs into high, intermediate and low risk of TdP. Human iPSCs are used to confirm in silico reconstructions of the electrophysiologic effects of drugs. Changes in the extracellular field potentials of active human SC-CMs using multielectrode array (MEA) platforms are observed as a result from drugs interaction [33]. One study published in 2015 has demonstrated the ability of hSC-CMs to detect delayed repolarization due to drugs exposure and holds promise to a new method to predict arrhythmogenic potentials [34]. There are still limitations when using human SC-CMs in drug safety testing because of their electrophysiological immaturity. Efforts are made in this direction to create more mature CMs from stem cells.

Another attempt to use human iPSC-CMs in prediction of drug-induced arrhythmia came from Japan Cardiac Safety Assessment (JiCSA). They studied the torsadogenic potential of 60 drugs and compare it with CiPA initiative, the results being extremely similar [35].

In the last years, a lot of studies have proposed a new drug induced torsadogenic risk system using human iPSC-CMs and the MEA system. Most of them compared the results with CredibleMeds®. The conclusion was that using human iPSC-CMs is reliable and has the potential to replace the hERG assay for torsadogenic risk prediction [36].

5. iPSCs IN CARDIO – ONCOLOGY

Cardiotoxic effects from cancer therapy using chemotherapeutic agents, including anthracyclines, antimetabolites, alkylating agents, tyrosine kinase inhibitors (TKIs), proteasome inhibitors (PIs) are a major cause of morbidity. It was necessary to develop preclinical models to predict human cardiotoxicity. The underlying mechanism of cardiotoxicity due to chemotherapeutic agents have not been fully elucidated until now. The most frequently used agents have the following cardiovascular effects: decline in left ventricle ejection fraction (LVEF), ischemia, cardiomyopathy, hypertension, vascular events, arrhythmias, myocarditis, etc.

Human iPSC-CMs have been used in cardiovascular research exploring the cardiotoxicity of different chemotherapeutic agents being a reliable alternative to invasive biopsy of human cardiac tissue. The most studied drug until now is doxorubicin, which belongs to anthracycline family. Doxorubicin inhibits the function of topoisomerase 2B (TOP 2B) causing cell death of cardiomyocytes. Cardiotoxicity of doxorubicin occurs in a dose-dependent manner [37]. A study published by Burridge et al. studied the different response to doxorubicin in human iPSC-CMs derived from healthy controls, patients treated with doxorubicin without cardiotoxicity (DOX) and patients with clinical cardiotoxicity due to doxorubicin (DOXTOX). The DOXTOX cells showed reduced cell viability, arrhythmic beating, and sarcomere disarray. Using human iPSC-CMs can unravel the molecular mechanisms of interindividual variation in toxicity induced by doxorubicin. It also holds promise for discovery of new cardio protectants such as N-Acetyl Cysteine (NAC) [38]. When comparing the effects of doxorubicin on 2D and 3D models derived from human iPSC-CMs, different cardiotoxic responses have been

shown, with an increased resistance in the 3D model [39]. Using human iPSC-CMs to create devices such "organ-on-a-chip" provides an opportunity to study cardiotoxicity of chemotherapeutics. In a study published by Zhang et al. doxorubicin induced cardiotoxicity, increased CK-MB levels and arrhythmias [40].

Monoclonal antibodies have revolutionized the treatment of cancer. Unfortunately, they also come with side effects, including cardiotoxicity. For example, trastuzumab promotes oxidative stress and downregulation of TOP2b gene expression in cardiomyocytes [41]. A recent study using human iPSC-CMs showed that trastuzumab is blocking the ErbB2/4 pathway that offers cardioprotection [42].

Using iPSC-CMs in chemotherapeutics testing may change the way drugs are screened, becoming a road to personalized medicine.

6. iPSCs AS A PLATFORM FOR CARDIAC DISEASE MODELING

Human iPSCs create patient-specific iPSC-derived cardiomyocytes *in vitro* as a platform for cardiac disease modeling. In this way, a lot of hereditary cardiac conditions like arrhythmias and cardiomyopathies can be studied.

6.1. Channelopathies

A lot of recent studies used iPSCs for exploring the fundamental mechanisms underlying proarrhythmic disease phenotypes like long QT syndrome, short QT syndrome, Brugada syndrome and catecholaminergic polymorphic ventricular tachycardia (CPVT). The maturity of iPSC-CMs is very important here so the molecular and structural characteristics, action potentials (APs), membrane currents and excitation-contraction coupling must be examined.

Long QT (LQT) syndrome is a life-threatening inherited disease characterized by a prolonged ventricular repolarization. There are three main subtypes of LQT syndrome: LQT1, LQT2 and LQT3 [43]. LQT1 syndrome is the most frequent and it's due to mutations in the KCNQ1 gene, encoding the α subunit of the K^+ channel responsible of I_{Ks}. The first study on LQT1 using human iPSC-CMs was published by Moretti et al. Action potentials of human iPSC-CMs were significantly prolonged in LQT1 probes compared to controls [44]. β-blocker (propranolol) was tested on iPSC-CMs from LQT syndrome patients showing beneficial effects on AP duration [45].

Described above, the combination of CRISPR/Cas9 with induced pluripotent stem cell technology can develop the study of hereditary cardiac arrhythmias. A study published by Makita et al showed that mutations in calmodulin genes caused early onset severe LQTS [46]. The mutations can be corrected by allele-specific knockdown using CRISPR technology, providing insights into new therapies [47].

Patient specific human iPSCs were also used to investigate CPVT. This arrhythmia is mainly caused by mutations in RYR2 (ryanodine receptor) or CASQ2 (calsequestrin). Human iPSC-CMs with these mutations showed an increased concentration of intracellular calcium and increased elementary Ca^{2+} release events after the addition of β adrenoreceptor agonist. Dantrolene was shown to ameliorate the disease, suppressing stress-induced arrhythmic events in CPVT human iPSC-CMs [48]. Besides dantrolene, carvedilol and flecainide were shown to have antiarrhythmic effects in cardiomyocytes derived from CPVT patients [49].

6.2. Cardiomyopathies

Besides cardiac arrhythmias, human iPSC-CMs have been used to study cardiomyopathies *in vitro*.

Familial dilated cardiomyopathy (DCM) is a disorder characterized by ventricular dilatation, systolic dysfunction, progressive heart failure and possibly development of arrhythmias. In a study published by Sun et al, the researchers evaluated the TNNT2 mutation (p.R173 W) in human iPSC-CMs from patients with familial DCM. This gene is expressed specially in the heart and regulates the contraction of cardiomyocytes. Comparing CMs from iPSCs carrying TNNT2 mutation with CMs from iPSCs of healthy controls, the first category exhibited an altered myofilament organization, altered Ca^{2+} handling and decreased contraction force. Some treatments for improving sarcomeric organization of CMs from patients with familial DCM were tested, showing that β-blockers (metoprolol) were protective [50].

Hypertrophic cardiomyopathy (HCM) is associated with abnormal thickening of the left ventricular myocardium, being one of the most common hereditary cardiac diseases. There are still no specific HCM therapies making this disease an attractive target for *in vitro* disease modeling. The pathogenic mechanism underlying the development of HCM was evaluated in a study published by Han L et al. Human iPSC-CMs from a patient with HCM caused by the MYH7 mutation were reported. Structural and functional abnormalities like increased intracellular calcium concentration and increased expression of HCM related genes and proteins were found in these cells. Calcium channel antagonists were proven to be benefic for human iPSC-CMs from HCM patients [51].

This finding indicates the potential of human iPSC-CMs for valid disease models and patient-specific therapies.

7. CARDIAC CELL THERAPY

Treatment efficiency in heart failure is limited for patients with severely decreased cardiac function. Therefore, cardiac cell therapy with human iPSC-CMs is a promising strategy for patients with end-stage heart failure. Transplanted

cardiomyocytes derived from pluripotent stem cells are expected to improve cardiac function.

A lot of studies have demonstrated the efficacy of cardiac stem cell therapy in small animal model. A study published by Rojas et al analyzed the engraftment and functional effects of iPSC-CMs in a murine model of myocardial infarction. iPSC-CMs were transplanted into the mice hearts 14 days after left anterior descending artery (LAD) ligation. Results showed that transplanted iPSC-CMs formed mature grafts within the myocardium, also significantly improved myocardial remodeling and pump function 28 days after LAD-ligation [52].

Recent studies have shifted towards large animal models. In a study conducted by Chong et al, one billion human embryonic stem cells-derived cardiomyocytes (ESC-CMs) were injected into the heart of adult pigtail macaques two weeks after induced myocardial infarction. Using a multi-drug regimen containing cyclosporine, methyl prednisone and basiliximab attenuated the immune rejection and improved survival of human ESC-CMs. However, transplanted human ESC-CMs did not survive at 140 days after transplantation so remuscularization as a mechanism for the better recovery of left ventricular function observed was excluded. A limitation of the study was the relatively small number of animals (32 monkeys) [53]. Extrapolating the experiment to the human heart, which is ten times bigger than a macaque's heart, an unrealistic number of human ESC-CMs would be needed to be injected into the human myocardium.

To overcome the poor survival of injected cells into the myocardium, patch-form cardiomyocytes have been tested in different studies. Engineered heart muscle from human ESC-CMs transplantation in a chronic myocardial infarction immunosuppressed rodent model led to high engraftment rates with no significant cell loss and progressive maturation of human cardiomyocytes. Also, no teratoma formation was observed. However, ESC-CMs engraftment could not be correlated with the functional cardiac improvement in the mice [54].

Lei Ye *et al.* reported engraftment of human iPSC-derived cardiomyocytes, endothelial cells and smooth cells into a porcine model of myocardial infarction. The 3D fibrin patch loaded with insulin growth factor (IGF) improved left ventricular function, reducing the infarct size, without inducing ventricular arrhythmias [55]. Anyway, researchers concluded that studies with longer follow-up periods are needed to ensure the benefits or potential side effects of the treatment.

The latest studies are focused on allogeneic transplantation models using iPSC-CMs, providing a major benefit in terms of immune rejection. Shiba et al performed an allogeneic transplantation experiment with iPSC-CMs from monkeys with major histocompatibility complex homozygosity. The grafted cardiomyocytes showed electrical coupling with the host cardiomyocytes and no evidence of immune rejection. The results are promising but further research to evaluate post-transplant arrhythmias is needed [56].

The first clinical transplantation of human ESC-cardiac progenitor cells embedded into a fibrin scaffold was performed to a 68 years old female patient, diabetic and with NYHA (New York Heart Association) III heart failure despite maximal medical therapy due to an extensive antero-lateral infarction. Three months after the surgery, the symptomatology improved, LVEF increased with 10% and a new-onset contractility was evident at echocardiography. No complications such as teratoma formation or arrhythmias were reported [57].

Cardiac myocytes derived from human ESCs are very similar to those derived from iPSCs so future approaches using iPSC-CMs for cardiac therapy should be studied.

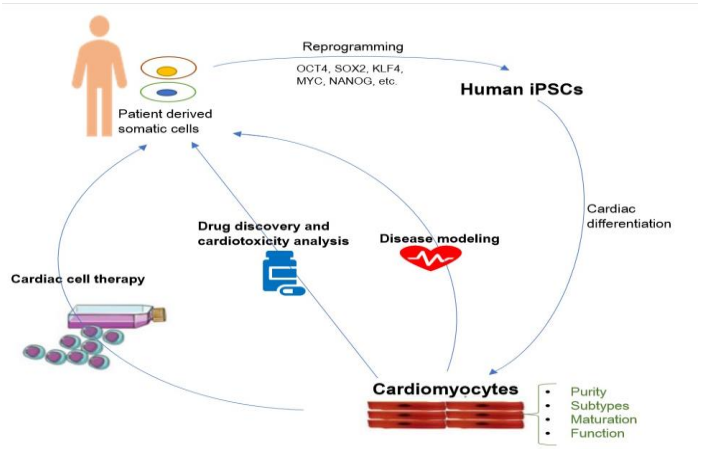

Fig.1. Application of human induced pluripotent stem cell-derived cardiomyocytes (iPSC-CMs) in drug discovery, disease modeling and cardiac cell therapy

CONCLUSIONS

Patient-derived iPSC-CMs provide a reliable model to study human heart tissue. They have been shown to play an important role in preclinical toxicity testing of drugs, disease modeling and brings hope for transplantation therapies in patients with end stage heart failure. iPSCs are a powerful tool that can provide a unique insight into therapeutic approaches for cardiac inherited disease management. Using novel techniques like CRISPR-Cas9 to genetically modify iPSCs will allow a high number of mutations to be studied in the next years. Despite their promising use in the future, there are still some obstacles that need to be overcome like improving maturity of human iPSC-CMs.

The future aims to create CMs in situ for heart regenerative therapy and with all the progress in the last years, this future may not be that far.

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APLICAȚII CLINICE PENTRU CARDIOMIOCITE OBȚINUTE DIN CELULE STEM PLURIPOTENTE INDUSE UMANE

REZUMAT

Celulele stem pluripotente induse (iPSCs) sunt un tip de celule ce pot fi generate direct din celule adulte și se pot diferenția spre mai multe linii celulare, inclusiv spre linia cardiomiocitara. Primele încercări de reprogramare au avut eficiență scăzută, pentru ca mai apoi să se obțină linii sigure, fără mutații de iPSCs. Această tehnologie oferă o oportunitate unică de a obține cardiomiocite pacient-specifice derivate din iPSCs. Această platformă permite utilizarea iPSCs pentru testarea medicamentelor, investigarea afectiunilor cardiace ereditare cât și pentru terapiile regenerative. Vom discuta în acest review progresul si posibilele aplicații ale celulelor stem pluripotente induse umane, inclusiv potențialul și limitările acestora.

Cuvinte cheie: cardiomiocite, celule stem embrionare, insuficiență cardiacă stadiu final, celule stem pluripotente induse, sindrom QT lung, torsada vârfurilor.

CAR-NK CELL-BASED THERAPY: AN ERA OF A NEW POTENTIAL IMMUNOTHERAPY

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ABSTRACT
Immuno-oncology field is in a continuous process of development by creation of revolutionized therapies as an alternative to the classical ones. Even though, at the beginning the immunomodulatory strategies were focused on using CAR-T cells as an alternative immunotherapy, the latest researches are focusing on developing an alternative immunotherapy such as CAR-NK. Natural killer cells are an important component of the immune system, having the capacity to secrete cytokines and chemokines. Because of this property, NK cells can kill the tumor cells and not only. In our review we are going to present the benefits and the latest results regarding therapies with NK cells, CAR-NK cells, also we are highlighting the importance of continuing studying them in order to improve the survival of the oncological patients.
Keywords: immunotherapy, chimeric antigen receptor (CAR), CAR-NK, tumor cells.

INTRODUCTION

Even though the conventional strategies used as therapies against cancers such as surgery, radiotherapy, chemotherapeutic agents offer substantial benefits, the negative effects of these therapies continue to be an important challenge [1,2]. Due to this, researchers have focused on developing new therapeutic alternatives in order to induce a potent response against tumor cells, with the association of fewer side effects [3]. Based on the important role of immune system to recognize and eliminate tumor cells, different immunotherapies have been developed [4]. In the last years, cancer immunotherapy has become an attractive and revolutionary strategy and showed its efficiency for treating cancer. This type of therapy utilizes the immune system in order to induce anti-tumor response by attacking tumor cells through natural mechanisms. For this, researchers have been developed different types of immunotherapies, many of them being assessed in clinical trials or being approved by the US Food and Drug Administration [2,5]. In the table below (Table I), we summarized all the immunotherapies approaches that have been studied till nowadays [6].

Table I: Classification of the immunotherapies

Immunotherapy	Description
Checkpoint inhibitors (CTLA-4, PD1, PDL1)	class of drugs that regulates immune system to increase its response against tumor cells
Agonists of co-stimulatory signals	have the role to bind to specific receptors present on the T cells surface and determine in a direct way activation, growth, differentiation of T cells in order to have an anti-tumor response
Adoptive cell therapy (TILs, TCR, CAR cells)	a cell based immunotherapy which has the role to manipulate immune cells, with enhancing their antineoplastic effect
Vaccines	cancer vaccines are an alternative immune therapy that uses dendritic cells, tumor cell lysate, neoantigens, nucleic acids to inhibit cancer progression and prevent its recurrence
Cytokines (IL, IFN, GM-CSF)	these immune regulators have the role to stimulate directly the immune system for an anti-tumor response

***Legend*:** CTLA4 cytotoxic Tlymphocyte associated protein 4, PD1 programmed cell death protein 1, PDL1 programmed Death ligand

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1, TILs tumor infiltrating lymphocytes, TCR T cell receptor, CAR chimeric antigen receptor, IL interleukin, IFN interferon, GM-CSF granulocyte-macrophage colon-stimulating factor. Adapted from Riley RS *et al. Nat Rev Drug Discov.* 2019;18(3):175–196 [6].

From all the immunotherapies we presented above, Adoptive Cell Therapy (ACT) (Figure 1) have started to gain interest, in recent years, because of the successful researches and studies regarding the applications of TIL therapies for melanoma and solid tumor, TCR-engineered cells for both hematological and solid tumor, CAR-T cells; latest being approved by the Food and Drug Administration (FDA) as treatment for B-cell malignancies [7].

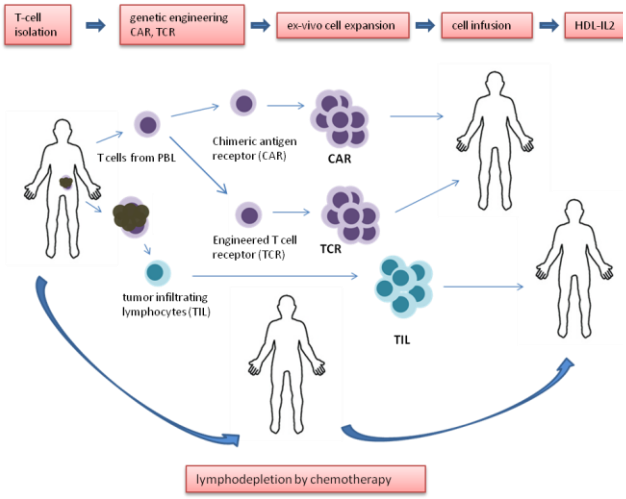

Fig.1. Classification of the adoptive cell therapies

Legend: Schematic representation of the main adoptive cell therapies: chimeric antigen receptor, engineered T cell receptor and tumor infiltrating lymphocytes. Adapted from Wolf B, *et al. Drug Saf.* 2019; 42(2):315-334 **[7].**

The principle of adoptive cell therapy is to treat the oncological patients using their own immune cells in order to have an anti-neoplastic response. For this, different approaches have been developed, such as:

- Tumor infiltrating lymphocytes therapy (TILs therapy): refers to using the lymphocytes infiltration (formed by a heterogeneous population of αβ T cells) presented in the cellular infiltration from the tumor microenvironment. This type of therapy refers to infusing back to the patient his own T cells which infiltrates normally the tumor, after those cells were isolated and expanded ex vivo. Prior infusion, the patient undergoes a lympho-depleting treatment. Even though, at the beginning, TILs were developed as an alternative immunotherapy with important result in treating malignant melanoma, the latest researches also showed the utility of using these lymphocytes as a prognostic marker [7,8].
- T cell receptor (TCR)-transduced T cell: TCR (Figure 2) is an alpha beta heterodimer receptor which has the property to bind to targeted antigens which are associated with the major histocompatibility complex proteins (MHC) [9]. This adoptive cell therapy that utilizes TCR refers to usage of autologous T lymphocytes which are modified *ex vivo* in order to present T cell receptors specific for the antigens we want to target.

Chimeric antigen receptor (CAR) therapy: is another immunotherapy which is based on producing, ex vivo, genetically engineered T cells that express chimeric antigen receptor. These modified T cells will be able to recognize tumor-associated antigens [7].

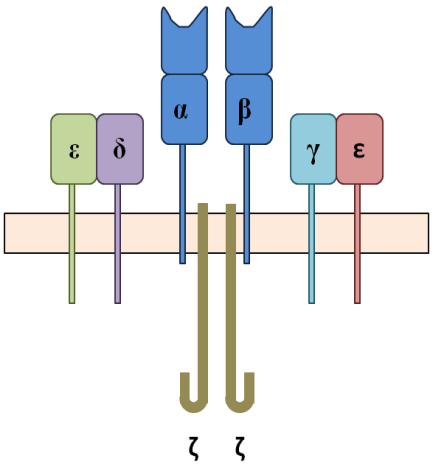

Fig.2. T cell receptor

Legend. T-cell receptor has in its structure the α and β chains, each of them having two extracellular domains, a variable domain and a constant region. The constant region is associated transmembrane with a CD3 complex with three dimers (CD3γ and CD3ε; CD3δ and CD3ε; CD3ζ). For an efficient activation of the cell, also there is required an accessory adhesion molecule expressed by T cell (CD4 for MHC class II and CD8 for MHC class I). Adapted from Sharpe M, et al. Model Mech. 2015;8(4):337-50 [10].

Within this review, we will summarize all therapies involving the use of chimeric antigen receptor (CAR) and natural killer cells (NK cells). Finally, we will discuss the utility of a new therapy - CAR-NK- as an alternative immunotherapy not only for cancer but also for other pathologies and the latest progresses in using this novel therapy.

CHIMERIC ANTIGEN RECEPTOR

As we presented above, there are three main adoptive cell therapies which are currently in research and we are

going to focus on the CAR cells therapies and their potential as an alternative immunotherapy.

Structure and generations of CARs

Because of TCR technology limitations to only recognize intracellular antigens associated with major histocompatibility complex, researchers developed a non-MHC restricted method. This new alternative is based on using genetically engineered lymphocytes T in order to express on their surfaces chimeric antigen receptors which can recognize a vary of antigens expressed at higher densities on the cell surface in a non-MHC-restricted manner. Also, CARs have the property to recognize other structure such as carbohydrates, glycolipids, increasing their ability to recognize a wide range of different targeted cells [11,12].

In 1989 this genetically engineered lymphocytes T were studied for the first time by Eshhar and his colleagues at the Weizmann Institute of Science in Israel [12]. They designed the first generation of CAR, consisted of an extracellular domain (ectodomain) represented by a variable fragment (scFv) which contain a region for antigen binding, a connecting sequence – hinge region- between ectodomain and transmembrane domain, a transmembrane domain and an intracellular domain (endodomain) which has attached a signaling domain that is derived from CD3 complex (CD3 ζ) (Figure 3). The ScFv ectodomain of CAR T cell represents the antigen-recognition region and consists of a variable light chain and heavy chains which are linked by a flexible linker, designed in recognizing and binding tumor-associated antigens expressed on the surface of tumor cells [13]. The most used linker is G4S which confers a series of properties such as flexibility and solubility. Also, the orientations of heavy chain, light chain and linker may influence the affinity and specificity of ScFv [12].

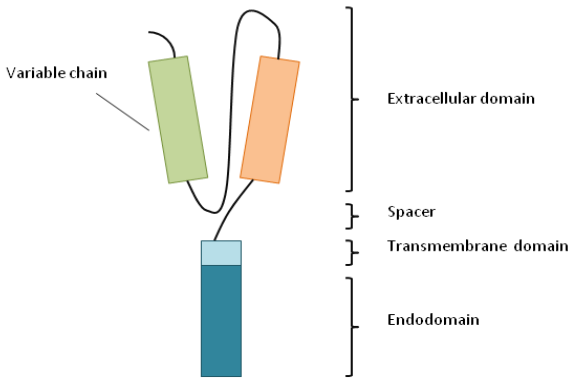

Fig.3. Structure of chimeric antigen receptor (CAR)

Legend. A schematic representation of the chimeric antigen receptors structure which is made of a variable chain (extracellular domain), a spacer, a transmembrane domain and an intracellular domain (endodomain) which has a variable form depending on the CAR generation. Adapted from Zhang C, et al. Biomark Res. 2017;5:22 [17].

Between the ectodomain and transmembrane region there is an extracellular structure called hinge region or spacer, which has the role to offer stability and flexibility for expression and activity of CARs. Also, there are research studies made by Guest *et al.*, Hudecek *et al.* which proves the influence of the spacer over the length and flexibility of the CARs [14, 15].

The transmembrane region has the role to connect the extracellular and intracellular domains and to offer CAR stability. It may be formed by a variety of molecules such as CD3-ζ, CD4, CD8, or CD28. Even though transmembrane domain wasn't considered an important structure, latest discoveries showed an influence over CAR-T cell function [13]. Solvado et al. have shown instability over time of first generation of CAR CD19 whose transmembrane domain contains CD3-ζ, in comparison with CAR CD19 with CD28 transmembrane domain [14]. Morin et al. also demonstrated the power of extracellular and intracellular CD28 domain to partially induce T cell activation [15, 16].

The endodomain (or the intracellular region) is the functional part of the CAR that has the role to transmit the signal intracellular in order to activate and induce proliferation over T cell [17]. This intracellular domain is, in general, represented by a CD3ζ domain, but its structure may differ according to CARs generations [12].

Thus, based on the endodomain structure, there have been developed four generations of CARs (Figure 4). The endodomain of the first generation of CAR is consisted of a single signaling structure which may derive from IgE (FcεRIγ) or CD3ζ chain. There have been made several *in vivo* studies which demonstrated a better rate in killing tumor cells for CAR containing CD3ζ endodomain, reason why almost all CARs that have been developed had in their structure a signaling structure derived from CD3ζ [16,17]. Because of the poor outcomes achieved after in vivo studies of the first generation of CARs properties, researchers developed a second generation of CARs.

The difference between the first and the second generation of CAR is the presence of a co-stimulatory signal (CD28-B7 system) associated to the CD3ζ domain for the second generation of CAR. The CD28/B7 co-stimulatory domain was added in order to determine a sufficient synthesis of Il-2 for a better activation of the T cells.

The desire to improve the T cell function and to optimize the design of CARs, a third generation of this type of receptors has been developed. This new generation of CARs has in their structure a multiple co-stimulatory domain (CD28, CD3ζ, OX40 or 4-1BB) [16].

The latest researches had led to development of a new and improved generation of CARs (TRUCKs) by using CAR-T cells as a transgenic product carrier for inducing a better anti-tumor response [18].

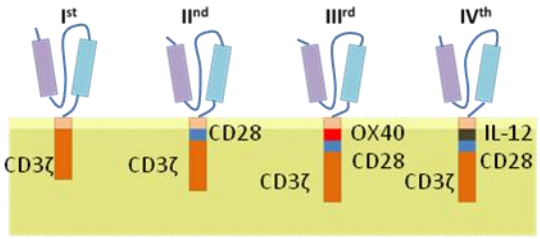

Fig.4. Generations of CARs

Legend. A representation of CARs evolution in all generations that have been developed. All generations contain an ectodomain, a transmembrane domain and an nedodomain which differs from generation to another.Aadapted from Zhang C, et al. Biomark Res. 2017;5:22 [17].

Production of CAR-T cells

Before the administration of the CAR-T cells to the patient, there are required several steps in order to obtain the desired product. From the patient's peripheral blood, leukocytes are isolated through leukapheresis. The next steps involve the separation of T cells from the leukocytes and their culture activation. Also, it may be performed a T cells subset CD4/CD8 separation using markers or specific antibody bead conjugates. T-cells isolated from the patient's blood are genetically modified using vector (viral or non-viral) (Table II) in order to integrate genetic material which, encode CAR, into T cell genome. This process will lead to the CARs expression on the T cells surface, cells that will be later expanded in culture in order to have enough cells for infusing back to the patient [20,21].

Table II. Transfection methods

Viral transduction	- Retroviruses - Adenoviruses - Adeno-associated viruses
Transposons	- Sleeping beauty system - Piggyback system
CRISPR/Cas9	
Non-viral transfer methods	- Non-viral transfer of plasmid DNA - IVT mRNA

Legend. Classification of all transfection methods used for inoculation of CARs genome inside the T cells. Adapted from Miliotou AN, et al. Curr Pharm Biotechnol. 2018; 19(1):5-18 [19].

Clinical applications of CARs

CAR-T cells therapy in blood malignancies

Many clinical trials showed that CAR-T cells therapy had the most the most effectiveness in relapsed or refractory B-cell malignancies (B-cell non-Hodgkin lymphoma, acute lymphoblastic and chronic lymphocytic leukemia), with a complete remission between 70% and 94%. After these results, many more trials have been conducted, showing promising results regarding usage of CAR-T cells therapy against targets as CD19, CD20, CD30 [19, 22]. The most studied target is CD19 because of its presence on the B cells, both normal and neoplasic ones, without being expressed on other tissues. Even though all clinical trials showed successful outcomes, CD19 CAR-T cells therapy induce B cell aplasia, aplasia which may be treated with immunoglobulin infusion [20,23]. Because of these successful results, FDA and EMA approved for the regular usage this therapy (Kymriah, Novartis) by using CAR technology for pediatric patients and young adults diagnosed with relapse or refractory Acute lymphocytic leukemia [19].

CAR-T cells therapy in solid tumors

Regarding CAR-T cells therapy for solid tumors, there are some limitations which make more difficult to treat these types of tumors. Some limitations are represented by:

- instability of tumor cells by stopping their expression of the antigens
- histopathological features of the cancer cells
- tumor heterogenicity
- tumor environment which may inactivate CAR-T cells
- inadequate ways of CAR-T cells transportation to the cancer sites.

Many clinical trials showed promising results in using this therapy for solid tumors by targeting types of proteins (epidermal growth factor receptor, human epidermal growth factor receptor 2, carcinoembryonic antigen, mesothelin) presented on the cancer cells surfaces, proteins which also haven an important role in tumorigenesis. In last years, in vivo/in vitro experiments with T cells transfected with this type of CARs were able to induce tumor apoptosis [8, 19, 24].

Challenges of CAR-T cells therapy

Even though CAR-T cells therapy has shown impressive anti-tumor results in clinical trials and in research field, there are some challenges to overcome in order to obtain a safe immunotherapy alternative:

- precision in targeting tumor cells
- prevention of mutagenesis and malignant transformation during viral vector transduction (genotoxicity)
- a better control of the activation and proliferation of CAR-T cells
- prevention of side effects caused by CAR-T cell infusion:
  - neurotoxicity
  - cytokine release syndrome (CRS)
  - B cell aplasia (CD19 CAR-T cell therapy)

- Systemic inflammatory response syndrome [19,23].

NATURAL KILLER CELLS

In order to have a better knowledge of CAR-NK, first, we must understand the biology and role of the natural killer cells, as well as their clinical applications; subjects we are going to approach in this chapter of our review.

The biology and role of the natural killer cells

Natural killer cells (NK cells/killer cells) are large granular lymphocytes which, together with eosinophils, basophils, phagocytic cells, and mast cells are part of the innate immune system and they also have an important role in defense against cancer and viral infections with a limitation of their spread. These cells originate from bone marrow, developing from the hematopoietic progenitor cells (HPCs) – $CD34^+$. Latest researches show that NK cells may also develop in secondary lymphoid tissues such as lymph nodes, tonsils, spleen and this is highlighted by the presence of NK cells subset CD56 bright – high density surface expression of CD56, which can be isolated from secondary lymphoid tissue [25-27].

According to their function and immunological phenotype, natural killer cells are divided in two main subsets: CD56dimCD16bright and CD56brightCD16dim, CD56 and CD16 being the NK cells surface antigens [25,28]. 90% of circulating NK cells express on their surface CD56dim and in response to target cell stimulation, they have the property to mediate cytotoxicity. The rest of the NK cells express CD56bright and have a greater ability to both secret and respond to cytokines. In comparison to lymphocytes (B and T), natural killer cells don't express on their surface specific receptors for different antigens. Thus, when an NK cells encounter a tumor cell which does not express a self MHC class I, there are induced several mechanisms in order to kill the tumor cell such as: expression of FasL or TNF- related apoptosis- inducing ligand (TRAIL) which induce death of tumor cells by interacting with their receptors (Fas and TRAIL receptors), releasing of cytoplasmic granules which contains granzymes and perforins.

Besides the presence of CD56 and CD16 on their surfaces, functionally, natural killer cells also express receptors that classified them as activating and inhibitory. Also, the interaction with other immune components may activate or inhibit NK cells activation and function. Cytokines (IL-2, IL-15, IL-12, IL-18), interactions with macrophage, dendritic cells, stromal cells have role in regulation of NK cell function. In contrast, secretion of transforming growth factor-beta (TGF-β), or IL-10 produced by acute myeloid leukemia (AML) are known to inhibit activation and function of NK cells [28].

NK cells have their unique ability to recognize target cells that don't express major histocompatibility complex (MHC) molecules on their surface [27]. Besides this ability, NK cells have a potent cytotoxic activity by releasing proteins (perforin, granzymes), can secrete cytokines and chemokines in order to regulate the immune system response [30]. Because of all these properties and the ability of fast killing, makes NK cells an important candidate for adoptive cell therapy [30].

Preparation of NK cells for immunotransfer

Methods regarding isolation, expansion and enhancing are necessary in order to use natural killer cells as a therapeutic source. Isolation and production can be done on NK cells obtained from different sites (Table III) [32].

Table III: Classification of potential NK sources

Umbilical cord blood
Adult lymphapheresis products
NK cells lines
Induced pluripotent stem cells (iPSC-NK)
Human embryonic stem cells (hESC-NK)

Legend. Classification of available resources used for isolation of natural killer cells. Adapted from Darji A, et al. *J Stem Cell Res Ther.* 2018; 8(3) [25].

First, leukocytes must be isolated through leukapheresis. After this process, with the help of CD56 beads, NK cells are isolated and then, infused back to the patient. In order to stimulate their expansion and activation, systemic cytokines (usually IL-2) are administrated.

Another alternative to autologous NK cells is the use of allogenic ones, over the last years being considered as promising alternative immunotherapy. Miller and his team were among the first researchers who demonstrated that this type of ex-vivo expansion of haploidentical NK cells and their administration after a lympho-depleting chemotherapy is a safe alternative without the risk of inducing graft *vs* host disease (GVHD) [28].

The latest approaches have focused on alternative ways to maintain and stimulate activation and expansion of NK cells. After the NK isolation, these cells must be expended and activated in culture cells medium enriched with cytokines such as IL-2, IL-15, IL-18, IL-21, type 1 Interferon. When will be an adequate number of cells, the patient will be infused with those cells [25,28,31].

More recent, researchers focused on developing alternative methods in order to produce larger quantities of purified and functionally active NK cells by using "feeder cells" such as: monocytes in the form of irradiated PBMCs, EBV-transformed lymphoblastoid cell lines, irradiated K562 cell that express membrane-bound IL-15/IL-22 and 41BB

ligand for a co-stimulation in gas-permeable expansion flasks [28-32].

Clinical applications of NK cells

When it comes to utilization of NK cells as a source of immunotherapy, several preclinical and clinical studies demonstrated the viability of NK cell lines to be used as a prognostic marker and therapeutic treatment in cancer [25-26].

There are evidences (experimental and clinical) which suggests the possibility of using NK cell as a prognostic marker in cancer. An 11 years study shows that low levels of NK in peripheral blood lymphocytes are correlated with an increased risk for cancer and the absence/low levels of these cells in the tumor infiltrating lymphocytes is a positive prognostic marker in multiple malignancies and the intratumoral presence of NK cells are correlated with improve outcome and delay of tumor progression [26, 28].

When it comes to hematological malignancies, there are multiple studies which suggest using NK cells as an alternative therapy, being associated with successful outcomes. All these results are proving its effectiveness unlike T-cell therapy [26]. In vitro were obtained the most successful results of using NK cells in different malignancies such as acute myeloid leukemia (AML), acute lymphocytic leukemia (ALL), multiple myeloma (MM) [30]. Also, same results were obtained for solid tumors (neuroblastoma, ovarian, renal cell, colon, gastric carcinoma) [25].

Even though natural killer cells may be a promising therapeutic alternative, for the treatment of solid tumors there are some challenges to overcome:

- Development of transport support of NK cells to the tumor site
- Ways of tumor capsule penetration in order to exert their function
- Tumor targets must be susceptible to NK-mediated cytotoxicity
- Altering the tumor microenvironment which inhibits NK cells function
- Finding antigen specific for each tumor cells [28].

Other pathologies

Though, NK cells therapy has been developed as a therapeutic alternative in different types of tumors, there are clinical trials which demonstrate the usage of this therapy with NK cells in viral infections (hepatitis, influenza, HIV). Also has been studied the role of natural killer cells in other diseases like asthma, diabetes, arthritis, multiple sclerosis. All these researches prove these cells, may be used as a potential therapy for other pathologies but it is necessary a better understanding of function and receptors of natural killer cells. [25].

CAR-NK

Using NK cells in order to express chimeric antigen receptor is an innovative immunotherapy, developed as a necessity for a better improvement of the NK cell clinical efficacy [32].

In the last past years, researchers have made important discoveries regarding genetic engineering immune cells used as therapy for treating cancer and not only. First, have been extensively used and studied the clinical potential of CAR-T cells, being successfully used in treating leukemia and lymphoma. But this novel therapy it is associated with several side effects and limitations, researchers focused in finding alternative immune cells in order to express chimeric antigen receptors in order to overcome this challenge. Because natural killer cells can kill their targets in a non-specific manner and are cytotoxic immune effectors, the latest researches have been done on using this type of cells as a base to express chimeric antigen receptors [28].

As we know from the previous chapter, natural killer cells are effectors of the immune system with a cytotoxic effect against tumor cells. Besides the cytotoxic effect, NK cells have multiple functions such as: degranulation, cytokine release, functions which are controlled by different signals from inhibitory receptors, activating receptors and heterodimeric C-type lectin receptor (NKG2A) with role in recognizing ligands present on the surface of targeted cells [30].

Because of their functions and efficiency NK cells have been used as an alternative for the classic oncological therapies [28, 32].

Only in preclinical tests have been demonstrated the feasibility of natural killer cells genetically engineered in order to express CARs, primary NK cells and NK-92 successfully expressing chimeric antigen receptors against several targets such as: CD19, CD20, CD44, Her2. *In vitro* and *in vivo*, CAR-NK cells efficiently mediate the killing of tumor targets. Also, Shimasaki and his team succeeded in testing expression of CD3ζ and 4-1BB signaling molecules (anti-CD19-BB-ζ) in human NK cells using mRNA electroporation as an alternative transfection, with the appearance of transfection result in 24 hours. Also, the levels of CARs expressed on NK cells were similar with levels of CAR-NK achieved through retroviral transduction [30].

CAR-NK therapy

The major advantage of using CARs is the possibility of equipping immune cells with the ability to recognize and kill their targets that are associated with MHC [32].

There are a series of advantages that shows the superiority of CAR-NK compared to CAR-T cells (see Table I)

- Autologous and allogenic natural killer cells have a limited persistence *in vivo*

- Low risk of life-threatening toxicity: cytokines release syndrome (CRS) because of the short rate of expansion of clonal NK cells and the immune mediated rejection of allogenic natural killer cells
- Preclinical and clinical trials have showed that NK cells do not cause GVHD
- The possibility to produce an off-the-shelf allogeneic product which may be used for immediate clinical use

When it comes to safety of the method, same concerns as for CAR-T cells are still available. Cytokine release syndrome, toxicity to normal tissues, on-target/off-tumors effects, tumor lysis syndrome, GVHD are relevant regarding the safety of CAR-NK therapy for oncological patients. Another challenge is genetic manipulation of NK cells. Evan though viral transduction is successful for T cells, unfavorable effects have been noticed on cell viability of transduced NK cells, reason that lead to optimization of viral transduction and electroporation technologies. Although all these problems regarding CAR-NK cells needs to be improved in order to have a safe and efficient therapy alternative, the biggest challenge remain finding the optimal target appropriate antigens specific for each tumor cells [30,32].

The main sources such as peripheral blood (PB), cord blood (CB), hematopoietic stem cell progenitors (HSCPs) are used for isolation of natural killer cells, cells which have been successfully genetically engineered to express chimeric antigen receptors [28].

CAR-NK cells in Leukemia and Lymphoma

In preclinical researches and clinical trials, have been studied the anti-tumor activity of CAR-NK cells that were engineered to target specific antigens such as CD19, CD20, CD33, CD138, CD319, CD3, CD5, CD123. From all these antigens, CD19 is the most studied and, in 2004 was presented data regarding NK-92 cell line harboring anti-CD19-CAR. Later, in 2016 were published results showing an increased cytotoxicity of anti-CD19 CAR-NK92 compared to unmodified NK92.

In acute myeloid leukemia studies highlighted the possibilities of targeting CD33 which is expressed by both healthy and malignant myeloid cells. Several approaches such as CD33-directed CAR-modified T cells with a scFV derived from gemtuzumab ozogamicin, CD33-directed NK-92 have been studied, both of them showing a potent cytotoxic activity against targeted cells. Because of CD123 expression in a high rate in malignant hematological cells and at low levels for hematopoietic stem cells, researchers focused on developing a therapy by targeting this antigen. Despite of its potential, anti CD123 CAR-T cells induced severe hematological off-target toxicities in mouse model. Therefore, have been developed an alternative to anti CD123 CAR-T cells - NK cells expressing anti CD123 CARs - which had shown *in vitro* activity against acute myeloid leukemia cell line and patient sample [33].

Solid tumors

Regarding multiple myeloma, there are studies showing evidence of efficiency of NK-92 cell line as a potential therapy in treating this type of cancer. Also have been successfully tested in vivo/in vitro anti CD319 and anti-CD138 CARs which were inserted into NK-92 and NK92MI (it is an NK92 IL2-independent) against primary and cell lines of multiple myeloma cancer cells. Recently, researchers are taking in consideration using SLAMF7-directed CAR-NK cells combined with CD38-directed antibody daratumumab as an alternative therapy for relapsed multiple myeloma.

In the past and present, it has often been shown that the NK-92 cell line can be effectively transduced with several different CARs against several malignancies for testing in preclinical approaches and currently in first clinical studies. CAR-NK-92 cells were quite successful in overcoming the tumor barrier and retargeted anti-tumor cytotoxicity against several resistant solid tumors, including epithelial cancers by targeting of human epidermal growth factor receptors (HER1 [ErbB1], HER2 [ErbB2]), neuroectodermal tumors by GD2, brain tumors by HER1 and HER2, and ovarian carcinomas also by HER2 [4, 6, 28]. However, there are some limitations to using this cell line [33].

THE FUTURE OF CAR-NK CELL THERAPY

The latest researches regarding a better understanding of NK cell biology and immunological function have paved the way to development of novel immunotherapies alternatives [1]. In the next years, there will be a greater interest regarding the improvement of tumor-specific NK cells efficiency, of gene editing techniques with developing better alternatives (CRISPR/Cas9, TALEN), targeting the tumor microenvironment, inhibiting the immune checkpoints and developing delivery biomaterials [2,3].

Although pre-clinical tests of CAR-NK therapy as potential therapy have begun in recent years, the promising results encourage continued testing and a later application of this therapy in clinical trials [4].

In this review we discussed the importance of adoptive cell therapies, the biology and function of chimeric antigen receptors, of natural killer cells and CAR-NK cells, as well as the importance of increasing focus on clinical applications and improvement the practicality of these genetically engineered immune components.

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TERAPIA CU CELULE CAR-NK: ERA UNEI NOI POTENȚIALE IMUNOTERAPII

REZUMAT

Domeniul imuno-oncologic se află într-un proces continuu de dezvoltare prin creearea de terapii revoluționare ca o alternativă la cele clasice. Chiar dacă, la început, strategiile imunomodulatoare s-au axat pe folosirea celulelor CAR-T ca o alternativa imunoterapeutica, cele mai recente cercetări se concentrează pe dezvoltarea unor terapii alternative cum ar fi CAR-NK. Celulele natural killer sunt o componentă importantă a sistemului imunitar, având capacitatea de a secreta citokine și chemokine. Datorită acestor proprietăți, celulele NK pot ucide celulele tumorale si nu numai. În cadrul review-ului nostru, vom prezenta beneficiile si cele mai recente rezultate legate de terapiile celulare cu NK, CAR-NK cele mai recente studii in ceea ce priveste terapia cu celulele NK, celulele CAR-NK, de asemenea punem in evidenta importanța continuării studiului lor pentru a îmbunătăți rata de supravietuiere a pacientilor oncologici.

Cuvinte cheie: imunoterapie, receptorul antigenic chimeric (CAR), CAR-NK, celule tumorale.

NEURODEGENRATION AND NEUROPROTECTION IN SEVERE RETINAL DYSFUNCTION

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ABSTRACT
The retina is a very complex structure, consisting in a thin multilayer of primarily neural cells derived from ectoderm. The retina can be assimilated as a photo-camera being highly specialized in perception, capture, signal initiation and transmission together with the visual stimuli from the "outside world "to the optic nerve and finally to the visual cortex of the brain. As a highly specialized ultrastructure with its very complex function, the pathological events in the retina can be schematized as: retina-visual pathway-photoreceptors-phototransduction-ganglion cells, horizontal cells (Amacrine cells)-interplexiform neurons. All those microstructures get affected by different aggressor either internal or external leading in 90% of the clinical situation to the vision loss. The present article tries to summarize the cellular and molecular mechanism in retina degradation and disfunction, as well by suggesting modern approaches and the contribution of some neuroprotective therapies in maintaining the optimal physiologically role of the retina in some retinal pathological conditions. Retinal neurodegeneration is an irreversible process which should be seriously taken into account having in view that, unfortunately, the corneal, macular or uveal degenerescence of disfunction can be partially solved, the degenerescence of the retina stays extremely delicate to be treated despite of all modern technology and therapeutically options. Retinal degeneration is a progressive neurological disorder caused by genetic mutations and/or environmental or pathologic damage to the retina; unfortunately, the problem is incurable.
Keywords: visual function, retina, neurodegeneration, retinal vascular degeneration, neuroprotection.

INTRODUCTION

Neurodegeneration plays a major role in the retinal dysfunction, which is still thought to be the main determinant of permanent disability, either minor, moderate or severe. The visual function stays a primordial one even during the different clinical stages of vitreous, uveal, macular and retinal damage. To foresee, to prevent and to treat any type of injury at these ocular levels, the cellular and molecular alteration are a very important prerequisite. Cellular dysfunctions occurred due to the changes in the retinal microenvironment lead from clinical complication to clinical complication and finally to blindness. The vascular compound of the retina should also be outlined as a contributor to all retinal pathologies. The administration of anti-vascular endothelial growth factor (anti VEGF) to suppress the vascular proliferation and exudation has highly impaired the prognosis of some unpleasant events [1].

Cellular disorders in the retina in a nutshell

It is very important to have a critical view on all compromising factors that plays a role in retinal neurodegeneration implying the severe dysfunction at the retinal level. We try briefly to illustrate the main attributes which should arouse attention: exogenous and endogenous. The synergic actin between those two factors and in between neural cells (retinal neurons and Mūller glial cells) via intracellular signaling, can cause, of course, neural functional disorders, abnormal gene expression and apoptosis. In this regards the retinoblastoma and as well other genetic condition of the eye like Retinal dystrophy with inner retinal dysfunction and ganglion cell anomalies stays the most life-threatening factor. Intracellular-inflammatory signals and ROS (reactive oxygen species) can be activated simultaneously: endogenous/exogenous. The pathological and disrupted generated signal will proliferate in all retinal structure conferring a maladaptive function by debilitating in time the patient. In the cases of elderly retinitis pigmentosa

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and retinoblastoma, ROS can generate de activation of mediatory intracellular effectors, such as: MAPK (mitogen-activated protein kinase), STAT (signal transducer activation of transcription proteins) and NF-kB (activated protein complex). If the condition is genetic, the intimate mechanism are very complex leading as well to a problematic clinical strategy sometimes.

Interleukin 6 (IL-6)-mRNA is expressed in the retinal ganglion cells. Pro-inflammatory signaling pathway can also exert a negative effect on retinal micro -vascularization. The retinal angiogenesis is also perturbed at this level. Retinal ganglion cells, expressing cytokines such as VEGF [2], are responsible also in an important condition called stress-induced retinopathy.

Most ocular diseases cause harmful neurodegenerative processes by expressing Gap 75 leading to the photoreceptors atrophy, cell death and impacting seriously the visual function. Calcium-gated voltage channels are also involved. The signaling cascade of the molecular events of Ca^{2+} channels antagonist D-cis diltiazem, disrupts the kinetic of rd 1 rod alteration, announcing the rescue of the scotopic vision [3].

Breakdown of the blood-retinal barrier

At the cellular and molecular level, the damage of blood-retinal barrier can be seen only post-mortem by anatomo-pathological remarks after the Evans blue test necessary to be performed to appreciate the blood-retinal barrier permeability. An immune adaptive response will be also triggered by the activation of the microglia/macrophages pathway which will destabilize the blood-retinal barrier favoring in this way moderate to severe hemorrhage. The demyelination of the optic nerve should be also mentioned and prevented by administrating of neuroprotective and neurotrophic factors.

Intracellular metabolic consequence in retinal cell death and the inflammatory burst can also lead to uveitis and age-related macular degeneration. During the retinal metabolic stress, the released glutamate will cause the death of neurons containing ionotropic glutamate with a negative consequence as an insufficient supply of nutrients to the optic nerve, uvea, and retina.

Retinal ganglion cells (RGC), need also to protect (neurotrophic/neuroprotective agents) the retinal pigment epithelium. There is no need to outline that RGC_s are a variety of a very vulnerable population. RGCs own a high energy demand, but in contrast to the retinal microvasculature, their network and structure is not so dense like the ones in the brain. On the other hand, we can talk about a natural compromise between the metabolic demands of the neurons and a optimal vascular supply [4]. In the pediatric population retinopathy of prematurity, and retinoblastoma claims an irreversible damage of all ocular microvasculature and retinal cells destruction.

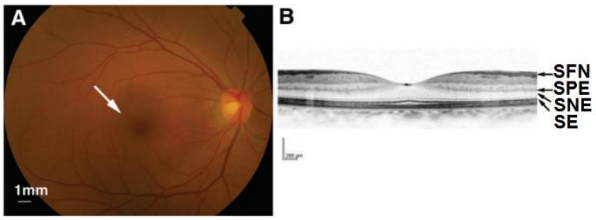

Fig.1. (**A**) Fundus photograph. An arrow shows the center of the retina, where photoreceptor cells and light stimuli are concentrated. (**B**) Optical coherence tomography (OCT) image showing a cross section of the central part of the retina. NFL, nerve fiber layer; OPL, outer plexiform layer; ONL, outer nuclear layer (photoreceptor cell layer); OS, outer segment. (Adapted after Ozawa, Sasaki *et al.* Current Pharmaceutical Design, 2012; 18: 51-56).

Other physiological attributes of the retinal cytoarchitecture impacting visual function and pathology

In the retina structure we found also multiple layers of interconnected neurons and also in the context of visual function, fovea plays an important role as well. Fovea is a central region of the human and primate retina consisting in a high number of cones cells responsible for an excellent visual acuity and the chromatic sense. Age-related macular degeneration (AMD) is a reversible loss of vision, the age being the most incriminated risk factor. Other metabolic conditions, such as: diabetes mellitus, hyperlipidemia, hypercholesterolemia, hyperuricemia, higher systemic blood pressure also affect the uveal and retinal microvasculature path. Cellular responses induced by retinal injury: alternations and dysfunctions in the retinal neurons and circuits, changes in photoreceptors and their synaptic connectivity, and here the clinician either a neurologist or ophthalmologist should be aware of the disease Rhodopsin-positive neuritis associate with gliotic Müller cell processes. The cell morphology and function as well the clinical features will significantly differ from a healthy individual to an individual in ill conditions.

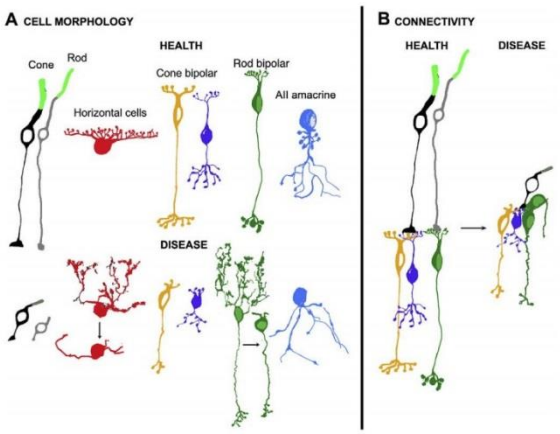

Fig. 2. Cellular remodeling during retinal degeneration. Schematic representation of the main changes in morphology (A) and

connectivity (B) that take place in retinal neurons during degenerative processes, regardless of the origin of the damage. (A) Signs of rod and cone degeneration are the reduction in the size of photoreceptors, the shortening of both outer and inner segments, and the loss of synaptic connections with second-order neurons. Bipolar cells display early retraction and loss of dendrites during retinal degeneration, with further sprouting of ON rod bipolar cell dendrites into the ONL in some degenerative diseases. In advanced degenerative processes, the retraction of dendrites may also occur. The axonal endings of bipolar cells are shortened. Horizontal cells retract their dendrites during retinal degeneration, although the sprouting of dendrites and axon terminals are frequent, with the formation of ectopic synapses in the ONL. Remodeling of AII amacrine cells involves the loss of lobular appendages in the OFF strata of the IPL in several retinal degenerative diseases. (B) Summary of the connectivity changes occurring in retinal neurons during the course of the degenerative process at the OPL. Rod bipolar cells make new contact with the remained cones. (Adapted after N. Cuenca et al. Progress in Retinal and Eye Research, 2014; 43)

Defragmentation and destruction (shortening of synaptic ribbon) are also threatening factors leading to retinal detachment. During the retinal damage a cellular remodeling may took place but needs to be still elucidated and defined.

Oxidative stress contributes as well to the retinal degeneration because retinal protein homeostasis modulates the degeneration process but, on the other hand regulates the action of some neuroprotective agents with an impact on retina, vitreous and optic nerve.

Therapeutic neuroprotective therapies

1. Anti-apoptotic therapy
2. Anti-growth-factor vascular extravasation therapy
3. Norgestrel
4. Antioxidant cocktails
5. Ginkgo biloba extract
6. Resveratrol
7. Quercetin
8. Cell-based therapies
9. Surgical procedures (laser Ar/Ne, Cryo precipitation, Cry ocoagulation)

There are some others natural adjuvant therapeutic options but the clinician can make an accurate selection to decide which patient can benefit of it and the optimal dosing/time effect.

CONCLUSIONS

Human retinal degenerative diseases are currently incurable and retinal degeneration, once initiated, is irreversible. Cell replacement through the transplantation of stem cell or differentiated retinal cell types and artificial vision were as well taken into account and there are clinical trials win those regards. Anyway, despite the efforts of researchers to identify a therapy capable of preventing retinal degeneration or restoring vision, current therapies entail difficulties that need to be addressed to achieve safe, effective treatments. In this context, the administration of antioxidants (alone or in cocktails), anti-apoptotic, anti-inflammatories, neurotrophic factors or viability factors unfortunately only slows the neurodegeneration of the retina by delaying retinal cell death, but fail to prevent the progression and prognostic of the disease.

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NEURODEGENERAREA ȘI NEUROPROTECȚIA ÎN DISFUNCȚIILE SEVERE ALE RETINEI

REZUMAT

Retina este o structură foarte complexă, constând într-un multistrat subțire de celule neuronale primare derivate din ectoderm. Retina poate fi asimilată ca o cameră foto fiind extrem de specializată în percepție, captare, inițierea semnalului și transmitere împreună cu stimulii vizuali de la „lumea exterioară" la nervul optic și în final la cortexul vizual al creierului. Ca o ultrastructură extrem de specializată, cu funcția sa foarte complexă, evenimentele patologice din retină pot fi schematizate ca: cale retină-vizuală-fotoreceptori-fototransducție-ganglion celule, celule orizontale (celule amacrine) - neuroni interplexiformi. Toate aceste microstructuri sunt afectate de un agresor diferit, fie intern, fie extern, ceea ce duce la 90% din situația clinică la pierderea vederii. Prezentul articol încearcă să rezume mecanismul celular și molecular în degradarea și disfuncția retinei, precum și prin a sugera abordări moderne și contribuția unor terapii neuroprotectoare la menținerea rolului fiziologic optim al retinei în unele condiții patologice ale retinei. Neurodegenerarea retinei este un proces ireversibil, care ar trebui luat serios în considerare, având în vedere că, din păcate, degenerescența retinei,corneei, maculei sau uveii poate fi parțial rezolvată, degenerescența retinei rămâne extrem de delicată pentru a fi tratată în ciuda întregii tehnologii moderne și opțiuni terapeutice. Degenerarea retinei este o tulburare neurologică progresivă cauzată de mutații genetice și / sau de deteriorarea mediului sau a patologiei retinei; din păcate, problema este incurabilă.

Cuvinte cheie: funcție vizuală, retină, neurodegenerare

TUBERCULOUS PLEURAL EFFUSION OR DEXTROPOSITION OF THE HEART? A CASE REPORT

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ABSTRACT
Pleural effusions are commonly encountered in clinical practice. Cardiac dextroposition is the horizontal displacement of the heart into the right hemithorax. Present case study is of an unknown congenital cardiac dextroposition, a female patient, aged 61 years, with no history of pulmonary or cardiovascular pathology, who was admitted with a suspicion of tuberculous pleural effusion. Chest X-ray (poster anterior view) showed the presence of a homogenous opacity occupying the half lower part of the left hemithorax, an image of hyperlucency rounded of a thick wall located next to the left hilum with the mediastinum shifted to the opposite side.
Keywords: tuberculous pleural effusion, dextroposition of the heart, chest X-ray.

INTRODUCTION

Pleural effusions are a common sight in clinical practice, for both respiratory specialists and others. [1] Identifying the cause for pleural effusion can be achieved in the majority of cases through a combination of physical examination, analysis of relevant clinical history, chest radiography and diagnostic thoracentesis. [2] Tuberculous pleural effusion constitutes the 2nd most common form of extra pulmonary tuberculosis, and in endemic tuberculosis areas it represents a frequent cause of pleural effusions. It is usually presented in acute form, with the most common symptoms being dyspnoea, fever, cough, and pleuritic chest pain. [3]

The definition of cardiac dextro-position is the presence of the heart, with the major axis of the heart in normal alignment, in the right hemithorax. No anatomic alteration is present in the heart itself, but rather only in its location.[4]

The dextroposition of the heart can be caused either by the congenital malformation of the organ, or of the traction of the organ over to the right side by extraneous mechanical causes such as tumors, the effusion into the left pleura, traction of right-sided pleuritic adhesions or cystic disease of the left lung.[5]

Dextroposition of the heart is different from true congenital dextrocardia, in which the apex of heart is pointed towards the right, and is formed by the left ventricle. In dextro-position the location of the left atrium and ventricle is on the left side of the heart chambers. The present clinical case showcases an unusual cause for cardiac dextroposition.

CASE REPORT

A female patient, aged 61 years, with no history of pulmonary or cardiovascular pathology, was referred to the clinic by the family doctor with a diagnostic of tuberculous pleural effusion under observation. She was admitted for physical asthenia, non-productive cough and dyspnoea.

Patient reported ongoing symptoms of mild non-productive cough and dyspnoea over the preceding 4 weeks.

She is a current tobacco smoker with a 15-pack-years history and denied drug usage or travel abroad in the preceding year. The patient's son was admitted to the clinic 3 months previously, with a diagnosis of pulmonary tuberculosis.

On examination, the patient was discovered to be of normal weight (IMC value of 22) with the following vital parameters values: a body temperature of 37.8 Celsius degrees, a heart rate of 89 beats/minute, respiratory rate 16/minute, blood pressure was 140/85 mmHg, and oxygen saturation measured by pulse oximetry was 98% on room air.

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Palpation revealed reduced vocal resonance on the half lower part of the left hemithorax, dullness was detected on percussion over the same area and auscultation revealed diminished vesicular breath.

Laboratory investigations performed, including full blood count, amylase determination, liver function tests, urea and electrolytes determination, were all within normal limits. TB-interferon γ release assay (TB-IGRA) result was positive.

Thoracic radiography (poster anterior view) revealed the presence of a homogenous opacity occupying the half lower part of the left hemithorax, an image of rounded hyperlucency of a thick wall located next to the left hilum with the mediastinum shifted to the opposite side (Figure 1).

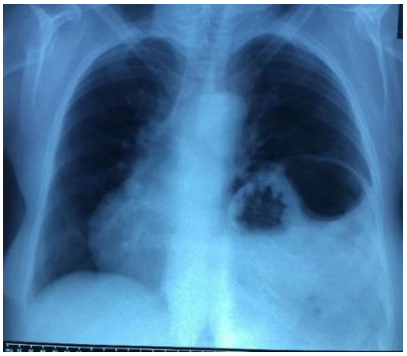

Fig. 1. Chest x-ray showing the presence of a homogenous opacity occupying the lower half part of the left hemithorax, an image of rounded hyperlucency of a thick wall located next to the left hilum and the mediastinum shifted to the opposite side.

Based on anamnesis, clinical examination, laboratory analysis, at the time of the investigation the diagnosis suspicion of tuberculous pleural effusion was raised. Thoracentesis was attempted, the result was negative for the diagnosis suspicion, therefore computer tomography of the chest and abdomen was performed.

Computer tomography revealed bilateral fibrosis lesions, bilateral inferior lobular cylindrical bronchiectasis, without pleural or pericardial effusion, ascension of the left hemidiaphragm, stomach and intestinal cords visible in the postero-inferior mediastinum (Figures 2 and 3).

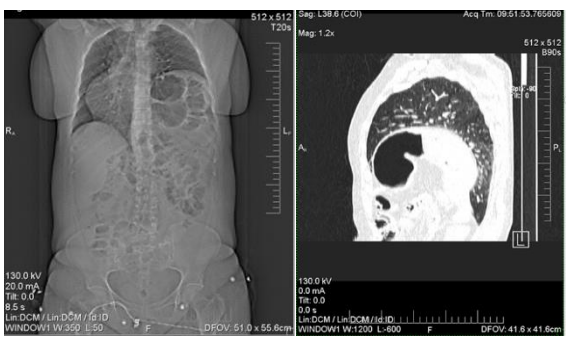

Fig. 2. Computer tomography showing, the ascension of the left hemidiaphragm, stomach and intestinal cords visible in the postero-inferior mediastinum.

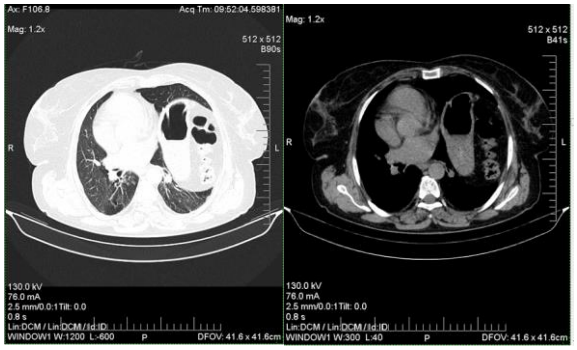

Fig. 3. Computer tomography showing bilateral fibrosis lesions, bilateral inferior lobular cylindrical bronchiectasis, without pleural or pericardial effusion.

Based on the clinical, biological, imagistic chart, the diagnosis of dextro-position of heart was established.

DISCUSSION

Pleural effusion represents an abnormal accumulation of fluid within the pleural space. It does not constitute a disease per se, but rather the complication of another underlying pathology [6]. The excess of fluid results from an overthrow of the equilibrium which is present across the pleural membranes. Thus, pleural effusion constitutes an indicator of a pathologic process, which can be of primary pulmonary origin, it can originate in another organ system, or it can be tied to a systemic disease. It is not a diagnosis in and of itself, and it can occur within a setting of either acute or chronic disease. Of the forms of extra-pulmonary tuberculosis, tuberculous pleural effusion represents one of the most common ones.

The patient was referred to the clinic, with a diagnosis of a possible tuberculous pleural effusion, since she was identified as a contact in the epidemiological investigation of her son - admitted to our clinic 3 months previous with a diagnosis of pulmonary tuberculosis.

Following the investigation, the diagnosis was negated by the absence of fluid on computer tomography and the ascension of the left hemidiaphragm, stomach and intestinal cords visible in the postero-inferior mediastinum and dextro-position of heart.

Dextro-position of the heart is classified as congenital or acquired. Congenital dextroposition of heart, in association with other cardiopulmonary anomalies, constitutes scimitar syndrome [7], with hypoplasia of the right lung [8]. Acquired dextro-position of heart is often seen in patients who underwent pneumonectomy, due to a multitude of factors: hyper expansion of the non-operated lung, the collapse of the pleura depending on the retraction of the intercostal space, the obliteration of the post pneumonectomy space which depends on both the reabsorption and the organization of the fluid it contains, elevation of the diaphragm.

In the present case, the suspicion is that the cause of the dextro-position of heart is congenital, since the patient had not underwent a cardiovascular examination or even a chest radiography ever before. However, none of the other usual cardiopulmonary anomalies were present. Thus, the explanation for dextro-position in the present case is most likely be due to elevation of hemidiaphragm with mediastinal shift.

Patients suffering from cardiac dextroposition are generally asymptomatic [9]. In the present case, the patient was admitted for dyspnoea, physical asthenia and non-productive cough, without any past medical history present.

Routine physical examination revealed the cardiac impulse identifying the cardiac apex closer to the sternum. Percussion performed on the heart borders confirmed the displacement of the heart to the right [10].

Chest radiography (poster anterior view) identified the cardiac silhouette, which was displaced to the right. This can sometimes be associated with the presence of chest wall deformities, or various other pathologies present in the diaphragm and the lungs.

In the present case, the chest X-ray revealed the presence of a homogenous opacity occupying the half lower part of the left hemithorax, an image of rounded hyperlucency of a thick wall located next to the left hilum with the mediastinum shifted to the opposite side. This raised the suspicion of pleural effusion, however, since thoracentesis was negative, further investigations were necessary. Computer tomography was performed, revealing ascension of the left hemidiaphragm, stomach and intestinal cords visible in the postero-inferior mediastinum and the heart displaced to the right.

CONCLUSION

Cardiac dextroposition represents the simple horizontal displacement of heart, to the right side of the chest, of congenital or acquired causes. The most frequent cause is mechanical, with acquired dextro - position of heart most commonly present in post-pneumonectomy patients. Patients suffering from cardiac dextroposition are generally asymptomatic. The diagnosis is confirmed by physical exam and complementary studies.

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REVĂRSAT PLEURAL TUBERCULOS SAU DEXTROPOZIȚIA INIMII? RAPORT DE CAZ

REZUMAT

Revărsatele pleurale sunt patologii frecvent întâlnite în practica clinică. Dextropoziția inimii reprezintă poziția orizontală a inimii în hemitoracele drept. Prezentul caz clinic prezintă o dextropoziție congenitală nedecelată, la o pacientă, în vârstă de 61 de ani, fără antecedente cunoscute de patologie pulmonară sau cardiovasculară, internată cu suspiciunea de pleurezie tuberculoasă. Radiografia toracică, incidență postero – anterioară, a arătat prezența unei opacități omogene care ocupă jumătatea inferioară a hemitoracelui stâng, o imagine de hipertransparență circumscrisă de un inel opac, situat lângă hilul stâng, cu deplasarea mediastinului de partea opusă.

Cuvinte cheie: revărsat pleural tuberculos, dextropoziția inimii, radiografie toracică.

THE ROLE OF VITAMIN D IN THE PATHOGENESIS OF MULTIPLE SCLEROSIS

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ABSTRACT

Multiple sclerosis (MS) is an autoimmune, inflammatory, chronic, demyelinating disorder of CNS, characterized by episodes of inflammation and focal demyelination with multiple localizations, disseminated in time, but also through a process of axonal degeneration that unfolds in parallel. The disease usually has an evolution with flares and remissions, but can also have a constant character of progression over time, which leads to accumulation of varied neurological deficits and different degrees of disabilities. It is considered to be the most frequent affection of the young adult after trauma, the onset age being between 20-40 years with a maximum at the age of 30 and is 2-3 times more frequent in women than in men, there is no scientific evidence to explain the increased incidence of the disease in females, the possible explanation being that women are generally more susceptible to inflammatory and immunological disorders. Although clinical studies on the administration and effects of vitamin D in various pathologies are limited, experimental and epidemiological data are sufficiently consistent and encouraging, reinforcing the hypothesis that hypovitaminosis D may be a risk factor for the onset of disease and, at the same time, a predictive factor for the development of multiple sclerosis. Although the doses of vitamin D used in clinical trials are quite different and vary between 100 IU/day and 14,000 IU/day, and the results were encouraging for all groups of patients under study, the administered doses should be determined for each subject in part taking into account a number of factors such as age, gender, associated comorbidities, environmental or genetic factors, the latter being able to interact and combine to cause disease. If we consider that this vitamin is involved in a large number of disease, including autoimmune disease and especially multiple sclerosis, significantly influencing the activity of regulating T lymphocytes, whose role is well known in the pathogenesis of the disease, it can be concluded that hypovitaminosis D becomes a major health problem and vitamin D treatment will become a simple and effective therapeutic option for prospective patients with multiple sclerosis which is known as the young adults disease, a disabling disease with strong negative impact on their quality of life. Therefore, recommending the administration of vitamin D to multiple sclerosis patients can be regarded as a wise, provocative and a sufficiently effective option, which is currently provisory, to achieve improvement health status in patients with this disease.

Keywords: Multiple sclerosis, autoimmune, inflammatory, chronic, demyelinating disorder of CNS, vitamin D.

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INTRODUCTION

Multiple sclerosis (MS) is an autoimmune, inflammatory, chronic, demyelinating disorder of CNS, characterized by episodes of inflammation and focal demyelination with multiple localizations, disseminated in time, but also through a process of axonal degeneration that unfolds in parallel. The disease usually has an evolution with flares and remissions, but can also have a constant character of progression over time, which leads to accumulation of varied neurological deficits and different degrees of disabilities. [1]. It is considered to be the most frequent affection of the young adult after trauma, the onset age being between 20-40 years with a maximum at the age of 30 and is 2-3 times more frequent in women than in men [2,12], there is no scientific evidence to explain the increased incidence of the disease in females, the possible explanation being that women are generally more susceptible to inflammatory and immunological disorders [1]. Multiple sclerosis has a prevalence of less than 1 per 100,000 inhabitants in the equatorial regions, 6 to 14 per 100,000 inhabitants in the US and southern Europe, 30 to 80 per 100,000 inhabitants in Canada, northern Europe and the northern US. The risk of developing the disease, according to some studies grows with the increase in latitudes, a fact confirmed by epidemiological research, so it is considered that African-Americans are at risk compared with the white population but both races present the same south-north risk gradient. These studies bring into question the fact that the environmental factor has an important role in the pathogenesis of Multiple Sclerosis, regardless of the genetic predisposition of the individual.(1) Also, there are epidemiological data indicating the existence of a direct causal relationship in the general population between latitude, exposure to solar radiation, vitamin D levels and the risk of Multiple Sclerosis as well as immunological data that showed vitamin D influences the activity of regulatory T lymphocytes, lymphocytes with important role in the pathogenesis of the disease [5].

As a result of the dissemination and random localization of lesions in the central nervous system, clinical manifestations can vary: retrobulbar optic neuritis, motor deficits with varied distribution (most common paraparesis), other signs of pyramid damage, sensitivity disorders in the form of paresthesia (most likely) or Lhermitte sign, impaired occulomotori nerves (internuclear ophthalmoplegia, the nerve paralysis of occulomotor III, IV, VI with diplopia), paralysis of the facial nerve, cerebelous syndrome, vestibular syndrome in particular vertigo, sphincter disorders (retention or urinary incontinence, compelling urge, constipation), fatigue, depression, cognitive impairment, sexual dysfunction [1,2,8,12].

Thus, depending on the evolution of the neurological signs and syndromes, four clinical forms are distinguished:

1. Relapsing- remitting MS (RRMS) represents the most common form of disease (60-70% of cases), being the apanage of the young age (30years) and is characterized by the appearance of the relapses, clinically reliable; the attack installs quickly (hours or days), the symptomatology can be submitted completely or incomplete, and there are no elements of progression of the disease between the attacks. Between the relapses there may be time intervals of months or even years [1,2,8,12].
2. Secondary Progressive MS occurs at a time when, in patients with RRMS, neurological deficits considered as relapses, are no longer resolved, thus occurring a continuous progression of the disease interrupted or not, sometimes with phases in flats. It is believed that a RRMS can turn into MSSP after approximately 10 years of evolution, each patient presenting a risk of approximately 2% each year to develop a MSSP [2,8,12].
3. The primary progressive MS form represents approximately 15% of all patients, being more frequent among patients over 40 years of age and characterized by progressive worsening of neurological deficits from their onset, with no remittance, possibly minor and temporary improvement, or very rarely, with periods in the flat, practically cannot describe the clinical attacks. According to demyelinating lesions localization, this type of disease affects mainly the spinal cord, from onset, rather than cerebral level [2, 8,12].
4. Progressive relapsing multiple sclerosis form, the rare form of illness, characterized by the existence of attacks, but which is not remitted from the beginning and has, from the onset, a continuous progressive evolution of the symptomatology. It can be considered as a particular form of evolution of SMPP or SMSP, being present in only about 5% of patients [2,8,12].

Although the disease pathogenesis remains somehow unknown because no etiological factor has been identified, it is considered that the epidemiological factor should matter and be included in these theories on the etiopathogenesis of the disease. Thus, multiple sclerosis, also known as plaque sclerosis is considered as having a multifactorial etiology, being the result of interaction between several factors: environmental, especially nutritional, vitamin D, geographic, infectious - possible infections with Epstein-Barr virus or genetic factors, giving the disease heterogeneous appearance regarding to clinical, histopathological and genetic aspects [3,5,6]. Other risk factors possibly involved in triggering multiple sclerosis are: age, gender, family history, race, smoking, sun exposure. Smoking increases the risk of illness, worsens neurological deficits and enhances handicap degree [3,4,6]. It therefore appears that some of these factors, such as infections, sun exposure and vitamin D levels, are potential risk factors, especially for the under-15 population [9].

Nutritional factors play an important role in the occurrence of the disease, so it is believed that a diet high

in animal fats increases the risk of developing MS as a result of the increase in cell membrane rigidity, the cell becoming vulnerable to various factors, and a diet rich in oils reduces membrane rigidity maintaining structural integrity of the cell. So, studies conducted in Asia, where the consumption of oils is superior to saturated fat, have shown an important decrease in the incidence of this disease, and a diet rich in polysaturated fatty acids and vitamin D decreased multiple sclerosis prevalence according to studies conducted by a group of Norwegian fishermen [10].

It has been reiterated the idea that hipovitaminosis D, as well as sun exposure, combined, or interacting with other environmental or genetic factors, represent risk factors for multiple sclerosis. It is known that skin exposure to ultraviolet radiation (UVB) is essential for the vitamin D biosynthesis, which is the major source of vitamin D for most individuals, and therefore sun exposure for short periods in the case of people living in areas over 40 degrees northern latitude or in temperate regions where the amount of ultraviolet radiation is low or in areas with short days and long nights longer than 6-8 months / year, the synthesis of vitamin D decreases [4,9,15]. Also, a diet rich in fish fat is an important source of exogenous vitamin D. [15]. There are two forms of vitamin D: vitamin D2 (ergocalciferol) that is produced by some plants and vitamin D3 (colecalciferol) synthesized in the skin of humans and animals, which is the most important source of vitamin D exogenous, the most active biological form (9). Vitamin D exerts a number of functions in the human body acting through the vitamin D receptors present in most human body tissues, apparently playing an immunomodulatory role in CNS. From this point of view, vitamin D levels, and especially hypovitaminosis D, present an increasing interest in the production and triggering of the disease, and the correction of this deficiency may become a new effective therapeutic target for multiple sclerosis [6,13,14].

Vitamin D is a steroidal hormone successively metabolized by UVB radiation in the skin, liver under the action of several vitamin D-25-hydroxylase enzymes, the most important of which is CYP2R1 and kidney in the proximal tube under the action of the 1-alpha hydroxylase enzyme (CYP27B1) to the active metabolite 1,25 dihydroxyvitamin D (CALCITRIOL), which in turn acts on tissues containing certain vitamin D receptors such as skin, muscle, bone, gonads, intestinal, CNS, microglia, macrophages, dendritic cells, activated monocytes and B and T lymphocytes, having a role in modifying transcription, in proliferation and differentiation of immune cells [4,15].

Therefore, hypovitaminosis D is one of the most studied environmental risk factors capable of producing MS, and the correction of this deficiency can become an important link in treating this disease [10]. Exogenous or produced vitamin D in the skin is transformed in the liver until 25-hydroxyvitamin D (25-OH-VITAMINE or COLECALCIFEROL) which represents its major circulating form and its plasma concentrations are influenced by vitamin D intake and exposure to sunlight, this metabolite being actually the one determined in the lab [4,15].

It is known that the fundamental role of this vitamin is to keep bone structures intact, to interfere with the metabolism of calcium and phosphorus, but it also exerts other general effects such as: acting in the regulation of cardiovascular system activity, anti-inflammatory, anti-proliferative effects (for certain types of cancer), modulators of neurotrophins, growth factors and neurotransmitters from mammalian CNS, immunomodulators, the latter being involved in the occurrence of autoimmune diseases such as type 1 diabetes, Chron disease, rheumatoid arthritis, and last but not least, multiple sclerosis [4,6,15].

The 25-OH-vitamin D plasma concentration of 80 nmol/l (30 ng/ml) is considered to be necessary both for maximum intestinal absorption of calcium and for the delivery of extra1-alpha-hydroxylase, which is present in more tissues, where it produces 1,25 dihydroxyvitamin D [15]. Because, following experimental and immunological studies, it has been found that vitamin D has a similar mode of action to interferon beta, it is assumed that it can have an important immunomodulatory effect [7,15]. Although it is known that the population of the northern countries is deficiency in vitamin D, there are studies which have shown that this deficiency may be present even more than was believed in sunny countries such as India and Australia [15]. There is a study, considered as a reference that looked at the risk of developing MS depending on the serum level of 25-OH-vitamin D in healthy individuals, meaning no clinical signs suggestive of MS. It was concluded that subjects with a high level of vitamin D, i.e. those with 99-152 nmol/L, had a significantly lower risk of developing the disease than those who had a low vitamin D level, ie between 15-63 nmol/L. Therefore, it appears that the risk of developing MS is lower provided that the serum level of 25-OH-vitamin D is maintained at levels above 100 nmol/l in children or adolescents. (11)

A small number of clinical trials have been performed that have enrolled patients with multiple sclerosis remitting remission, in which 25-OH- vitamin D dosages have been performed and which have shown an apparent number of patients, over 83% of them had serum levels of 25-OH-vitamin D below 75 nmol/l, and 17% of patients had 25-OH-vitamin D deficiency, i.e. below 25 nmol/l. Also, more than 95% of the patients analyzed never reached the serum level of 100 nmol/l of 25-OH-vitamin D. Thus, it can be concluded that most multiple sclerosis patients have low serum vitamin D levels, and it has been estimated that nearly three-quarters of multiple sclerosis cases could be avoided if vitamin D plasma levels were above 100nmol / L(10). In the study conducted by Mahon and colleagues who followed multiple sclerosis patients treated with 1000 IU/day of vitamin D for 6 months, it was observed that in fact the concentrations of (TGF-β1) transforming growth factor beta 1 increased significantly during the course of the study, without influencing the levels of tumor necrosis factor alpha

(TNF-alfa), interferon gamma (IFN-gamma) or interlekin 3 (IL-13) after vitamin D treatment. It may be considered that TGF-β1 serum levels associated with elevated serum levels of vitamin D may favor the recovery or improvement of the status of MS patients. (7; 16) Canadian researchers also demonstrated that the use of high doses of vitamin D, approximately 14,000 IU/day, for a period of 6 months - 1 year, do not produce hypercalcemia or notable side effects, even if the vitamin D serum level is 400nmol/l [15].

Although clinical studies on the administration and effects of vitamin D in various pathologies are limited, experimental and epidemiological data are sufficiently consistent and encouraging, reinforcing the hypothesis that hypovitaminosis D may be a risk factor for the onset of disease and, at the same time, a predictive factor for the development of multiple sclerosis. Although the doses of vitamin D used in clinical trials are quite different and vary between 100 IU/day and 14,000 IU/day, and the results were encouraging for all groups of patients under study, the administered doses should be determined for each subject in part taking into account a number of factors such as age, gender, associated comorbidities, environmental or genetic factors, the latter being able to interact and combine to cause disease [15]. If we consider that this vitamin is involved in a large number of disease, including autoimmune disease and especially multiple sclerosis, significantly influencing the activity of regulating T lymphocytes, whose role is well known in the pathogenesis of the disease, it can be concluded that hypovitaminosis D becomes a major health problem and vitamin D treatment will become a simple and effective therapeutic option for prospective patients with multiple sclerosis which is known as the young adults disease, a disabling disease with strong negative impact on their quality of life [5].

Therefore, recommending the administration of vitamin D to multiple sclerosis patients can be regarded as a wise, provocative and a sufficiently effective option, which is currently provisory, to achieve improvement health status in patients with this disease.

AUTHOR CONTRIBUTION

The authors contributed equally to the manuscript and share first authorship.

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ROLUL VITAMINEI D ÎN PATOGENIA SCLEROZEI MULTIPLE

REZUMAT

Scleroza multiplă (S.M.) este o afecţiune autoimună, inflamatorie, cronică, demielinizantă a SNC, caracterizată prin episoade de inflamaţie şi demielinizare focală cu localizări multiple, diseminate în timp, dar şi printr-un proces de degenerescenţă axonală care se desfasoară în paralel. Boala are de obicei, o evoluţie cu pusee şi remisiuni, dar poate avea şi un caracter constant de progresivitate în timp, ceea ce conduce la acumulare de deficite neurologice variate şi la dizabilităţi de grade diferite. Deşi studiile clinice în ceea ce priveşte administrarea şi efectele vitaminei D în diverse patologii sunt limitate, totuşi datele experimentale şi epidemiologice sunt suficient de consistente şi în acelaşi timp încurajatoare întărind ipoteza că hipovitaminoza D poate fi un factor de risc pentru declanşarea bolii şi în acelaşi timp un factor predictiv de evoluţie al sclerozei multiple. Cu toate că dozele de vitamina D folosite în studiile clinice sunt destul de variate şi diferite între 100 UI/zi până 14.000 UI/zi, iar rezultatele au fost încurajatoare pentru toate loturile de pacienţi luaţi în studiu, dozele administrate ar trebui stabilite pentru fiecare subiect în parte tinând cont de o serie de factori precum vârsta, sexul, comorbidităţile asociate, factorii de mediu sau genetici, aceştia din urmă putând interacţiona şi combina astfel încât să determine boala. Dacă avem în vedere faptul că această vitamină este implicată în apariţia unui numar destul de mare de afectiuni, inclusiv bolile autoimune şi în special scleroza multiplă , influentând semnificativ activitatea limfocitelor T reglatoare, al căror rol este bine cunoscut în patogenia bolii, se poate afirma că hipovitaminoza D devine o problemă majoră de sănătate, iar tratamentul cu vitamina D va deveni o opţiune terapeutică simplă şi eficientă, de perspectivă, pentru pacienţii cu scleroză multiplă care este cunoscută ca fiind boala adultului tânăr, o boală dizabilitanta şi cu un puternic impact negativ asupra calitătii vieţii acestora. De aceea, recomandarea administrării de vitamina D la bolnavii cu SM poate fi privită ca o opţiune înţeleaptă, provocatoare şi suficient de eficientă, momentan provizorie, care sa aibă ca rezultat îmbunătăţirea stării de sănătate a pacienţilor cu această boală.

Cuvinte cheie: Scleroza multiplă, autoimună, inflamatorie, demielinizantă a SNC, vitamina D.