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CUPRINS

CONTENTS

A FOCUSED REVIEW OF THE IMMUNE RESPONSE IN ELECTROCHEMOTHERAPY

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Keywords: electrochemotherapy, abscopal effect, gene electrotransfer, electroporation

Abbreviations:
ECT - electrochemotherapy
EP- electroporation
Insp-ECT - International Network for Sharing Practices on Electrochemotherapy
ICD - immunogenic cell death
DCs – dendritic cells
DAMPs - damage-associated molecular patterns
PRRs - pattern recognition receptors
TLR - toll-like receptors
TAA - tumor associated antigen
NLR - nucleotide-binding oligomerization domain (NOD) - like receptors
RLR - Retinoic acid-inducible gene I (RIG- I) - like receptors
CRT - calreticulin
HSP - heat-shock proteins
ATP - adenosine triphosphate
HMGB1- high-mobility group box-1
IFN - interferon
IL- interleukin
ER - endoplasmic reticulum
ROS - reactive oxygen species
GET- gene electrotransfer
EGT - electrogenetherapy
APC - antigen presenting cell
CD – cluster of differentiation

Abstract

Electrochemotherapy has been established since 2018 as an mainstream major cancer treatment. Electrochemotherapy is based on reversible electroporation and concomitant low-dose chemotherapy administration to target both cutaneous and subcutaneous tumor types . Standardized protocols, including the revised ESOPE (2018)[1] and NICE (2014)[2] guidelines, support electrochemotherapy (ECT), which is practiced in over 150 medical centers across Europe. The Insp-ECT (International Network for Sharing Practices on Electrochemotherapy) study[3] reported an average 80% objective response rate for multiple cutaneous tumor types following ECT treatment. The emergence of checkpoint inhibitors in immunotherapy, such as PD-L1 molecules, has highlighted the immune system's role in combating malignancy[4]. Consequently, the immune system's contribution to ECT's effectiveness, including impacts on untreated distant tumors, has garnered interest. Some studies attribute the lack of local recurrence after ECT to immunological mechanisms involving DAMPs molecules like calreticulin and HMGB1, while others argue that this response is insufficient for a systemic abscopal effect[5]. Ongoing research explores combining ECT with immuno-stimulants (including gene electro-transfer of immune molecules) to enhance therapeutic outcomes[6]. This review summarizes reports on the immune response's role in ECT's clinical efficacy and recent electro/immune therapy combinations.

Overview

Electrochemotherapy (ECT) is a local procedure that combines reversible electroporation (EP) of cellular membrane with low-dose chemotherapy. The intrinsic cytotoxicity of a non-permeant or poorly permeant chemotherapeutic drug delivered intravenously or injected intratumorally, is highly increased by the exposure of cells to controlled electric pulses which

permeabilize the cell membrane and allow an increased diffusion of the drug inside the cell[7]. To be treated efficiently, the whole tumor must be exposed to the drug molecules and simultaneously to electric field pulses having an intensity above a specific threshold value which reversibly permeabilize the cytoplasmic membrane.

The steps of an ECT procedure are presented in Fig 1.

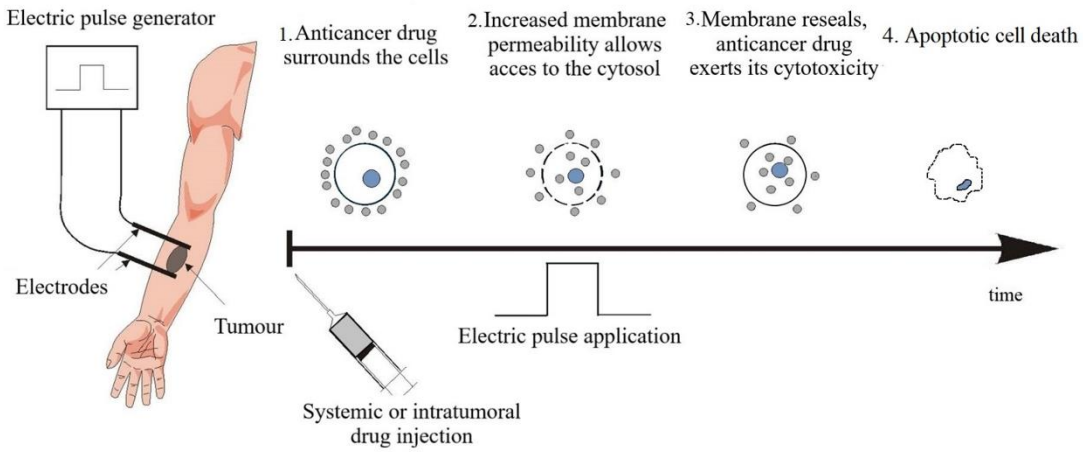

Fig 1 Schematic representation of ECT procedure: the drug is injected in bolus, after a waiting period of 20 minutes electroporation is performed on one or multiple nodules, after the cell membrane reseals, the drug exerts the cytotoxic effect ending with the apoptotic/mitotic cell death. (Adapted after Miklavcic, D.,-2012) [8]

This standardized treatment requires the use of medical approved devices (like CliniporatorTM, IGEA [10]) which ensure a minimal invasiveness, a fast application of electric field, and a simple and clear usability. The method became more widely used after the recent reports from Insp-ECT (International Network for Sharing Practices on Electrochemotherapy) about the remarkable efficiency of ECT in large cohorts of patients: 80% objective response rate in the case of all oncological histologies, and 60–70% complete remission rate following only one treatment[11].

Some of the major benefits of ECT include: the scarce chance of drug resistance occurrence in case of repeated treatments, a reduction of chemotherapy dosage and the subsequently increased quality of life index.

During EP pulses application the cells within the targeted tumor area are subjected to transient structural changes which can be modulated by using electric pulses with precisely controlled parameters (number, shape, duration, and repetition frequency, direction of the electric field), electrode geometry, and electrode position with respect to the tumor. The pulsing procedure given in ESOPE consists in 8 square wave pulses of 100 μs, resulting in an electric field of 1000V/cm between electrodes at adequate distances, with variations in protocol depending on tumor size, drug administration pathway, electrode type and the CliniporatorTM machine used[11]. When choosing the geometry and positioning of electrodes one must consider that the whole tumor should be exposed to an electric field above the permeabilizing threshold and as homogenously as possible[8]. The normal tissue around the tumor exposed to ECT is less affected since the division rate of healthy cells is slower compared to cancer cells and the membrane repair capacity is better preserved[12].

During ECT, therapeutic drug concentrations are obtained in tumor cells, resulting in a several fold increased cytotoxicity[13].This allows the use of lower dosages of bleomycin and cisplatin, compared to standard chemotherapy, enabling to repeat the procedure several times and avoiding the risk of occurrence of major side effects of the drugs (for bleomycin: nausea and vomiting, pneumonia, pulmonary fibrosis, hematological toxicity, skin reactions; for cisplatin: nausea and vomiting, hair loss, impeded wound healing, temporary vision loss, infections, hematological toxicity).

The implications of immune activity during ECT administration have been investigated through various in vitro, in vivo, and clinical studies, which indicate that the response to ECT differs significantly between immunodeficient and immunocompetent cases. Additionally, the role of the immune system in the progression of various solid tumors following combined ECT-immunotherapy treatment is being increasingly studied, with positive outcomes such as the absence of local recurrence being observed.[14].

ECT and immune response

A series of studies have demonstrated that electrochemotherapy induces immunogenic cell death markers, such as adenosine triphosphate, calreticulin, heat-shock proteins, and high-mobility group box-1, which are crucial for generating

tumor-specific cytotoxic T cells[15, 16]. The CD8+ T cells involved are specifically generated and can potentially target cancer cells that are resistant to other treatments. Studies on ECT in immunodeficient mice also support the immune response theory: the oedema and tumor regression after ECT delivery was significantly reduced in T lymphocyte-deficient mice compared to immunocompetent ones. ECT-induced local oedema encourages the infiltration of dendritic cells and lymphocytes into the affected area [17], which are found in all tumors treated with electrochemotherapy post-therapy. Furthermore, electrochemotherapy aids in the maturation of existing Langerhans cells within the tumor, prompting their movement to the tumor-draining lymph nodes and triggering a robust peripheral release of anti-tumor monocytes. It was previously thought that electrochemotherapy only provoked a localized anti-tumor immune response and had no effect on distant untreated nodules, indicating that the immunogenic cell death induced by electrochemotherapy was not strong enough to eradicate distant tumors. However, recent findings indicate that combining ECT with immunostimulants like IL-2-based immunotherapy can potentially treat both ECT-targeted and distant nodules18.

Inflammatory responses

Reversible electroporation (EP) alone, without the addition of chemotherapy, does not result in significant cell death or non-thermal tumor ablation[14]. However, it does induce immunological effects. The secretion of pro-inflammatory mediators, such as TNF-α, IL-1β, and other cytokines from the electropermeabilized cells, drives the recruitment of immune cells to the electroporated regions. This results in an inflammatory microenvironment in the treated area [17], characterized by a high inflammatory cellular infiltration (such as macrophages, dendritic cells, and polymorphonuclear leukocytes). Additionally, the presence of antigen-presenting cells in the electroporated area leads to the upregulation of APC maturation markers such as the F4/80 antigen or molecules of the type II major histocompatibility complex class. Interestingly, studies have shown that APC and PMN infiltration also occurs when electroporation is combined with DNA injection[18], enhancing immunization in DNA vaccination contexts. Additionally, the release of ATP following EP supports the hypothesis that EP promotes the chemoattraction of DCs and their precursors, facilitating their differentiation and maturation into antigen-presenting DCs[17]. Another pro-immunological effect of EP is observed at the vascular level, where acute vasoconstriction of blood vessels[14], particularly prolonged in tumor capillaries, facilitates the extravasation of immune cells and erythrocytes.

Induced immunogenic cell death

The pathway of cell death triggered by a treatment is crucial for its therapeutic success, as it influences the effectiveness of the systemic anti-tumor immune response. The way in which tumoral cells die due to electrochemotherapy depends on the specific mechanism of action of the chemotherapy. In this course of actions the host's immune response is vital in overcoming tumor resistance [14]. Four main types of cell death have been classified based on the tumor type and chemotherapeutic drug used: (i) mitotic cell death (with bleomycin), (ii) apoptotic cell death (with bleomycin), (iii) necroptosis (with bleomycin, cisplatin, and oxaliplatin), and (iv) pyroptosis-like immunogenic cell death (with bleomycin) [19].

One apoptosis type generated by various oncological treatments (such as chemotherapeutic drugs, oncolytic viruses, physicochemical therapies (cryotherapy), photodynamic therapy or radiotherapy) is the immunogenic cell death (ICD) [20, 21]. The ICD main mechanism of action is based on the release of damage-associated molecular patterns (DAMPs) from affected tumor cells. Critically during immunogenic cell death, the increase of reactive oxygen species and the subsequent stress in the endoplasmic reticulum (ER) leads to the increased level of (DAMPs). These DAMPs include the cell surface display of calreticulin (CRT) and heat-shock proteins (HSP70 and HSP90), as well as the extracellular release of molecules such as adenosine triphosphate (ATP), high-mobility group box-1 (HMGB1), type I interferons (IFNs), C-X-C motif chemokine ligand 10 (CXCL10), and members of the interleukin-1 (IL-1) cytokine family.)[22]. These molecules act as activators and thus are able to boost tumor-specific immune responses. ECT-released DAMPs bound on a series of risk-detecting receptors (or pattern recognition receptors (PRRs) such as toll-like receptors (TLRs) or NOD-like receptors (NLRs)[23]. These receptors are constitutive found on innate immune cells (such as dendritic cells (DCs), monocytes, and macrophages), and their activation leads to the activation of T lymphocytes that are thus able to eliminate tumor cells via phagocytosis. These processes may be supported and further enhanced by additional molecular events, such as the exposure of other ER chaperones on the cancer cell surface (including HSP90), and the secretion of various immunostimulatory cytokines like IL-1β and IL-17 by different immune cell types, thus facilitating DC recruitment into the tumor bed (also stimulated by ATP), tumor antigen engulfment by DCs (also stimulated by calreticuline), and optimal antigen presentation to T cells (also stimulated by HMGB1) [15]. ECT-treated melanoma patients have shown dendritic cell infiltration as a local response to the oncological treatment [24].

This ICD process discussed above can be complementary described as a balance between calreticulin (CRT) and CD47 upregulation on the tumor cell membrane. The CD47 is a widely expressed cell surface protein that regulates phagocytosis by innate immune cells, acts as a non- phagocytosis main signal addressed to macrophages and opposes the CRT which acts here as one of the phagocytosis main signal, as seen in Fig 2. [25].

To conclude, electrochemotherapy (ECT) induces mitotic death in the regions with both electric field and drug concentration coverage, with the apoptosis cascade triggered by tumor-associated antigens release from electroporated cells. The balance between calreticulin and CD47, as a crucial role/ and/or key mechanism of action played in the immune response should be a focus of future studies on this ECT driven immunogenic cell death. It is worth noted that/Notably, cells that receive suboptimal

ECT, such as surviving cancer stem cells or chemo-resistant cells, are later identified and eliminated by the cellular immune system, specifically by primed effector T cells p(via their earlier exposure to TAAs)[6]. These findings highlight the activation of the immune system following ECT, which may explain the absence of local recurrence and support the rationale for combining ECT with immunotherapy.

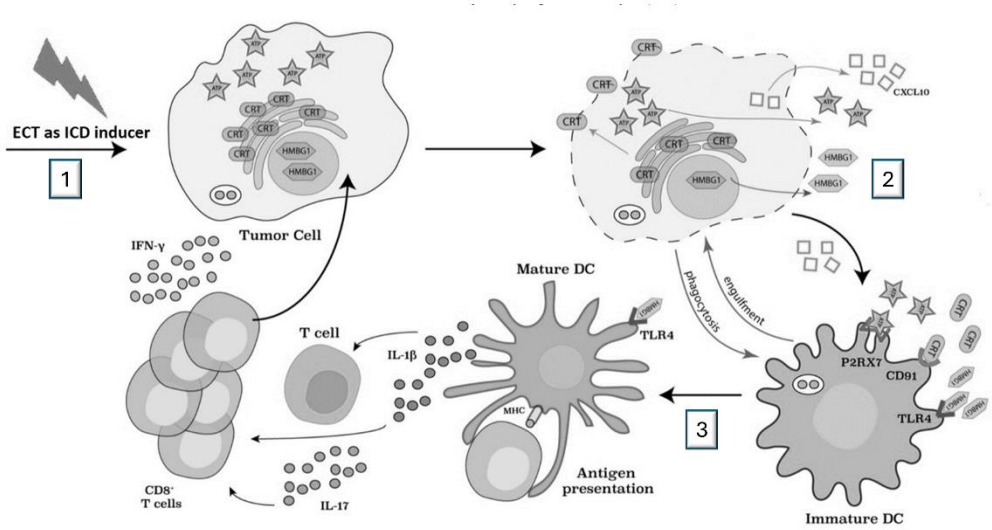

Fig. 2 Schematic representation of ECT as an ICD inducer in the micro-tumoral environment and the complex process of generation of tumor-specific cytotoxic T cells : 1) ECT application; 2) alterations in the tumor cell membrane inducing a specific immune reaction followed by the subsequent release of soluble mediators into the tumor microenvironment which in turn are bounding on dedicated receptors expressed by APC such as dendritic cells, and 3) presentation of tumor-associated antigens, leading to activation and proliferation of type T cytotoxic lymphocytes (CD8+)

Note: the number of various immune cells types in place at the time of electric pulses application influences the impact of ECT in efficient removal of tumor cells. (Adapted after Terenzi et al., Journal of Inorg. Biochem. 2016[26])

Emerging co-therapies applied with ECT

Although electrochemotherapy has an efficient direct effect for solid tumors, the anti-tumor immune response it elicits is insufficient to eradicate distant tumors. Emerging combination treatments, including checkpoint inhibitors and immunomodulators, are now being employed to enhance the immunological response induced by ECT, thereby promoting a systemic anti-tumor effect.

ECT addresses immune evasion mechanisms when combined with immunotherapy

While immunogenic cell death (ICD) induced by electrochemotherapy (ECT) promotes a beneficial immune response, tumors also have developed immune evasion mechanisms that are crucial for cancer progression.

Studies have highlighted the role of tumor neoantigens in the immune recognition of cancer cells. Tumor cells that present specific mutant antigens or fail to express antigens can evade immune detection. Immune editing shape tumor cell antigenicity during tumor attacks and reductions creating surviving resistant cells that contribute to the development of new cancer cell sub-populations.

Among several strategies employed by the tumoral cells to silence the immune response : (i) reducing the expression of MHC-1 to prevent recognition by immune cells, (ii) diminishing NK cell attacks by lowering the expression of NK cell receptors and activators, (iii) immune suppressor release that create an immunosuppressive microenvironment, iv) immunoediting ability of immunocompetent T cells creating cancerous resistant cell sub-populations[18, 27]. Examples of mechanisms by which tumor cells escape immune detection are illustrated in Fig. 3.

The immunosuppressive microenvironment is realized mainly by altering the induction of immune checkpoint receptors (example given CTLA-4, PD-L1) and secretion of anti-inflammatory cytokines by tumoral cells (such as interleukins (IL-10) or metabolic enzymes like indoleamine 2,3-dioxygenase (IDO) [28]). A weak antitumor T cell activity is thus created which promotes the expansion of regulatory T cells (Treg) and myeloid-derived suppressor cells (MDSCs). Furthermore, macrophages are polarized towards the tumor-promoting M2 phenotype, also known as tumor-associated macrophages (TAMs).).[28]. Therefore, treatments aimed at enhancing the immune response have become a promising approach in the

oncology field.

Recent advances in immunotherapy have shown promising results in cancer treatment. By combining immunotherapy with electrochemotherapy (ECT) new possibilities have been offered for long-term cancer eradication by enhancing and sustaining anti-tumor immunity.[29]. Immunotherapy has recently become one of the primary treatment options for melanoma. Most of the data on the efficacy of combining immunotherapy with other treatments have been gathered from patients with this low-survival-rate disease[30].

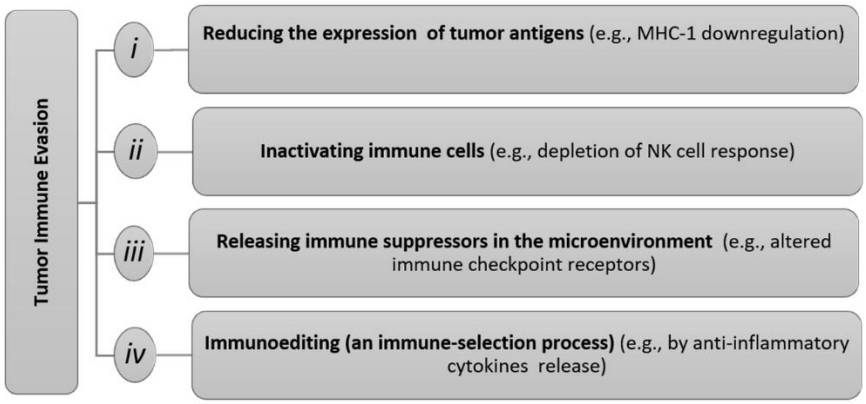

Fig. 3 Schematic representation of the ways in which tumor cells escape the immune system detection. (Abbreviations: NK, natural killer T lymphocyte; MHC-1, major histocompatibility complex molecules)

In a case report, combining BLM-ECT with ipilimumab, a monoclonal antibody targeting cytotoxic T lymphocyte-associated protein 4 led to a complete clinical response in a patient with multiple cutaneous melanoma metastases. Additionally, the appearance of vitiligo-like lesions specifically around the ECT treatment sites indicated that ipilimumab enhanced the immune activation initially triggered by ECT [11]. In another case report, the combination of BLM-ECT with nivolumab, an anti-programmed cell death protein 1 (PD-1) inhibitor, used as a fifth-line treatment, showed no evidence of cutaneous or visceral disease at a 4-year follow-up. This resulted in an extended remission period for a patient with advanced metastatic melanoma[31].

In a retrospective analysis patients treated with both ipilimumab and BLM-ECT, an objective response was observed in 67% of them (n=15), with 27% achieving a complete response and 40% a partial response. However, this study's limitation was the lack of a control group receiving only ipilimumab[32]. Another retrospective trial compared the effectiveness of BLM-ECT combined with ipilimumab to BLM-ECT combined with PD-1 inhibitors (pembrolizumab or nivolumab) in treating unresectable or metastatic melanoma. The ipilimumab group showed a systemic overall response rate (ORR) of 19.2%, while the anti-PD-1 group exhibited a systemic ORR of 40%. The study concluded that ECT combined with PD-1 inhibitors was more effective than ECT with ipilimumab in terms of overall response[33]. These findings are promising for the treatment of malignant melanoma, highlighting the need for more prospective trials to fully assess this combination's potential. Currently, a phase II interventional trial of ECT combined with pembrolizumab is underway[34].

ECT and electrogene therapy

DNA vaccination and cytokine-based anti-cancer therapies represent the most advanced strategies of gene electrotransfer (GET), also known as electrogenetherapy (EGT), which aims to stimulate anti-tumor immunity. GET involves delivering plasmid DNA (pDNA) or small interfering RNA (siRNA) molecules to various tissues, including tumors, through electroporation. This process allows for the transfer of therapeutic genetic material, enabling gene therapy to restore specific cellular functions and instruct cells to produce therapeutic or immunogenic proteins endogenously[35].

GET, which can be administered either intratumorally or peritumorally into the skin, involves in vivo gene electrotransfer of plasmid DNAs (pDNAs) encoding immunomodulatory molecules like cytokines, chemokines, and adjuvant sequences. Additionally, DNA vaccines carrying tumor-specific or tumor-associated antigens (TAAs) can be used alone or combined with chemotherapeutics for tumor treatment. The choice of approach depends on the immunogenicity of the tumors and the immune status of the organism[36, 37].

Plasmids up to 15 kilobases, edited with a Kozak sequence, are used for electrotransfer-based gene treatments in eukaryotic cells. Gene expression is regulated by environmental and developmental factors, with regulatory elements located at various distances from the gene. In gene therapy, coding sequences must be minimized. Tissue-specific promoters, which limit expression to specific tissues and reduce off-target effects, are commonly used in clinical trials, though their expression

levels are typically lower than those of viral promoters. Cell-specific targeting is also achievable through nuclear import sequences[38]. Regulable promoters, which can be controlled by an external factor, have significant potential but should be designed for inducible rather than repressible expression to minimize patient exposure to the inducing agent.

Electrotransfer of mRNA is commonly used to engineer antigen-presenting cells in vitro, inducing antitumor activity when injected into a patient. RNA interference can suppress gene expression after direct electroporation-mediated delivery of sequence-targeted small RNAs. Double-stranded small interfering RNA and stem-loop containing microRNA, derived from endogenous RNAs, are 20–30 base pair oligonucleotides that are processed in the cytoplasm to cleave and degrade specific target mRNAs. Since ubiquitous and resilient ribonucleases degrade cytoplasmic RNA, this must be considered when using electroporation as a delivery method. Pattern recognition receptors identify specific pathogen components, such as bacterial endotoxin and nucleic acids, triggering an inflammatory response and/or cell death. Gene therapies, including those involving viruses, plasmids, and oligonucleotides, can be detected by these receptors and may be mistaken for pathogen invasion, leading to unregulated inflammatory responses.[36].

Immune stimulation through EGT has shown significant promise for cancer treatment due to its safe administration [39], enhanced DNA uptake, and adjuvant effects. The stress condition induced by EGT stimulates local production of inflammatory cytokines, activating the innate immune response and recruiting M1 macrophages with anti-tumor activity. It also enhances adaptive immunity by attracting lymphocytes and inflammatory cells to the electroporated areas [27].

One of the most studied applications of EGT is the transfer of an immune gene coding for IL-12, which promotes the cytotoxicity of immune cells in both the innate and adaptive immune systems, fostering an antitumor type 1 cytokine environment. IL-12 promotes the secretion of IFN-γ from NK cells and T lymphocytes, enhancing the function of antigen-presenting cells by increasing class I and II MHC molecule expression. IL-12 also has anti-angiogenic properties[27]. Although recombinant IL-12 has shown systemic toxicity during clinical testing, the first clinical trial of GET with a plasmid encoding IL-12 in 2008 proved this method is safe and controlled. Studies using IL-2 or IL-12 have demonstrated antitumor effectiveness on distant untreated tumors and long-term anti-tumor memory [40].

Combining ECT (electrochemotherapy) with immunostimulatory EGT offers a promising strategy to enhance local antitumor response and induce an abscopal effect. Recent studies [36] have focused on combining BLM-ECT with peritumorally administered IL-12 GET. A study on three murine tumor models showed that IL-12 GET enhances the antitumor effect of ECT in poorly immunogenic B16F10 melanoma, while ECT alone is more effective in achieving an abscopal effect and long-term immunity in more immunogenic 4 T1 and CT26 tumors[41].

Investigating the immune response associated with ECT and immunomodulatory GET should include examining regulatory T cells (Tregs). In the tumor environment, Treg-induced immune suppression significantly hinders anticancer responses targeted by immunotherapeutic strategies[39] . Recent findings suggest that combining Treg depletion with immunotherapy based GET in the B16F10 melanoma model could reduce systemic metastasis and improve survival rates[41].

Short interfering RNA (siRNA) is also being explored as a viable oncological treatment. For example, siRNA targeting VEGF, an angiogenic factor, has been used to suppress tumor growth[42]. Similarly, siRNA targeting cyclin B1 has shown promising effects in inhibiting tumor cell proliferation and could be clinically validated in combination with other strategies, such as T cell targeting and downregulation of PD-1 or CTLA-4 [43].

ECT and calcium electroporation

Calcium electroporation (Ca EP) is a safe, cost-effective cancer treatment that introduces high calcium concentrations into cells via electroporation, causing ATP depletion and cancer cell death. A randomized double-blind phase II study showed its safety and efficacy in treating cutaneous metastases and recurrent head and neck cancer[44]. The efficacy is comparable to BLM-ECT for small tumor metastases, with 84% objective response for BLM-ECT and 72% for Ca EP, with no statistical difference at a one-year follow-up. Further clinical trials are needed to validate these findings[7].

Although primarily a local therapy, calcium electroporation (Ca-EP) has been shown to trigger a systemic immune response. In vivo studies revealed that Ca-EP treatment of colon cancer tumors in immunocompetent mice led to a complete response and long-lasting anti-tumor effects, while immunocompromised mice did not exhibit the same response. Additionally, in vivo Ca-EP increased the systemic release of pro-inflammatory cytokines, and in vitro Ca-EP elevated the release of High Mobility Group Box 1 protein (HMGB1), an important DAMP associated with immunogenic cell death (ICD)[45].

These findings indicate that Ca-EP can activate immune stimulators and induce a systemic immune response. Another in vivo study showed that combining calcium with irreversible electroporation (which permanently permeabilizes cell membranes with high-intensity electric fields) resulted in more T-cells and fewer suppressor cells[46]. Furthermore, a case report documented a systemic response following treatment with BLM-ECT and Ca-EP in a patient with disseminated malignant melanoma[47].

These studies suggest that Ca-EP can induce a systemic immune response, either alone or in combination with ECT. However, further research is necessary to understand the mechanisms of immune activation and to explore the potential of

Ca-EP and immunotherapy combinations.

Conclusions

The immune system plays a crucial role in cancer progression and treatment response, making therapies that activate the immune response a promising approach in oncology. EP-based therapies have gained attention in recent years due to their high efficacy rates and lack of severe side effects, showing local immune activation and markers of systemic response. However, there is no consensus on the abscopal effect of immune activation from ECT.

Various immunotherapies have been recognized as effective treatments for many solid tumors, including melanoma. Both immunotherapy and ECT have achieved efficacy levels that warrant their inclusion in the standardized anti-tumor treatment panel. Consequently, combining immunotherapy with ECT requires more attention to further improve patient clinical response, quality of life, long-term local and systemic remission, and life expectancy.

The combination of ECT with gene electrotransfer (GET) of immunomodulatory molecules is currently under study and showing encouraging results. Promising in vitro, in vivo, and clinical case reports indicate the potential of calcium electroporation (Ca-EP) to stimulate the release of specific molecules that trigger immunogenic cell death (ICD).

Despite these advances, the complex mechanisms behind the systemic immune response implications in ECT and Ca-EP are not yet fully understood, necessitating further research to determine their long-term impact on patient clinical outcomes.

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RĂSPUNSUL IMUN ÎN ELECTROCHIMIOTERAPIE. SCURTĂ SINTEZĂ

REZUMAT

Electrochimioterapia (ECT) a fost stabilită din 2018 ca unul din tratamentele principale împotriva cancerului. ECT se bazează pe electroporarea reversibilă a membranelor celulare și administrarea concomitentă de chimioterapie în doze mici pentru a viza atât tipurile de tumori cutanate, cât și cele subcutanate. Protocoalele standardizate, inclusiv ghidurile revizuite ESOPE (2018) și NICE (2014), susțin electrochimioterapia (ECT), care este astfel practicată în peste 150 de centre medicale din întreaga Europă. Studiul Insp-ECT (International Network for Sharing Practices on Electrochemotherapy) a raportat o rată medie de răspuns obiectiv de 80% pentru mai multe tipuri de tumori cutanate după tratamentul ECT. Apariția inhibitorilor de puncte de control în imunoterapie, cum ar fi moleculele PD-L1, a evidențiat rolul sistemului imunitar în combaterea malignității. S-a dezvoltat aşadar interesul acordat contribuției sistemului imunitar la eficacitatea ECT, inclusiv impactul acesteia asupra tumorilor îndepărtate netratate. Unele studii atribuie lipsa recurenței locale după ECT mecanismelor imunologice care implică participarea moleculelor DAMPs precum calreticulina și HMGB1, în timp ce altele susțin că acest răspuns este insuficient pentru un efect abscopal sistemic. Cercetările în curs explorează combinarea ECT cu imuno-stimulante (inclusiv electro-transferul genelor al moleculelor imune) pentru a îmbunătăţi rezultatele terapeutice. Această sinteză rezumă rapoartele privind rolul răspunsului imun în eficacitatea clinică a ECT și combinațiile recente între electrochimioterapie / imunoterapie.

Cuvinte cheie: electrochimioterapie, efect abscopal, transfer electro-genic, electroporare

CARDIAC ELECTROPHYSIOLOGY OF ARRHYTHMOGENESIS: IMPACT OF HABITUAL CIGARETTE SMOKING ON THE INDEX OF CARDIAC ELECTROPHYSIOLOGICAL BALANCE IN APPARENTLY HEALTHY AFRICAN YOUNG MEN

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Abstract

Aim and Background Studies have shown the effects of smoking on ECG parameters. However, investigation into the effect of habitual smoking on iCEB within young African population as a relatively new and more accurate marker for arrhythmogenesis beyond QT and QT_C is warranted.

Methods 30 apparently healthy subjects with no history of smoking constitute the non-smokers or control group, while subjects with cigarette smoking habit with non-specific symptoms were enrolled to the smoker group. Anthropometric, demographic and ECG parameters were obtained.

Results The mean values for height, weight, BSA and BMI were 1.73 ± 0.07 m, 63.96 ± 7.64 kg, 1.75 ± 0.13 and 21.82 ± 3.02 kg/m^2 respectively. The ECG heart rate (bpm) increased significantly among smokers' group compared to non-smokers' group (73.67 ± 13.41 vs. 67.97 ± 6.70; $p < 0.05$). Specifically, indices of cardiovascular balance iCEB and corrected index of cardiovascular balance iCEBc showed significant shortening among smokers' group compared to non-smokers' group (4.49 ± 0.65 vs. 5.31 ± 0.94; $p < 0.001$ and 5.14 ± 1.55 vs. 6.02 ± 1.65; $p < 0.04$ respectively). However, QT and QTc did not differ significantly between smokers' group compared to non-smokers' group (353.00 ± 31.31 vs. 361.70 ± 35.53; $p > 0.05$ and 401.30 ± 92.28 vs.408.00 ± 77.30; $p > 0.05$ respectively).

Conclusion Both iCEB and iCEBc decreased significantly in apparently healthy smokers compared with non-smokers. This may suggest chronic and habitual smokers' susceptibility to non-Torsades de Pointes-mediated ventricular arrhythmias with or without QT-prolonging genetic variations.

Keywords Arrhythmia, Cardiac electrophysiology, iCEB, Smokers, Nicotine, Arrhythmogenesis

Introduction

Cigarette smoking remains a top risk factor for certain cardiovascular (CV) disorders [1]. Specifically, exposure to cigarette smoke causes various hemodynamic and cardiac electrophysiological deteriorations through complex mechanisms both in the short and long runs [2–4]. Nicotine, a major constituent of cigarette smoke, was reported to delay ventricular repolarization, release catecholamines into circulation, activates sympathetic nervous system, and to prolong membrane repolarization via direct blockage of inward K^+ channels in ventricular myocardium [5].

Furthermore, nicotine, together with carbon monoxide and other oxidative agents, was suggested to be casually related to the development of myocardial fibrosis in different cardiac compartments [2].

Research outcomes have shown that the 12-lead electrocardiography (ECG) could provide valuable data concerning increased cardiac arrhythmogenesis, such as increased QT and corrected QT (QTc) intervals, PR interval, Tpeak to Tend interval (Tp-Te), dispersion of QT interval, and dispersion of P wave [6–12]. On the other hand, index of cardiac electrophysiological balance (iCEB), namely the ratio of QT to QRS (QT/QRS) calculated from 12-lead ECG, is a novel and simple ECG marker that may predict ventricular arrhythmogenesis [13, 14]. It is suggested that the iCEB is a surrogate marker of the cardiac wavelength λ (λ = effective refractory period (ERP) x conduction velocity (CV)), and an ultimate representation of the balance between cardiac repolarization and depolarization [13]. For this reason,

either an increased or decreased iCEB values relates to certain ventricular arrhythmic events.

The index of Cardiac Electrophysiological Balance (iCEB) is a novel non-invasive marker which reflects the balance between depolarization and repolarization of the cardiac action potential. iCEB is evaluated by dividing QT interval by QRS duration (QT/QRS). In a study, using a different model, iCEB was shown to be a strong predictor of drug-induced cardiac arrhythmias (10).

In the light of the afore-mentioned premises, we intended to assess the status of iCEB in otherwise healthy people with habitual cigarette smoking compared with healthy non-smoker subjects.

Materials and Methods

Recruitment of the study subjects

A total of 60 subjects were subdivided into smokers' group and non-smokers' group participated in the study following an initial screening process. Volunteers without smoking habit for the study were evaluated clinically to exclude symptomatic systemic diseases that could adversely affect the cardiovascular status were grouped among non-smokers, while volunteers with cigarette smoking habit with non-specific symptoms were enrolled to the smoker group.

All the study participants had no previous history of major clinical problem. Smoking habit was defined as at least 3 cigarette smoking per day for at least 1 year. All of the participants were subjected to a comprehensive physical examination, and echocardiographic and ECG evaluation to inquire probable cardiac disorders. The exclusion criteria were set as follow: history of cardiovascular disease, diabetes mellitus, hypertension, cerebrovascular disease, chronic kidney failure and chronic inflammatory disease, endocrine disorders, acute infections, and chronic medication usage.

Anthropometry Measurements

Height and weight measurements

The standing heights were measured using a stadiometer (Liam Medical England, Model: NO. RGZ-160) and following the methods described elsewhere [15-17]. Each participant was assessed while in good standing posture on the foot rest of the device with minimal clothing without shoes but with the head facing forward, shoulders relaxed, arms hanging loosely on both sides, palms facing forwards, feet together, and knees straight.

The height for each subject was taken when the movable headboard was lowered to touch the crown of the head. The measurements were taken to the nearest 0.5 cm.

The weighing scale was checked for zero balance before each use. Subjects were instructed to empty pockets and remove shoes and any apparel that could interfere with weight measurements. Only light clothing was permitted. The subjects then stood on the scale looking straight ahead, relaxed, and motionless without leaning on any object or the wall. Weight measurements were taken when the scale stabilized and recorded to the nearest 0.5 kg.

The body-mass index (BMI) was calculated as weight in kilograms divided by the square of height in meters.

Calculation of Body Mass Index (BMI) and Body Surface Area (BSA)

BMI was calculated as weight in kilograms divided by the square of height (in metres). BMI was expressed in kg/m^2 .The BSA was calculated using Mosteller formula [18]: BSA (m^2) = (weight (kg) × height (cm)/3600)$^{1/2}$.

Standard 12-Lead Electrocardiogram Recordings

Each participant's standard 12-lead resting electrocardiogram (ECG) was recorded in the supine position, during tranquil respiration. Each subject provided both written and verbal informed permission. To eliminate electromagnetic interference and increase the quality of the ECG, materials such as wrist watches, jewellery, coins, and cell phones were removed from the body. The chest and limbs were exposed, and ECG electrodes were placed in accordance with an internationally recognized methodology [19]. The chest lead recordings (V_1-V_6) were obtained utilizing conventional techniques of attaching six electrodes to specific anatomical sites on the anterior chest wall:

V_1 at the 4th intercostal space right sternal edge
V_2 at the 4th intercostal space left sternal edge
V_3 at the point mid-way between V_2 and V_4
V_4 at the 5th intercostal space left mid-clavicular line
V_5 at the 5th intercostal space left anterior axillary line, and
V_6 at the 5th intercostal space left mid-axillary line.

The six limb leads (I, II, III, aVF, aVL, and aVR) were recorded, using four electrodes connected to the distal end of each limb, by following the conventional limb electrode placement method [19].

The standard 12-lead ECGs were recorded at a speed of 25 mm/s and calibration signal of 10 mm/mV. The electrocardiogram were printed out and the waves, intervals, and the segments were manually obtained from the rhythm strip (lead II) [12].

The QT interval was then corrected for heart rate (QTc) using Bazett's formula: QTc = QT/(RR). The following are the definitions: R-R interval, the time interval between two consecutive R waves; PR interval, the time interval from the start of the P wave to the start of the QRS complex; QT interval, the time interval from the start of the Q wave to the end of the T wave where it intersects with the isoelectric line; and, iCEB, the ratio of QT to QRS (QT/QRS); and, iCEBc, the ratio of QTc to QRS (QTc/QRS).

Informed Consent The study complied with the Helsinki Declaration. Both written and oral consents were obtained from all subjects prior to and during the study.

Statistical analysis

The statistical analysis was implemented by using SPSS (Version 26 for Windows, SPSS Inc., Chicago, USA). Categorical variables of the subjects were given in numbers and percentages; whereas continuous variables were expressed as mean ± SD and median (25–75 interquartile range). Quantitative data were assessed for normality by using Kolmogorov-Smirnov test. While normally-distributed variables were compared using independent sample t-test, the variables which show non-normal distribution were compared by the help of MannWhitney U test. p value was considered statistically significant, if it is < 0.05.

Table 1

Descriptive Statistics of Demographic and Anthropometric Data of Participants (n = 60)

Variable	Smokers (n = 30) (Mean ± SD)	Non-Smokers (n = 30) (Mean ± SD)	Combined Data (n = 60) (Mean ± SD)	Min	Max
Age (years)	22.71 ± 1.61	21.50 ± 2.44	22.50 ± 2.39	17	30
BMI (kg/m²)	22 ± 2.15	20.96 ± 1.47	21.48 ± 1.90	17.73	26.95
BSA (m²)	1.79 ± 0.12	1.71 ± 0.13	1.75 ± 0.13	1.46	2.04
Height (cm)	173.80 ± 7.26	171.20 ± 7.60	172.5 ± 7.48	156	187
Weight (kg)	66.40 ± 7.46	61.52 ± 7.13	63.96 ± 7.64	49	83

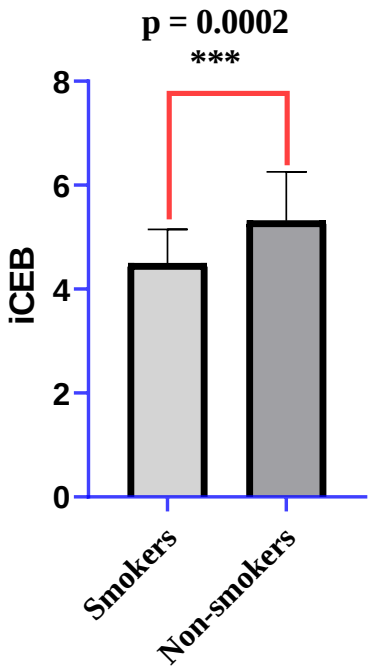

Figure 1 – Index of cardiac electrophysiological balance (iCEB) between smokers and non-smokers

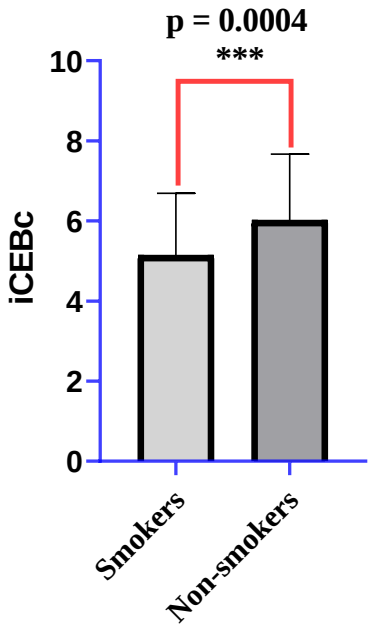

Figure 2 – Corrected index of cardiac electrophysiological balance (iCEBc) between smokers and non-smokers

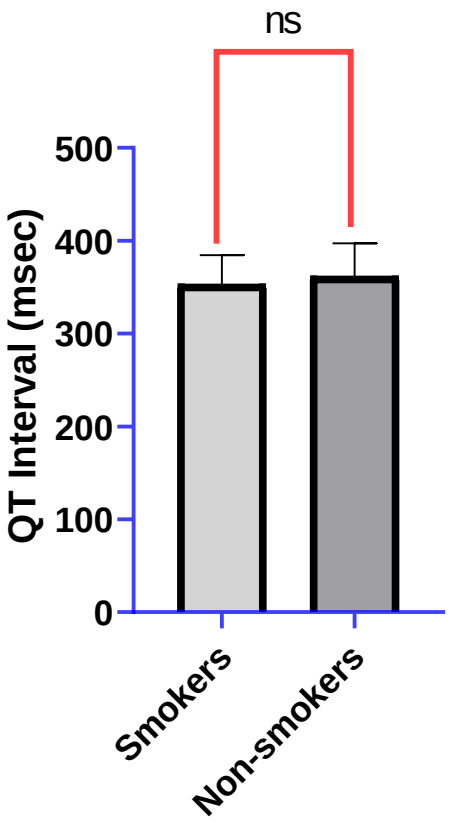

Figure 3 – QT interval between smokers and non-smokers

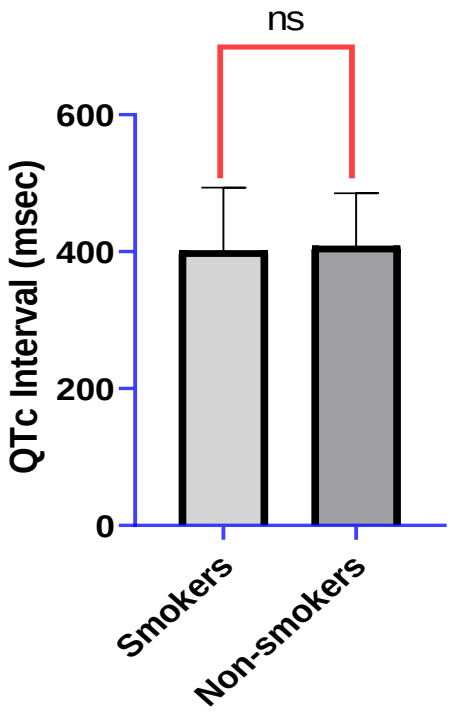

Figure 4 – Corrected QT (QTc) between smokers and non-smokers

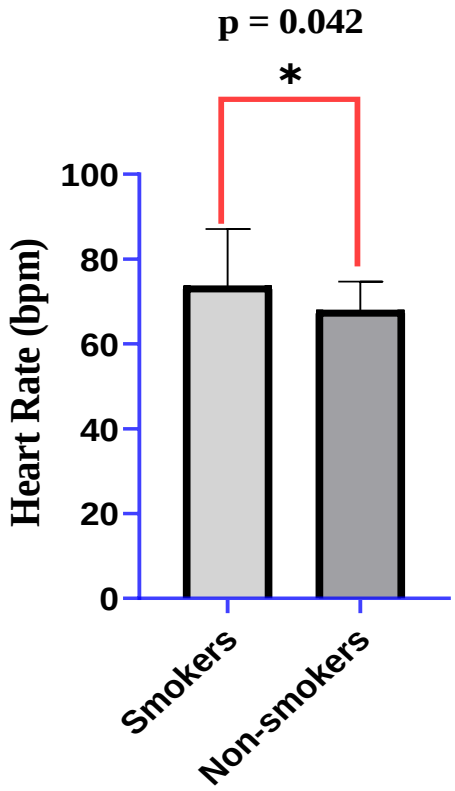

Figure 5 – Comparing the resting heart rates (HR) between smokers and non-smokers

iCEB: index of cardiac electrophysiological balance
iCEBc: index of cardiac electrophysiological balance with heart rate correction
'ns' indicate the comparison is not statistically significant
p values in bold with asterisk indicate the comparison is statistically significant

Results

A total of 60 subjects volunteered and were enrolled for this study. Among these, 30 subjects who were apparently healthy with no history of smoking constitute the non-smokers or control group, while subjects with cigarette smoking habit with non-specific symptoms were enrolled to the smoker group. The combined age range of the participants (n = 60) was 17 – 30 years. Among all participants, the mean values for height, weight, BSA and BMI were 1.73 ± 0.07 m, 63.96 ± 7.64 kg, 1.75 ± 0.13 and 21.82 ± 3.02 kg/m^2 respectively.

Assessment of ECG surrogate markers of cardiac wavelength λ between smokers' group and non-smokers' group

Indices of cardiovascular balance iCEB and corrected index of cardiovascular balance iCEBc showed significant shortening among smokers' group compared to non-smokers' group (4.49 ± 0.65 vs. 5.31 ± 0.94; $p < 0.001$ and 5.14 ± 1.55 vs. 6.02 ± 1.65; $p < 0.04$ respectively).

Assessment of Heart rate, QT and QTc markers between smokers' group and non-smokers' group

The heart rate showed a significant increase among smokers' group compared to non-smokers' group (73.67 ± 13.41 vs. 67.97 ± 6.70; $p < 0.05$). However, there were no significant differences in QT interval and QTc values between smokers' group compared to non-smokers' group (353.00 ± 31.31 vs. 361.70 ± 35.53; $p > 0.05$ and 401.30 ± 92.28 vs.408.00 ± 77.30; $p > 0.05$ respectively).

Discussion

The results of the present study show that both iCEB and iCEBc were significantly lower among habitual smokers compared with the non-smokers. Secondly, mean resting heart rate was increased, but surprisingly, the baseline QT interval and QTc were not significantly different between smokers and non-smokers group which is a direct contrast to findings elsewhere.

Chronic smoking has been a robust risk factor for future cardiovascular events. Moreover, habitual smoking brings about a chronic inclination to decreased vagal tone, blunted baroreflexes, and increased sympathetic autonomic tone [20]. This unopposed escalation in sympathetic autonomic tone is implicated to a certain extent in all cardiovascular and hemodynamic complications of smoking, and even sudden cardiac death [20, 21]. A specific reflection of this unopposed chronic sympathetic activation could simply be exemplified by the increase in basal heart rate in chronic cigarette smokers compared with that of non-smokers. Venkatesh *et al.,* [22] revealed in their study a faster basal heart rate in chronic smokers than that of non-smokers. Sharma *et al.,* [23] evaluated ECG changes in 150 chronic smokers and also reported a significant increase in resting heart rate in the smokers compared with non-smokers. In another recent and large-scale study encompassing a total of 141,317 subjects, Linneberg *et al.,* [24] found a significant relationship between chronic cigarette smoking and greater resting heart rate. On the contrary, they could not reveal any causal relationship between chronic smoking and higher blood pressure or development of a new hypertension. In this regard, our study findings seem consistent with the previous reports.

QT interval is the time between ventricular depolarization (giving rise to the "QRS complex") and its repolarization (giving rise to the "T wave"). Since the QT interval changes with heart rate, hence it is often stated as the QT interval corrected for heart-rate (QTc) using Bazett's method.

QT and QTc intervals encompass the complete period of ventricular depolarization and repolarization and their prolongation portends risk for Torsades de Pointes- (TdP-) mediated ventricular arrhythmia occurrence and sudden cardiac death [7]. Current data is conflicting with regard to the ultimate change in QT interval in chronic smokers, where some studies suggested an increase [25], some others reported no change [23, 26, 27] or even decrease in mean QT interval [23, 28] in apparently healthy smokers. Contrary to QT interval, studies investigating the effects of cigarette smoking on QTc interval revealed more consistent results in favour of a significant increase in mean QTc in smokers as compared to non-smokers [21, 23, 26, 28]. In our study, mean QT interval did not change significantly between the smokers and non-smokers. In contrast, we revealed a significant prolongation in baseline QTc interval in the smoker group compared with the non-smoking controls, which we thought was compatible with the previous reports.

iCEB is a relatively new non-invasive ECG parameter may prove useful in predicting TdP and non-TdP mediated ventricular arrhythmic events, far beyond sole QT and Tp-Te intervals [13,14]. In their animal study conducted 2013, Lu *et al.,* [13] introduced this simple parameter and suggested that a marked increase in iCEB could potentially exert a TdP mediated arrhythmogenic effect, while a marked decrease could give rise to non-TdP mediated ventricular arrhythmias. Thereafter, Robyns *et al.,* [14] showed in their clinical study a close and significant relationship between invasive cardiac electrophysiology study-derived (EPS-derived) ventricular ERP and ECG-derived QT interval, thus introducing a new ECG-derived surrogate marker of cardiac wavelength. They further reported that such respective drugs and clinical conditions conducive to TdP-mediated ventricular arrhythmias as sotalol and possessing mutations of congenital long QT syndrome increased both of iCEB and iCEBc, whereas some other conditions such as Brugada syndrome and use of flecainide which were conducive to non TdP-mediated ventricular arrhythmias decreased the same parameters [14].

Our study clearly shows a significant reduction in ICEB between smokers group and the control group. iCEBc was similarly also lower in the smokers group compared to the control group. On the basis of our study findings, we may speculate that chronic cigarette smoking may pose an additive risk of non TdP-

mediated ventricular arrhythmias to those habitual smokers who may inherently been carrying long QT syndrome mutations as Africans. Finally, these findings suggest that ECG monitoring should be required and included as part of routine health check-up for smokers at all levels of healthcare.

Conflict of interest statement

Authors wish to declare that this study possess no conflict or competing interests.

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ELECTROFIZIOLOGIA CARDIACĂ A ARITMOGENEZEI: IMPACTUL FUMATULUI OBIȘNUIT DE ȚIGĂRI ASUPRA INDICELUI DE ECHILIBRU ELECTROFIZIOLOGIC CARDIAC LA TINERII AFRICANI APARENT SĂNĂTOȘI

Rezumat

Scopul și contextul Studiile au arătat efectele fumatului asupra parametrilor ECG. Cu toate acestea, este justificată investigarea efectului fumatului obișnuit asupra iCEB în cadrul populației africane tinere ca un marker relativ nou și mai precis pentru aritmogeneză dincolo de QT și $_{QTC}$.

Metode 30 de subiecți aparent sănătoși fără istoric de fumat constituie grupul de nefumători sau de control, în timp ce subiecții cu obiceiul de a fuma țigări cu simptome nespecifice au fost înscriși în grupul de fumători. S-au obținut parametrii antropometrici, demografici și ECG.

Rezultate Valorile medii pentru înălțime, greutate, BSA și IMC au fost 1,73 ± 0,07 m, 63,96 ± 7,64 kg, 1,75 ± 0,13 și, respectiv, 21,82 ± 3,02 $^{kg/m2}$. Ritmul cardiac ECG (bpm) a crescut semnificativ în rândul grupului de fumători comparativ cu grupul de nefumători (73,67 ± 13,41 vs. 67,97 ± 6,70; $p < 0,05$). În mod specific, indicii de echilibru cardiovascular iCEB și indicele corectat de echilibru cardiovascular iCEBc au prezentat o scurtare semnificativă în rândul grupului de fumători comparativ cu grupul de nefumători (4,49 ± 0,65 vs. 5,31 ± 0,94; $p < 0,001$ și 5,14 ± 1,55 vs. 6,02 ± 1,65; $p < 0,04$, respectiv). Cu toate acestea, QT și QTc nu au diferit semnificativ între grupul de fumători comparativ cu grupul de nefumători (353,00 ± 31,31 vs. 361,70 ± 35,53; $p > 0,05$ și 401,30 ± 92,28 vs. 408,00 ± 77,30; $p > 0,05$ respectiv).

Concluzie Atât iCEB cât și iCEBc au scăzut semnificativ la fumătorii aparent sănătoși comparativ cu nefumătorii. Acest lucru poate sugera susceptibilitatea fumătorilor cronici și obișnuiți la aritmii ventriculare mediate de non-Torsades de Pointes cu sau fără variații genetice de prelungire a QT.

Cuvinte-cheie Aritmie, Electrofiziologie cardiacă, iCEB, Fumători, Nicotină, Aritmogeneză

NEUROGLOBIN, A HEME PROTEIN WITH NEUROPROTECTIVE FUNCTION

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ABSTRACT
Neuroglobin is a relatively recently discovered heme protein whose roles, although incompletely defined, may be related to oxygen delivery to nerve cells, neuroprotection against hypoxia/ischemia, antioxidant defense, and stimulation of neuronal survival. The aim of this brief review is to present the functional roles of neuroglobin in light of published data, showing its relevance to the pathophysiology of some neurologic disorders and the mechanisms through which it could provide neuroprotection.
Keywords: neuroglobulin, oxidative stress, neuroprotection

INTRODUCTION

In the family of proteins involved in oxygen transport and storage, in addition to the well-known hemoglobin and myoglobin, a new structurally and functionally related globin was identified in humans and mice by Burmester et al. [1]. It was named neuroglobin (Ngb) because it is mainly expressed in the brain of these vertebrate species [1]. Ngb has been identified not only in neurons but also in primary cultured astrocytes isolated from the neonatal mouse brain [2], in reactive astrocytes under various pathological circumstances [3, 4], in the eye (retina) [5, 6] and in the endocrine tissues (adenohypophysis, adrenal glands, testis, β-cells of pancreatic islets of Langerhans) [6, 7, 8, 9]. In the mouse brain, Ngb has been detected in endothelial cells and pericytes of the neurovascular unit, where it may play a role in reducing blood-brain barrier (BBB) leakage [10].

The parts of the human brain where Ngb is widely distributed are: the cerebral cortex, cerebellum, thalamus, hypothalamus, hippocampus and olfactory bulb [4, 8, 11]. Likewise, Ngb is expressed in brain areas involved in the sleep-wake cycle, and its immunoreactivity was demonstrated to oscillate with the sleep-wake cycle, revealing that its expression may be regulated by sleep [12]. Analysis of Ngb immunoreactivity in the mouse indicated a focal distribution, with the most intense staining in the medial vestibular nucleus and paraolivar nucleus, a more reduced extent in the thalamic and subthalamic regions and cortex, and an absent immunoreactivity in the hippocampus and corpus callosum [9]. Regional variation in Ngb protein expression could be related to variation in hypoxia tolerance of different regions of the mouse brain [13]. Di Giulio et al. reported the presence of Ngb in the carotid bodies of male rats under normoxic conditions and showed that chronic intermittent exposure of animals to hypoxia for 12 days significantly increased Ngb levels compared to control rats exposed to room air [14]. Previous research showed that Ngb is restricted to the cytoplasm of neurons [9, 15], while later the presence of Ngb was reported in mitochondria [16, 17, 18] and in the nucleus [17, 18] as well. The roles of Ngb in the cytoplasm and nucleus of cells could be different [10].

STRUCTURAL ASPECTS OF NEUROGLOBIN AND ITS AFFINITY FOR OXYGEN

The structure of Ngb has been highly conserved throughout evolution [6, 8, 9, 15]. There are only small differences in amino acid positions between human and mouse Ngb [6, 8]. Semenova et al. developed a system for the biosynthesis, isolation, and purification of recombinant human Ngb, which allowed the production of protein to facilitate the study of its psysicochemical properties [19]. Human Ngb is a monomeric heme protein, made up of 151 amino acids, with a molecular weight of approximately 17,000 Da [1, 8, 19, 20]. The globin chain consists of eight α-helices, named from A to H [1, 8, 19, 21]. The heme group is located between helices E and F [8, 22]. The iron in the Ngb heme is hexacoordinated [8, 9, 19], compared to the heme iron of hemoglobin and myoglobin, which is pentacoordinated [8, 15, 19, 21], in the absence of an external ligand [15, 18]. The six iron ligands of Ngb are the four N atoms of the heme plane [8, 22], a histidine residue (HisF8), which binds heme iron proximally [8, 21, 22], and a histidine residue (HisE7), which binds to iron distally [8, 21, 22]. Substitution of the distal histidine residue with Val renders human Ngb unable to form the bis-His conformation, leading to a reduced ability to protect neurons against oxidative stress-induced cell death [18], while its replacement with leucine or glutamine results in a stable five-coordinated conformation, which reduces nitrite to nitric oxide (NO) much faster than the wild type of Ngb [23]. Iron can exist in either divalent (ferrous) or trivalent (ferric) form [18, 24]. In addition to oxygen, ferrous Ngb can bind carbon monoxide (CO) and NO [9, 16, 21, 25], but these gaseous ligands have to displace the HisE7 residue to be bound by iron [15, 26, 27].

The oxygen affinity of this reversible oxygen-binding protein is similar to that of myoglobin [6, 9, 26], although lower [1, 25, 28], but higher than that of hemoglobin [1, 26, 28]. The P_{50} (partial pressure of oxygen (P_{O2}) at which Ngb is 50% saturated with oxygen) of human ferrous Ngb at pH 7.0 and 25.0°C ranges from 0.9 mmHg to 10 mmHg [22]. Oxygen binding by Ngb is thought to be connected to the redox state of the cells [6, 15]. Ngb binds oxygen at high P_{O2} and unloads it at low P_{O2} [15, 29]. The concentration of Ngb is correlated with mitochondrial distribution and P_{O2} [5, 15]. Ngb can regenerate NAD+ under anaerobic conditions, participating in production of ATP [6]. Hypoxia leads to the accumulation of NADH, which stimulates the release of oxygen from Ngb [6]. However, because of its low concentration in the brain [1, 15] and the high oxygen consumption of neurons, it has been questionable whether the main function of Ngb is to bind or store oxygen [25, 27],

as in the case with myoglobin, the concentration of which in red muscle fibers is much higher [25]. One explanation for the necessity of Ngb in neurons is related to the longer diffusion distance between the blood-brain capillaries and neuronal mitochondria, compared with the shorter diffusion distance in other tissues [15].

INSIGHTS INTO THE FUNCTIONAL ROLES OF NEUROGLOBIN

The primary functional role of Ngb is likely in connection with neuronal oxygen homeostasis, supplying oxygen to metabolically active nervous tissue [6, 15, 26]. However, increasing experimental evidence is accumulating that Ngb may perform other tasks important for neuronal functioning and survival. Ngb may act as an oxygen sensor [18, 30], a scavenger of oxygen and nitrogen radicals [8, 15, 31], a NO reductase [23], a neuroprotector against hypoxic/ischemic insults [20, 32], an enhancer of the neuronal apoptotic threshold [8, 15, 24], a regulator of neuronal energy metabolism [28] and a promoter of neurogenesis [10, 33, 34].

Sun et al. showed that the expression of this globin was enhanced by exposing mouse neuronal culture to hypoxia *in vitro* and mice cerebral cortex to focal ischemia *in vivo* [20]. Neuronal survival after hypoxia was reduced when Ngb expression was inhibited, but was increased by overexpression of Ngb [20]. This experiment indicated that Ngb might be a hypoxia-inducible factor whose action is to protect neurons against ischemic hypoxia damage [20]. Although it is not clear how this neuroprotection is achieved, it can be speculated that Ngb acts similarly to myoglobin in muscles, binding oxygen and delivering it to neuronal mitochondria [20]. Another research revealed that hypoxic postconditioning increased Ngb expression in the CA1 subregion of the hippocampus after transient global cerebral ischemia (tGCI) in adult rats [32]. Moreover, inhibition of Ngb expression with Ngb antisense oligodeoxynucleotide suppressed the neuroprotective effect of hypoxic postconditioning, whereas Ngb overexpression reduced neuronal damage after tGCI [32]. Hypoxic postconditioning increased the level of Na^+/K^+-ATPases β1 subunit (Atp1b1) in CA1 neurons after tGCI [32]. Upregulation of Ngb maintained the membranous level of Atp1b1 and reduced the glutathionylation of Atp1b1 by inhibiting the production of reactive oxygen species (ROS), thus preserving the activity of the ion pump [32].

Yu et al. evaluated the role of Ngb-Cyt1 (cytochrome c1) binding in oxygen-glucose deprivation (OGD)/reoxygenation-induced neurotoxicity and ROS production in primary neurons and concluded that Ngb binding to the mitochondrial complex III subunit Cyt1 suppressed ROS production and reduced OGD-induced neurotoxicity [35]. Ngb has been shown to be localized in the mitochondria of primary-cultured mouse cortical neurons, and its mitochondrial concentration was significantly increased after OGD, which may indicate the translocation of Ngb from the cytoplasm to the mitochondria through the mitochondria permeability transition pore (mPTP) opening [16]. The translocation was blocked by inhibitors of the mPTP and the voltage-dependent anion channel (VDAC) [16]. The increased mitochondrial distribution of Ngb may contribute to neuroprotection [16].

It is hypothesized that Ngb can sense the intracellular redox state of the cell by modifying its three-dimensional structure [30]. Evidence has been provided that human Ngb functions as an oxidative stress-responsive sensor, and oxidative-stress induced structural changes in Ngb are required to protect neurons against oxidative damage [18]. Oxidative stress determines the conversion of the oxygen-bound ferrous form of Ngb, characteristic to normoxemic conditions, to the ferric bis-His conformation [18]. The oxidized form of Ngb (ferric bis-His Ngb) binds to flotilin-1, a lipid raft microdomain-associated protein, and to the α-subunits of heterotrimeric G proteins (Gα(i/o) that are activated by ROS [18]. Ferric bis-His Ngb acts as a guanine nucleotide dissociation inhibitor for Gα(i/o) and supresses oxidative-stress mediated reduction of cAMP to prevent neuronal death [18].

Experimental cerebral ischemia induced in mice by transient middle cerebral artery occlusion (tMCAO) revealed that the distribution of Ngb was not limited to neurons and astrocytes, but was also expressed in platelet-derived growth factor receptor β (PDGFRβ)-positive pericytes of the neurovascular unit [10]. After stroke, the concentration of Ngb was significantly decreased in the infarct core, and Ngb and PDGFRβ-positive pericytes were detached from the cerebral blood vessels, while in the infarct penumbra, PDGFRβ-positive pericytes expressing Ngb were increased compared to their amount in the infarct core [10]. Furthermore, BBB leakage after tMCAO was reduced in cerebral vessels with Ngb-positive PDGFRβ pericytes around the vasculature of BBB/or neurovascular unit, that shows that Ngb might be related to pericyte neuroprotection during reperfusion injury after stroke [10].

The neuroprotective function of Ngb could hypothetically be achieved through several mechanisms, such as decreasing ROS production, improving mitochondrial function and inhibiting neuronal apoptosis [8, 32, 33, 36]. The antiapoptotic activity of Ngb could be related to the interaction with one of the electron carriers of the mitochondrial respiratory chain, cytochrome c (Cyt c), which is released into the cytoplasm when the mitochondrial outer membrane becomes permeable [19, 24, 32]. By binding Cyt c, Ngb blocks the Cyt c–dependent apoptotic pathway [19, 24]. Ngb inhibits Cyt c-induced caspase 9 activation, and thereby, prevents caspase 3 activation that causes cell death [24]. It has been postulated that the crucial role of expressing high levels of Ngb is to prevent accidental neuronal apoptosis caused by intracellular stress signals associated with physiological neuronal functioning that might lead to mitochondrial damage [24].

In ischemic mouse neurons, the expression and distribution of Ngb has been associated with axonal regeneration after ischemic reperfusion process [37]. Overexpression of Ngb increased axon length in normal cultured cortical neurons and

OGD/reoxygenation treated neurons by activating the p38 MAPK signaling pathway [37]. The oxygen-binding site (His^{64}) of Ngb is the major regulatory site for p38 activation [37].

Using murine neuropathological models of traumatic brain injury, cerebral malaria, experimental autoimmune encephalitis, and kainic acid-mediated epileptic seizures, DellaValle et al. reported that Ngb is localized not only in neurons but also in reactive astrocytes, compared with control mice, where Ngb was identified only in neurons [3]. In addition, Ngb positive astrocytes were identified in regions of the most severe damage and in scar–forming astrocytes [3]. Analysis Ngb immunoreactivity in post-mortem human brain tissue from subjects who died of brain traumatism provided evidence that Ngb was less expressed in acute brain traumatic injury and significantly more in subacute and chronic brain trauma, and that Ngb expression in astrocytes can persist for 12 months after brain trauma [4]. The detection of Ngb in astrocytes after brain injury could be an endogenous protective mechanism that enhances neuronal survival [17]. However, in another study on mouse brain sections, the authors observed the expression of Ngb protein in neurons, but not in astrocytes, of brain structures affected by age-related neurodegenerative diseases [11]. It was shown that there is a decrease in Ngb expression in the rat cerebral neocortex, hippocampus, cerebellum, and caudate-putamen with age, which may be related to the increased susceptibility to neurodegenerative diseases with aging [11].

Another function of Ngb in the nervous system could be that of ROS scavenger [31, 36]. The antioxidant potential of recombinant human Ngb was demonstrated *in vitro* against several free radicals (superoxide anion, hydrogen peroxide, hydroxyl radical), which were scavenged with a capacity comparable to that of vitamin C for superoxide anion and hydrogen peroxide [31]. This characteristic may explain the neuroprotective role of Ngb and its functional benefit in neurological diseases associated with oxidative stress. During acute hypoxia, Ngb maintained Cyt c in a ferrous state (Fe^{2+}) by utilizing intracellular antioxidants [38]. Depletion of antioxidants leads to activation of nuclear factor erythroid 2-related factor 2 (Nrf2) and hypoxia-inducible factor 1α (Hif-1α) that mediate the neuronal survival response [38]. However, if hypoxia is prolonged, it results in oxidation and degradation of Ngb, decrease in Nrf2 and Hif-1α, and release of pro-apoptotic Fe^{3+} Cyt c into the cytosol that activates apoptosis [38].

Ngb also binds NO and defends against NO-induced neurotoxicity [15, 36]. NO is a signaling molecule involved in the regulation of many biological processes in the central and peripheral nervous system [39]. In physiological amounts, it is neuroprotective, but if secreted in excess, NO has a neurotoxic effect, forming noxious compounds, called reactive nitrogen species (RNS), which, like ROS, damage cells and promote neurodegenerative diseases [39]. In both neurons and astrocytes, NO decreases ATP production by inhibiting mitochondrial respiration [40]. It causes nitrosylation of mitochondrial cytochrome oxidase [41] and reversibly inhibits electron transport at reduced P_{O2}, in addition, hypothetically contributing to neuroprotection [23]. Cultured mouse astrocytes exposed to NO caused rapid and reversible depletion of intracellular glucose, due to stimulation of glucose utilization, and led to lactate accumulation, effects that were detected even at low concentrations of NO [41]. Nonetheless, under low oxygen conditions, Ngb can reduce nitrite to NO [15, 23, 42]. Tiso et al. reported that the redox-sensitive surface cysteines 55 and 46 of deoxygenated human Ngb regulate heme coordination and the rate of nitrite reduction to NO, and this rate is maximal in the five-coordination state of Ngb [23]. It is considered that under normal functioning (oxygenation) conditions, when the intracellular concentration of oxidized glutathione (GSSG) is low but the level of reduced glutathione (GSH) is high, the Ngb disulfide bond is not formed and nitrite reductase activity is low [23]. Conversely, under oxidative stress conditions, disulfide bond formation is promoted, Ngb reductase activity increases, leading to the formation of NO and inhibition of mitochondrial ROS production [23].

The neurogenesis-promoting effect of Ngb was evidenced both in neural progenitor cells (NPC) and in the brain of mice after middle cerebral artery occlusion [33]. Overexpression of Ngb stimulated the proliferation of cultured NPCs and their differentiation, while in mice it caused an increase in the number of neuroblasts and newly differentiated immature neurons after transient focal ischemic stroke [33]. This action has been shown to be mediated through the Wnt signaling pathway [33]. In adulthood, neurogenesis can be activated in stroke [33]. Ngb was barely detected in human embryonic stem cells *in vitro*, whereas its expression in the rat subventricular zone was evident in neural stem cells and further differentiated neurons [34]. Ngb is expressed early during neurodevelopment, suggesting that it may regulate neural cell survival or death [34]. It has been reported that the density of Ngb-positive neurons in the cerebral cortex of mice tends to decrease after birth compared with the prenatal period, and Ngb levels were enhanced in response to mild hypoxia [29]. Ngb could regulate oxygen delivery during neocortical development in mice [29]. High expression of Ngb in the developing and neonatal brain is associated with increased lipogenesis and glycogenesis during these times of brain development [28].

Ngb activity may be related to neuronal energy metabolism as well, where inhibition of AMP-activated protein kinase (AMPK) and stimulation of neuronal anabolism have been reported [28]. Studies on transgenic mice overexpressing murine Ngb and on the murine hippocampal cell line (HT22 cells) showed that ATP, lipid and glycogen content were increased in HT22 cells compared to control cells, whereas in mouse cortical neurons overexpressing Ngb, the glycogen synthase was activated and the AMPK pathway was inhibited [28]. Overexpression of Ngb can improve mitochondrial activity, increase ATP and glycogen content, factors that may contribute to neuroprotection [28].

Endogenous CO, released by metabolism of heme, interacts with Ngb and may exert an anti-neuroinflammatory function [43]. The mouse microglial cell line BV-2, initially treated with lipopolysaccharide (LPS) to induce inflammation and subsequently exposed to CO, revealed reduced inflammation by decreasing inducible NO synthase (iNOS) expression and

NO and tumor necrosis factor-α (TNF-α) production, as well as and increasing interleukin-10 (IL-10) secretion [43]. It was observed that the anti-inflammatory effect of CO was correlated in time with CO-induced upregulation of Ngb, and knockdown of Ngb reversed the anti-inflammatory action of CO and decreased oxygen consumption of microglial cells, suggesting that the anti-inflammatory outcome of CO and its modulation of microglial metabolism are Ngb-dependent [43].

This succinct overview of the potential functions of Ngb shows that it is a protein with multiple roles in the activity of the nervous system, both in physiological and pathological conditions, among which that of neuprotection is essential.

CONCLUSIONS

Ngb is a heme protein that has been identified in several cell types and in a wide range of nervous structures. Ngb expression may be variable depending on the stage of brain development. In humans, it may fulfill several functional roles, in addition to increasing oxygen availability to neurons. Promoting nerve cell defense against hypoxia and oxidative stress by removal of ROS and RNS, protecting against NO-induced neurotoxicity, and regulating of neuronal death or survival might be some of them. As Ngb is a protein discovered not long ago, more research is required to better understand its specific actions in the nervous system and to elucidate the mechanisms that stimulate its expression in ischemic neurological diseases associated with glucose and oxygen deprivation.

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NEUROGLOBINA, O PROTEINĂ HEM CU FUNCȚIE NEUROPROTECTOARE

REZUMAT

Neuroglobina este o proteină hem descoperită relativ recent, ale cărei roluri, deși incomplet definite, pot fi legate de livrarea de oxigen celulelor nervoase, neuroprotecția împotriva hipoxiei/ischemiei, apărarea antioxidantă și stimularea supraviețuirii neuronale. Scopul acestei scurte recenzii este de a prezenta rolurile funcționale ale neuroglobinei în lumina datelor publicate, arătând relevanța acesteia pentru fiziopatologia unor tulburări neurologice și mecanismele prin care ar putea asigura neuroprotecție.
Cuvinte cheie: neuroglobina, stres oxidativ, neuroprotecție

Impact of Nonalcoholic Fatty Liver Disease and Type 1 Diabetes on the Right Side of the Heart in Young Patients

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Abstract

Nonalcoholic fatty liver disease (NAFLD) is a leading cause of chronic liver disease globally and encompasses a spectrum of liver disorders, ranging from simple steatosis to nonalcoholic steatohepatitis (NASH), which can progress to severe conditions like liver fibrosis, cirrhosis, and hepatocellular carcinoma. Studies in the literature have shown that in addition to an increased prevalence of left ventricular remodeling and left ventricular diastolic dysfunction in patients with NAFLD, right ventricular and atrial dysfunction may also be associated. This study aims to assess right heart side function by transthoracic echocardiography in young patients diagnosed with nonalcoholic fatty liver disease. This study is a prospective, descriptive, and comparative analysis of right ventricular and atrial function in young adults with nonalcoholic fatty liver disease, utilizing conventional and modern imaging methods, including speckle-tracking echocardiography. The study group included 79 patients aged 15-45 years diagnosed with nonalcoholic fatty liver disease. This group of patients was divided into two blots, one which included 35 participants with NAFLD alone and a second blot which included 44 patients who, in addition to liver disease, also had type 1 diabetes mellitus. The control group included a total of 80 healthy subjects in the same age group as patients diagnosed with NAFLD. The study results showed a significantly higher BMI in the NAFLD (30±3 kg/m²) and NAFLD+T1D (27±6 kg/m²) groups compared to the control group (23±3 kg/m², p<0.0001). While TAPSE, S', and RV GLS showed no significant differences between groups, FAC was reduced in NAFLD (36±7% vs. 45±7%, p<0.0001), and RAEF was significantly lower in NAFLD (70±27%) compared to control (115±53%, p=0.0007) and NAFLD+T1D (107±46%, p=0.01), highlighting impaired atrial and right ventricular function. In conclusion, GLS, TAPSE, and S' did not show statistically significant differences between groups, suggesting preserved systolic function across cohorts. However, FAC was significantly lower in the NAFLD group compared to both the control and NAFLD+T1D groups, indicating impaired right ventricular function in NAFLD regardless of coexisting diabetes. Additionally, the reservoir function of the right atrium was reduced in patients with NAFLD, highlighting atrial dysfunction in this population.

Introduction

Nonalcoholic fatty liver disease (NAFLD) is a leading cause of chronic liver disease globally, with an estimated incidence of 47 cases per 1,000 people and a prevalence of 32%, higher in males (40%) compared to females (26%) [1]. NAFLD encompasses a spectrum of liver disorders, ranging from simple steatosis to nonalcoholic steatohepatitis (NASH), which can progress to severe conditions like liver fibrosis, cirrhosis, and hepatocellular carcinoma if untreated [2]. Its development involves complex interactions between genetic predispositions, environmental influences such as diet and exercise, and metabolic factors like obesity, insulin resistance, and lipotoxicity [3].

NAFLD is closely associated with components of metabolic syndrome, including obesity, insulin resistance, dyslipidemia, and hypertension [4]. Currently, there is a growing body of evidence suggesting that NAFLD is not just a simple marker of cardiovascular disease and is even involved in its pathogenesis [5,6]. When NAFLD progresses, many important changes occur in the liver, leading to increased insulin resistance, the production of atherogenic lipids, and the release of proinflammatory, prooxidant, prothrombotic, and vasoactive substances into the bloodstream. All these changes may negatively influence the risk of cardiovascular disease, contributing to the development of cardiac complications in patients with non-alcoholic fatty liver disease [7]. NAFLD is an independent risk factor for cardiovascular disease and is associated with endothelial dysfunction [8], a higher prevalence of vulnerable coronary plaques [9,10], and a higher level of indicators of

subclinical atherosclerotic disease, i.e., increased carotid intima-media thickness [11]. Patients with NAFLD are more likely to die from cardiovascular complications rather than liver disease [12].

Although nonalcoholic liver disease is associated with impaired left ventricular function [13-17], the impact on right ventricular function remains unclear. A study involving 32 adults with NAFLD demonstrated that the presence of NAFLD was associated with impaired right ventricular diastolic function but without impaired systolic function [18]. Furthermore, NASH score appears to be an independent predictor of right ventricular function [19].

The right ventricle (RV) has a complex geometric shape and is wrapped around the left ventricle, which allows it to shorten in systole but also to benefit from interventricular interdependence during left ventricular contraction [20]. The myocardial fibers of the RV are organized in two main orientations: they are arranged circumferentially in the superficial layers and longitudinally in the deeper layers. This arrangement leads to a contraction that progresses from the inlet to the outlet and from the free wall to the septum. Unlike the left ventricle, RV contraction primarily depends on longitudinal shortening rather than twisting and rotational movements [21].

Echocardiography offers both qualitative and quantitative RV function assessment. Standard methods include 2D imaging, M-mode, Doppler, Tissue Doppler Imaging (TDI), and color flow mapping. Advanced techniques like strain and 3D echocardiography provide robust evidence for diagnosis and prognosis [22]. A comprehensive approach is often needed as no single parameter suffices, supported by established protocols and datasets

Studies in the literature have shown that in addition to an increased prevalence of left ventricular remodeling and left ventricular diastolic dysfunction in patients with NAFLD; right ventricular dysfunction may also be associated.

This study aims to assess right ventricular function by transthoracic echocardiography in young patients diagnosed with nonalcoholic fatty liver disease.

Materials and methods

This study is a prospective, descriptive, and comparative analysis of right ventricular function in young adults with nonalcoholic fatty liver disease, utilizing conventional and modern imaging methods, including speckle-tracking echocardiography. The study was conducted at the Craiova County Emergency Hospital Cardiology Clinic between November 2016 and September 2020. The study adhered to the ethical and deontological principles outlined in the Declaration of Helsinki. All participants were fully informed about the study's purpose and procedures and provided their consent for voluntary participation.

Study population

Patients with nonalcoholic fatty liver disease

The study group included 79 patients aged 15-45 years diagnosed with nonalcoholic fatty liver disease. This group of patients was divided into two blots, one which included 35 participants with NAFLD alone and a second blot which included 44 patients who, in addition to liver disease, also had type 1 diabetes mellitus.

Patients were included in the study according to the following criteria: patients diagnosed with non-alcoholic fatty liver disease using ultrasound criteria, patients aged 15-45 years, patients with an echocardiographic window favorable for further data processing, and patients who signed informed consent at inclusion. Among the exclusion criteria were patients diagnosed with viral hepatitis, patients with alcohol-induced liver disease, patients with autoimmune liver disease, patients with congenital liver diseases (hemochromatosis, Wilson's disease, alpha-1-antitrypsin deficiency, polycystic ovary syndrome), patients with

drug-induced liver disease, patients diagnosed with hypertension, patients with sinus rhythm changes other than premature beats, patients with ischemic or valvular heart disease

Control Group

The control group included a total of 80 healthy subjects in the same age group as patients diagnosed with NAFLD. Normal subjects were recruited from students, hospital employees, or their relatives.

Methodology of transthoracic echocardiography

To perform the imaging study, all subjects were echocardiographically evaluated in the echocardiography laboratory of the Cardiology Center of the Craiova County Emergency Hospital, with a single echocardiographic machine (Vivid S6, GE Vingmed Ultrasound, Horten, Norway).

According to the laboratory's internal protocol, all echocardiographic examinations were stored, measuring three cardiac cycles, in DICOM (Digital Imaging and Communications in Medicine) format and postprocessed offline using EchoPAC version 8.0 (GE Healthcare).

The echocardiographic image was acquired at the end of three cardiac cycles using the M5S two-dimensional transducer (GE Healthcare, Horten, Norway), with a frequency between 1.5 and 4.5 MHz.

Echocardiographic evaluation of the RV

- Right ventricular area in telesystole and telediastolic RV was measured from apical 4-chamber incidence by manually tracing the endocardium and then indexed to body surface area (cm2/m2).
- The fractional area change (FAC) of the RV was calculated according to the formula:

FAC (%)= 100 x (AVDd-AVDs)/AVDd

- TAPSE- represents the systolic excursion of the tricuspid annulus plane systolic excursion toward the apex during systole (mm). This parameter was obtained using M-mode.
- Systolic longitudinal contraction velocity St was obtained using pulsed tissue Doppler imaging by placing the sample at the lateral tricuspid annulus from the apical 4-chamber incidence. We highlighted the positive, systolic St wave, which reflects the velocity of displacement of the annulus toward the apex during systole, measuring RV longitudinal function.

Echocardiographic evaluation of the right atrium

- Telediastolic and telesystolic volumes of the right atrium were assessed in two-dimensional mode, using the apical 4-chamber apical section, by the air-length method, manually tracing the right atrial contour and measuring the right atrial length. The above volumes were then used to determine the right atrial ejection fraction, an indicator of reservoir function.

Echocardiographic speckle tracking assessment

Dedicated software was required to analyze the speckle tracking of patients in the study groups. Thus, after image acquisition offline, myocardial deformation parameters were assessed using the EchoPac program, version BT12, GE-Vingmed, Horten, Norway.

Given the peculiarities of the RV, determined by its thinner wall and different shape, the speckle tracking analysis of the RV involved the evaluation of its most important component, namely the global longitudinal deformation and, in particular,

that of the free wall.

The overall longitudinal strain of the RV was obtained using apical section 4C by manually tracing the endocardial contour of the free wall and interventricular septum (6-segment model) followed by post-processing.

The RV-free wall longitudinal strain was obtained using the same apical 4C section by manually tracing the endocardial contour of the RV-free wall (3-segment model), followed by data postprocessing.

Statistical Analysis

All numerical data were presented as mean and standard deviation. Data were analyzed using Graph Pad software (version 9, La Jolla, CA, USA). For statistical analysis, we used the Student t-test to compare the means of two data groups. In all cases, the value of *p* was calculated, and it was considered that there was a statistically significant difference between the means of the compared groups if it was less than 0.05.

Results

Descriptive analysis of patients studied

This study included 79 patients diagnosed with nonalcoholic fatty liver disease, of which 35 patients had liver involvement, and 44 patients had type 1 diabetes mellitus in addition to liver involvement. The control group included 80 subjects.

Subjects with NAFLD had a significantly higher mean age (38±5) compared to the control group (29±5) ($p<0.001$), but in NAFLD + T1D (31±8), no significant differences were observed between the two groups ($p=0.1$).

Body mass index (BMI) was significantly higher in people with NAFLD (30±3) and NAFLD + T1D (27±6) compared to the control groups; in both cases, the differences were highly statistically significant ($p<0.0001$).

In the NAFLD group, the mean systolic blood pressure value was significantly higher (126±8 mmHg) compared to the control group (117±7 mmHg), with a highly statistically significant difference ($p<0.0001$). Also, in the NAFLD + T1D group, the mean systolic blood pressure value was similar (126±14 mmHg) and significantly higher than in the control group (117±7 mmHg) ($p<0.0001$).

In the NAFLD group, diastolic blood pressure was higher (75±9 mmHg) compared to the control group (68±5 mmHg) ($p<0.0008$). In the NAFLD + T1D group, the mean diastolic blood pressure was even higher (80±7 mmHg) compared to the control group (68±5 mmHg); the difference is highly statistically significant ($p<0.0001$). These results are seen in Table 1.

Table 1. Characteristics of patients with NAFLD and NAFLD + T1D compared to control groups

Variable			*p*			*p*			*p*
	NALFD	**Control**		**NALFD+T1D**	**Control**		**NALFD**	**NALFD+T1D**	
Age	38±5	29±5	<0.001	31±8	29±5	0.1	38±5	31±8	0.1
BMI	30±3	23±3	<0.0001	27±6	23±3	<0.0001	30±3	27±6	<0.0001
BPs	126±8	117±7	<0.0001	126±14	117±7	<0.0001	126±8	126±14	>0,9

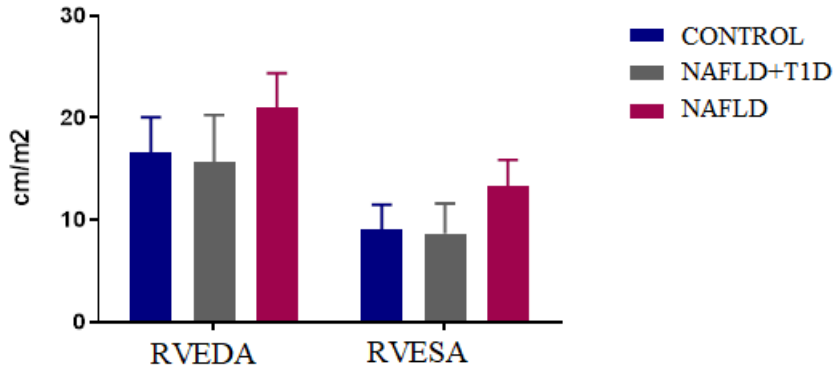
BPd	75±9	68±5	<0.0008	80±7	68±5	<0.0001	75±9	80±7	0.2

Height-cm, Weight-kg, BMI=body mass index-kg/m², BPs-systolic blood pressure, BPd - diastolic blood pressure

Echocardiographic evaluation of the RV

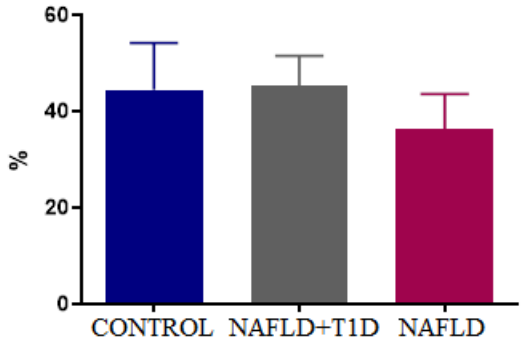
The calculation of RV area revealed that patients with NAFLD had the highest value of this parameter, which was statistically significant compared to the group of patients with type 1 diabetes mellitus and healthy subjects (Figure 1, Table 2, Table 3). Subjects who also had type 1 diabetes mellitus did not have a statistically significant value compared to the control group, but there was a significant difference compared to the group of patients who had only nonalcoholic fatty liver disease (Figure 1, Table 2).

Figure 1. *Distribution of subjects in the study groups according to RV area.*



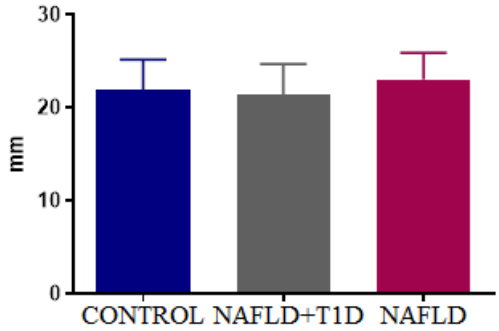
A comparison of the FAC RV showed that patients with NAFLD had statistically significant values compared to the control group and the group of patients with associated diabetes mellitus (Figure 2, Table 2). In contrast, there were no statistically significant differences when comparing the group with liver disease and associated diabetes to the control group (Figure 2, Table 2).

Figure 2. *Distribution of subjects in study groups according to the FAC RV*



There were no statistically significant differences between the three groups when comparing tricuspid annular plane systolic excursion (TAPSE) that expresses the right ventricular longitudinal contraction function (Figure 3, Table 2).

Figure 3. *Distribution of subjects in the study groups according to TAPSE*



By determining the S' wave in the free wall of the RV using tissue Doppler imaging, we observed no statistically significant difference in the patients in the study groups (Figure 4, Table 2).

Figure 4. *Distribution of subjects in the study groups according to the velocity of systolic longitudinal contraction S't.*

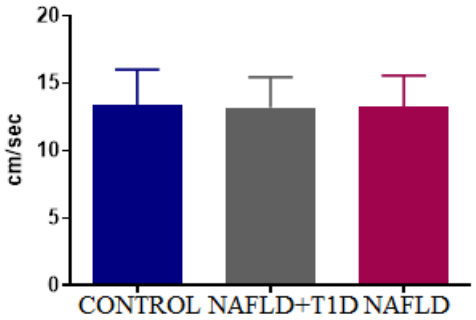


Table 2. Conventional echocardiographic parameters of the RV

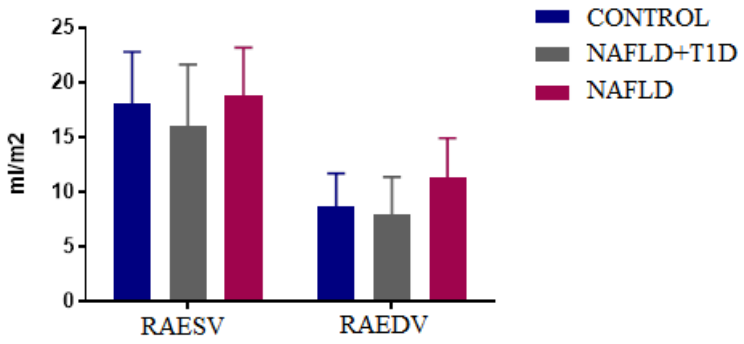
Variable				*p*			*p*			*p*
		NALFD	**Control**		**NALFD+T1D**	**Control**		**NALFD**	**NALFD+T1D**	
RVEDA		20±3	16±3	<0.0001	15±4	16±3	0.5	20±3	15±4	<0.0001
RVESA		13±2	9±2	<0.0001	8±2	9±2	0.9	13±2	8±2	<0.0001
FAC		36±7	45±7	<0.0001	45±6	45±7	0.9	36±7	45±6	<0.0001
TAPSE		23±2	21±3	0.3	21±3	21±3	0.7	23±2	21±3	0.1
S'		13±2	13±2	>0.9	13±2	13±2	>0.9	13±2	13±2	>0.9

RVEDA - RV end-diastolic area, RVEDA - RV end-systolic area, FAC – fractional area change, TAPSE - tricuspid annular plane systolic excursion, S' - systolic longitudinal contraction velocity

Echocardiographic evaluation of the right atrium

Indexed right atrial volume measurements did not show a statistically significant difference in patients with nonalcoholic fatty liver disease or associated type 1 diabetes mellitus (Figure 5, Table 3).

Figure 5. *Distribution of subjects in the study groups according to right atrium volumes.*



Assessment of right atrial reservoir function using right atrial ejection fraction showed statistically significant value among patients with NAFLD compared to the control group. In contrast, no statistically significant differences were observed between the patients with type 1 diabetes mellitus and the control group (Figure 6, Table 3).

Figure 6. *Distribution of subjects in the study groups according to right atrial ejection fraction.*

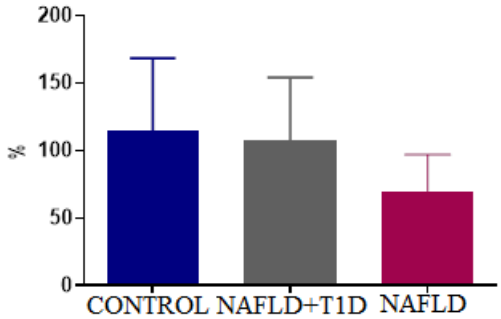


Table 3. Distribution of subjects in the study groups according to echocardiographic parameters of the right atrium

Variable	NALFD		*p*	NALFD+DZ		*p*			*p*
	NALFD	**Control**		**NALFD+T1D**	**Control**		**NALFD**	**NALFD+T1D**	
RAEDV	11±3	8±2	0.07	7±3	8±2	0.9	11±3	7±3	0.01
RAESV	18±4	18±4	0.06	15±5	18±4	0.05	18±4	15±5	0.06
RAEF	70±27	115±53	0.0007	107±46	115±53	0.6	70±27	107±46	0.01

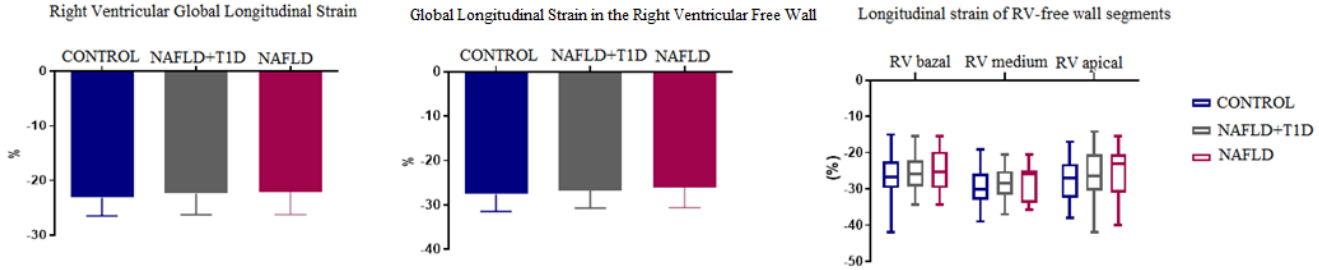
RAEDV - right atrial end-diastolic volume, RAESV- right atrial end-systolic volume, RAEF - right atrial ejection fraction

Right ventricular speckle tracking echocardiographic evaluation

This study focused on analyzing myocardial strain in the RV. It included an assessment of the overall longitudinal strain and the longitudinal strain specifically in the right ventricular free wall, examining its basal, middle, and apical segments.

The statistical analysis of the overall longitudinal strain of the RV indicated no statistically significant differences between the groups. Similarly, no significant differences were found in the analysis of the longitudinal strain of the free wall or the longitudinal strain of the RV free wall segments (Figure 7, Table 4).

Figure 7. *Distribution of subjects in the study groups according to the value of longitudinal strain of the RV (%)*



Analysis of mechanical dispersion in the RV showed no statistically significant differences in patients with NAFLD and patients with associated type 1 diabetes mellitus (Figure 8, Table 4).

Figure 8. *Distribution of subjects in the study groups according to the value of right ventricular mechanical dispersion (ms)*

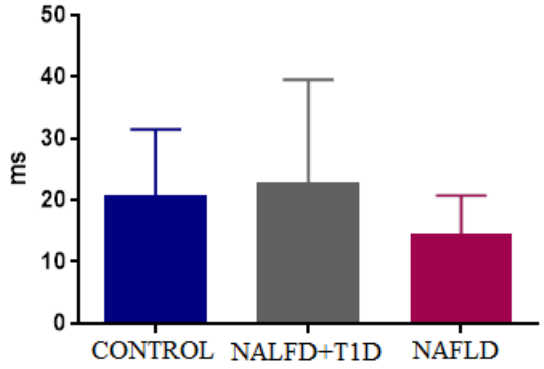


Table 4. Values of longitudinal strain and mechanical dispersion in the RV

Variable	NALFD		p	NALFD+T1D		p			p
	NALFD	Control		NALFD+T1D	Control		NALFD	NALFD+T1D	
GLS	-22±4	-23±3	0.5	-22±3	-23±3	0.5	-22±4	-22±3	0.9
LS wall	-26±4	-27±3	0.2	-26±3	-27±3	0.5	-26±4	-26±3	0.7
LS bazal	-25±5	-26±5	0.7	-25±4	-26±5	0.8	-25±5	-25±4	0.9
LS medium	-27±5	-29±4	0.3	-28±4	-29±4	0.7	-27±5	-28±4	0.8
LS apical	-25±6	-27±5	0.4	-26±5	-27±5	0.3	-25±6	-26±5	0.9
RVMD	14±6	20±10	0.1	22±16	20±10	0.5	14±6	22±16	0.04

GLS - global longitudinal strain, LS wall - free wall longitudinal strain, LS basal - basal segment longitudinal strain, LS medial - medial segment longitudinal strain, LS apical - apical segment longitudinal strain, RVMD - right ventricular mechanical dispersion

Discussion

Echocardiographic assessment of right ventricular function

Although fascinating because of the complexity and importance of its anatomy and functions, the RV was the "forgotten" or "misunderstood" ventricle for many centuries, not recognizing its pathophysiological significance in the human organism. Recently, Rudski and Afilalo pointed out that the RV is no longer considered the forgotten chamber but only the incompletely understood ventricle [23].

The more anterior RV position in the chest limits its visualization by echocardiography. Its complex geometric shape, in contrast to the ellipsoid shape of the left ventricle, makes it difficult to assess its function by conventional echocardiography. The RV has prominent trabeculations, making the clear delineation of the endocardium difficult. Finally, the muscle fibers of the RV are arranged mainly longitudinally so that most of the contraction occurs in this plane. Therefore, the pattern of right ventricular contraction is characterized by a free wall movement toward the septum, a longitudinal movement of the base toward the apex, and bulging of the ventricular septum into the right ventricular cavity [24-26].

The fractional area change is a marker of global systolic function, as echocardiographically visible regions represent 80% of the RV. It has been found to correlate best with right ventricular ejection fraction determined by magnetic resonance imaging, as well as with adverse events in patients with myocardial infarction and pulmonary hypertension [27, 28]. FAC is calculated as the difference between the end-diastolic and end-systolic areas, divided by the end-diastolic area, and expressed as a percentage. An abnormal FAC is a value less than 35% (29).

Tricuspid annular systolic plane excursion (TAPSE) is another echocardiographic parameter used to assess right ventricular systolic function and is the method most commonly used in practice due to its ease of assessment. TAPSE measures the distance the lateral tricuspid valve annulus moves during systole, assessed in millimeters using M-mode

echocardiography [21]. It does not consider the contribution of the ventricular septum and/or outflow tract to right ventricular performance [30,31].

S' is the peak systolic velocity of the lateral tricuspid annulus measured by pulsed-wave tissue Doppler imaging (PW-TDI) (21). Like TAPSE, it depends on the ultrasound beam's angle and the heart's loading conditions. S' reflects the longitudinal function of the right ventricular base and has a moderate correlation with MRI-derived right ventricular ejection fraction (RVEF) [32].

Strain is a parameter that quantifies myocardial deformation and is defined as the deformation of an object compared to its original shape or the change in length of a myocardial segment relative to its original length, expressed as a percentage [32]. **Right ventricular longitudinal strain** (RVLS) is assessed using 2D speckle tracking. It relies on high-quality imaging of the endocardial and myocardial borders, typically obtained in the RV-focused four-chamber view with a high frame rate [21]. Global RVLS can be calculated using three segments (RV free wall) or six segments (free wall and interventricular septum), which are more standardized than segmental values. RVLS is less dependent on angle, load, or translational motion and is more reproducible than TAPSE and S' [21]. It also effectively detects subclinical dysfunction and has established prognostic value [34]. However, RVLS does not account for the contribution of the right ventricular outflow tract (RVOT), and results may vary depending on vendor-specific software [21]. Some authors have shown that global longitudinal strain in the right ventricular free wall correlated better with ejection fraction measured by magnetic resonance imaging than with total global longitudinal strain [35].

Echocardiographic assessment of right atrial function

The atria serve as buffer chambers, receiving blood from the venous system in a regulated manner and delivering it efficiently to the ventricles. In the absence of an atrium, the corresponding ventricle faces a significant hemodynamic burden to maintain cardiac function [36]. The right atrium (RA) supports cardiac performance through three main roles: as a reservoir during ventricular systole, as a conduit during early diastole, and as a (booster) pump during late diastole [37]. Assessing RA size using volumetric measurements is considered more accurate than linear methods due to RA enlargement's irregular and asymmetric nature [37]. Two-dimensional echocardiography assesses the right atrial size and estimates volume by making geometric assumptions. This approach has limitations in assessing the size of non-spherical atria. In addition, errors due to foreshortening and off-axis image planes are inherent to 2DE. Despite these limitations, 2D parameters can be easily measured and are an important part of the echocardiographic assessment to provide a quick and simple impression of RA deviation [37].

Several studies have shown that non-alcoholic fatty liver disease is a risk factor for cardiovascular disease, especially coronary heart disease, independent of traditional risk factors such as age, gender, obesity, obesity, dyslipidemia, hypertension, and smoking [38, 39]. At the same time, cardiovascular disease is one of the most common causes of death in patients with nonalcoholic fatty liver disease [40]. Therefore, clinicians must be aware of the importance of cardiovascular risk stratification in this category of patients with liver disease. Corey and colleagues [41] have shown that, in addition to traditional risk factors, cardiovascular disease is independently associated with low serum albumin and serum sodium levels in patients with NAFLD with advanced fibrosis.

The literature has scarce data on right ventricular systolic function in patients with NAFLD. Given the anatomic position of the liver and RV, the impact of NAFLD on right ventricular function is of interest for clinical practice.

Our study showed that the right ventricular systolic function assessed by conventional echocardiography is not impaired in young adults with NAFLD nor those with associated type 1 diabetes mellitus, which agrees with previous reports [18, 42].

Hepatomegaly, a hallmark of advanced NAFLD, can mechanically influence cardiac structures due to its anatomical proximity to the heart. While studies emphasize left ventricular dysfunction in NAFLD (43), evidence points to the possibility of subclinical RV involvement, including impaired strain parameters and diastolic abnormalities [18]. These effects are often undetectable with conventional echocardiography, highlighting the need for advanced imaging techniques to evaluate RV performance comprehensively in this context.

When we talk about the right atrium, we can say that the heart cavity receives the least attention in echocardiography. However, the right atrium provides significant information, its dimensions increasing in certain pathologic conditions while also providing prognostic information. Our study found slightly larger right atrial volumes in patients with NAFLD but no statistically significant difference. The right atrial reservoir function was also reduced in patients with nonalcoholic fatty liver disease, the difference in this case being significant compared to the control group but also to patients with associated type 1 diabetes mellitus. We could not compare our data with the literature, as there are no studies evaluating right atrial function in patients with nonalcoholic fatty liver disease.

The majority of studies in the literature have been directed toward the assessment of left ventricular function and remodeling in patients with nonalcoholic fatty liver disease [13-17], while only a few studies have analyzed right ventricular structure and function in this category of patients. In the study of Sunbul et al. [19], who investigated 90 patients with NAFLD (mean age 45.2+9.8 years), it was shown that there were statistically significant differences in S', TAPSE, and GLS values compared to the control group (45 subjects with a mean age of 44.5+7.2 years). Even though these values were normal, they were lower than the control group. In our study, we found that these values were normal compared to the normal values reported in the guidelines [29, 35], but we did not obtain a statistical difference between groups. The differences between our study and Murat et al.'s could be due to the unique characteristics of the patients involved, the type of echocardiograph used, and the deferrals related to the sonographer, highlighting the crucial role of human expertise in medical research.

In a study by Styczynski and colleagues [44] involving a cohort of 171 subjects with morbid obesity and no cardiovascular pathology, patients were divided into three groups based on liver biopsy results: those with NASH isolated hepatic steatosis and no hepatic steatosis. The NASH group had concentric remodeling of the left ventricle and hyperdynamic circulation characterized by increased cardiac output. Regarding the right ventricle, TAPSE values showed no significant differences between the groups and remained within the normal range. These findings are consistent with the results of our study, which also showed no significant differences in TAPSE values among the groups analyzed (control, NAFLD+T1D, NAFLD), with all values within normal limits. Although not a significant difference, the mean TAPSE value was higher in patients with NAFLD; this could be explained by the increase in preload due to increased cardiac output and activation of the Frank-Starling mechanism. It should not be neglected that this group had the highest body mass index compared to the other two groups, and obesity is correlated with increased cardiac output [45].

Conclusions

The study provides valuable insights into the evaluation of right heart function in young patients diagnosed with NAFLD and type 1 diabetes, contributing to a relatively limited body of literature currently available on this topic. The study offers important insights into evaluating right heart function in young patients with NAFLD and type 1 diabetes, adding to the currently

limited literature on this subject.

GLS, TAPSE, and S' did not show statistically significant differences between groups, indicating preserved systolic function across the cohorts. However, FAC demonstrated significant differences, being lower in NAFLD compared to both the control group and the NAFLD+T1D group, suggesting impaired right ventricular function in NAFLD independent of coexisting diabetes GLS, TAPSE, and S' did not show statistically significant differences between the groups, indicating that systolic function was preserved across the different cohorts. However, FAC exhibited significant differences, being lower in the NAFLD group compared to both the control group and the NAFLD+T1D group. This suggests that right ventricular function is impaired in NAFLD, independent of any coexisting diabetes.

The reservoir function of the right atrium has been reduced in patients with nonalcoholic fatty liver disease, but new echocardiographic techniques may improve the evaluation of the right atrium in nonalcoholic fatty liver disease.

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IMPACTUL BOLII HEPATICE GRASE NONALCOOLICE ȘI AL DIABETULUI DE TIP 1 ASUPRA PĂRȚII DREPTE A INIMII LA PACIENȚII TINERI

Rezumat

Boala ficatului gras non-alcoolic (nonalcoholic fatty liver disease - NAFLD) este o cauză principală a bolilor cronice de ficat la nivel global și cuprinde un spectru de tulburări hepatice, variind de la steatoză simplă la steatohepatită non-alcoolică (NASH), care poate progresa către condiții severe precum fibroza hepatică, ciroza și carcinomul hepatocelular. Studiile din literatură au arătat că, pe lângă o prevalență crescută a remodelării ventriculare stângi și a disfuncției diastolice ventriculare stângi la pacienții cu NAFLD, disfuncția ventriculului drept și atrială poate fi, de asemenea, asociată. Acest studiu își propune să evalueze funcția părții drepte a inimii prin ecocardiografie transtoracică la pacienți tineri diagnosticați cu boala ficatului gras non-alcoolic. Acest studiu este o analiză prospectivă, descriptivă și comparativă a funcției ventriculului drept și atriului drept la tineri adulți cu NAFLD, utilizând metode imagistice convenționale și moderne, inclusiv ecocardiografia speckle-tracking. Grupul de studiu a inclus 79 de pacienți cu vârste cuprinse între 15 și 45 de ani diagnosticați cu NAFLD. Această grupă de pacienți a fost împărțită în două loturi, unul care includea 35 de participanți cu NAFLD singur și un al doilea lot care includea 44 de pacienți care, pe lângă boala hepatică, aveau și diabet zaharat de tip 1 (DZ tip 1). Grupul de control a inclus un total de 80 de subiecți sănătoși din aceeași grupă de vârstă ca pacienții diagnosticați cu boala ficatului gras non-alcoolic. Rezultatele studiului au arătat un IMC semnificativ mai mare în grupurile NAFLD (30±3 kg/m²) și NAFLD + DZ tip 1 (27±6 kg/m²) comparativ cu grupul de control (23±3 kg/m², p<0.0001). În timp ce TAPSE, S' și RV GLS nu au arătat diferențe semnificative între grupuri, FAC a fost redus în NAFLD (36±7% vs. 45±7%, p<0.0001), iar RAEF a fost semnificativ mai mic în NAFLD (70±27%) comparativ cu grupul de control (115±53%, p=0.0007) și NAFLD+T1D (107±46%, p=0.01), evidențiind o funcție afectată a atriilor și ventriculului drept. În concluzie, GLS, TAPSE și S' nu au arătat diferențe statistic semnificative între grupuri, sugerând o funcție sistolică păstrată în cadrul cohortelor. Cu toate acestea, FAC a fost semnificativ mai mic în grupul NAFLD comparativ cu atât grupul de control, cât și grupul NAFLD + DZ tip 1, indicând o funcție ventriculară dreaptă afectată în boala ficatului gras non-alcoolic, indiferent de diabetul coexistent. În plus, funcția de rezervor a atriului drept a fost redusă la pacienții cu boala ficatului gras non-alcoolic, evidențiind disfuncția atrială în această populație.

PERCEIVING BIOTERRORISM: THE INTERSECTION OF MEDICAL INSIGHT AND PUBLIC SECURITY CULTURE

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This paper is part of a broader research project developed within the author's master's thesis, conducted under the Master's program at the "Mihai Viteazul" National Intelligence Academy (ANIMV), Faculty of Intelligence, in the field of Information Management in Counter-Terrorism, under the supervision of Associate Professor Dr. Ioana Leucea.(leucea.ioana@animv.eu)

Abstract: The COVID-19 crisis has transformed the perception of biosecurity, highlighting the need for a holistic approach—one that encompasses strategic risk management practices to defend against biological threats. At the same time, it has reshaped understandings of biosafety, drawing attention to the healthcare system's capacity to respond effectively to crisis situations. Given the similarities between the outbreak of an infectious disease and a bioterrorist attack, preparedness for both types of biological risks requires the strengthening of healthcare systems, particularly through infrastructure capable of treating large numbers of patients simultaneously. Specifically, a bioterrorist attack demands the presence of medical personnel equipped with specialized knowledge about pathogenic agents and their effects on the human body. This paper aims to assess physicians' perceptions regarding their level of preparedness, the resilience of the healthcare system, and the need for reforms and additional professional training in the event of a biological risk incident. **Material and Method:** A structured questionnaire was designed to evaluate Romanian physicians' perceptions of bioterrorism, their self-reported knowledge in this domain, and their level of confidence in the national healthcare system. The survey was distributed to a sample of 350 physicians across Romania, with 203 valid responses collected, yielding a response rate of 58%. The questionnaire was developed using the Google Forms platform and disseminated via online channels. Data processing was performed using Microsoft Excel; the XLSTAT package was used to perform complex statistical tests (Chi square test, calculation of the rho Spearman correlation coefficient). **Results and Conclusions:** Approximately 50% of respondents acknowledged insufficient knowledge of the pathogens classified as bioterrorism threats, while over 58% reported lacking adequate information about the specific treatments for illnesses caused by such agents. Furthermore, 42% of physicians did not consider the development of biological agents to be scientifically difficult, suggesting an awareness of the technological advancements in the fields of synthetic biology and genetic engineering. One of the study's most relevant findings highlights a marked discrepancy between the theoretical knowledge and the practical readiness of physicians in relation to biological threats. While a considerable number of respondents report an awareness of the potential risks associated with bioterrorism, their actual preparedness levels remain low, and participation in specialized training programs is limited. This disconnect between knowledge and operational capability poses a significant concern, as timely and well-coordinated responses during a real biological incident are critical for avoiding systemic disruption and ensuring effective crisis management
Keywords: Biological risk, bioterrorism, biosecurity

Introduction

The health, social, and demographic dimensions are fundamental components of national security. During the COVID-19 pandemic, as biological risk became a tangible event, the healthcare system assumed an active role in national security, with its vulnerabilities impacting society as a whole.

The crisis reshaped perceptions of both biosecurity—emphasizing the need for a holistic risk management approach against biological threats—and biosafety, highlighting the importance of healthcare system resilience in crisis scenarios. Given the similarities between infectious outbreaks and bioterrorist attacks, preparedness for both requires a strengthened health infrastructure capable of managing mass casualties.

A bioterrorist attack, in particular, demands medical personnel with specialized knowledge of pathogenic agents and their effects. COVID-19 should be viewed as a catalyst for reform, and its analysis through the lens of national security must aim to influence policymakers toward the development of public strategies that enhance the healthcare system's crisis response—regardless of whether the biological threat is natural or man-made.

Amid speculations linking the pandemic to viral bioterrorism, the need to clearly distinguish between different types of biological risk has become increasingly evident.

While health and security are fundamental national priorities that may, under certain circumstances, warrant exceptions to the protection of human rights, pandemics often elude clear classification as either natural or technological threats. Rather, they represent transnational phenomena—comparable in scope and complexity to challenges such as climate change, artificial intelligence, or biological weapons. Accordingly, pandemics may be conceptualized in two primary ways: first, as a form of global catastrophic—or potentially existential—risk; and second, as an acute public health emergency at the national level, capable of justifying the implementation of heightened security measures **[1].**

The coronavirus crisis has reshaped the way transatlantic partners approach biosecurity at the intersection of health and security, prompting renewed discussions ranging from the role of institutions such as NATO in the context of bioterrorism threats, to increased investments in unconventional security aimed at planning for, mitigating, and countering the challenges posed by pandemics. **[2]**.

As a result, many nations incorporate medical intelligence within organizational frameworks, seeking to develop indicators to predict chronological patterns of infectious disease spread, estimate the impact on demographics, the supply and demand of pharmaceuticals in the affected country, the need for foreign assistance, vaccination requirements for international travel,

border control measures for disease surveillance, and assessments of the infected country's military readiness.**[3]**.

Bioterrorism is defined as the deliberate use of biological agents—such as bacteria, viruses, and toxins—against humans, animals, or crops, with the intent to intimidate civilian populations or authorities and to advance political, ideological, or religious agendas. Its primary effects include disease, death, and the contamination of water, food, and soil. During the Cold War, potential biological weapons were identified based on characteristics such as pathogenicity, lethality, aerosol stability, and ease of production and weaponization. In 2002, the U.S. Centers for Disease Control and Prevention (CDC) classified biological agents into three categories—A, B, and C—based on ease of dissemination, disease severity, and mortality. Category A includes high-priority agents like Bacillus anthracis, Yersinia pestis, and Ebola virus. Category B includes moderately easy-to-disseminate agents such as ricin and Brucella species, requiring enhanced diagnostics. Category C comprises emerging pathogens with limited population immunity, such as Hantavirus and Nipah virus. Toxins—produced by various organisms—are non-infectious biological molecules that can cause severe, often irreversible, physiological damage through inhalation, ingestion, or absorption, and are also considered bioterrorism agents due to their neurotoxic and cytotoxic effects. **[4, 5]**

Remarkable advances in cellular and molecular biology—while enabling innovations in the control and treatment of various diseases—also present a darker counterpart, shaping a new form of threat linked to the intentional use of these tools to design biological weapons and potentially trigger pandemics.

The scientific literature documents numerous ways in which biological weapons could be genetically engineered for enhanced potency: insertion of new genes, genetic recombination, alterations in gene expression, creation of novel synthetic pathogens with minimal genomes, and human-induced antigenic shifts in viruses, among others. The intended outcomes include increased pathogenicity aimed at targeting humans in general, specific susceptible ethnic populations, or agricultural systems—plants and animals alike—resulting in widespread social and economic disruption. **[6]**

The threat of a bioterrorist event requires healthcare professionals to be prepared to manage mass-casualty incidents caused by biological weapons. Rapid identification of the causative agent and appropriate patient management steps are critical to minimizing morbidity and mortality. Since biological agents—particularly infectious ones—can mimic naturally occurring diseases, healthcare workers play a key role in early detection. Effective response demands familiarity with both epidemiology and containment measures. Enhancing diagnostic and therapeutic capabilities, along with implementing well-structured response plans, is essential. Training healthcare personnel and other frontline responders is increasingly recognized as a necessary component of bioterrorism preparedness. **[7]**.

The detection of a biological or toxin-based attack is inherently challenging, as its clinical presentation often mirrors that of a typical epidemic. Regardless of whether the biological event is deliberate or naturally occurring, preparedness requires a unified approach—centered on strengthening the healthcare system. This includes ensuring infrastructure capable of managing large-scale patient care, securing access to protective equipment and medical supplies, and, when necessary, developing new therapeutic solutions. **[7, 8]**

Materials and Methods

Building on the arguments presented, a 10-item questionnaire was developed. The first two questions focused on respondents' professional level and medical specialty, in order to characterize the study sample. The remaining eight questions addressed key aspects related to the research topic and were structured using a 5-point Likert scale (1. To a very great extent / 2. To a great extent / 3. To a small extent / 4. To a very small extent / 5. Not at all), with an additional option (6. Don't know / Prefer not to answer).

The questionnaire included the following items:

1.Your professional level: Senior physician / Specialist / Resident

2.Your medical specialty: Medical / Surgical / Other / ICU-Emergency

3.Are you familiar with the pathogens currently identified as bioterrorism threats?

4.Do you consider a bioterrorism event likely to occur in Europe in the future?

5.Are you familiar with the biological effects of such agents and the treatment of related illnesses?

6.Do you consider the scientific development of new biological weapons to be difficult?

7.Do you believe there are similarities between natural pandemics and bioterrorism?

8.Based on your post-pandemic experience, do you consider state institutions to be resilient in the face of biological threats?

9.Do you consider postgraduate training courses on bioterrorism to be necessary and relevant?

10.Do you believe the Romanian healthcare system is prepared for a biological risk event?

The questionnaire was distributed to 350 physicians across Romania, of whom 203 responded, resulting in a 58% response rate. It was created using Google Forms and disseminated online.

The data were processed using Microsoft Excel (Microsoft Corp., Redmond, WA, USA) with the XLSTAT add-on (Addinsoft SARL, Paris, France). Responses were recorded in Excel files and analyzed statistically to identify correlations between respondents' demographic/professional profiles and their answers to the questionnaire.

Data processing involved descriptive analysis of the sample based on various parameters, along with graphical representation. This was performed using Microsoft Excel, utilizing tools such as Statistical Functions, Pivot Tables, Charts, and options from the Data Analysis menu. For more advanced statistical testing (Chi-square test, Fisher's exact test), commands from the XLSTAT module were employed.

All subjects in this study submitted their participation agreement.

Results and Suggestions

As previously mentioned, the first two questions were intended to characterize the study group.

The cohort consisted of 203 physicians, distributed by professional level as follows: 51.23% senior physicians, 25.62% specialists, and 23.15% residents.

According to their medical specialty, the cohort was composed of 58.62% physicians from medical specialties, 30.05% from surgical specialties, and 11.33% from other fields, including 1.97% in intensive care (ICU) and 1.48% in emergency medicine.

A majority representation from medical specialties may indicate a focus on the prevention and treatment of infectious diseases. These physicians should be aware of the risks associated with bioterrorism and engage in continuing education programs to be adequately prepared to respond effectively in the event of a biological attack.

The high percentage of senior physicians suggests that most respondents have extensive experience in the medical field, which may positively influence their ability to assess biological risks.

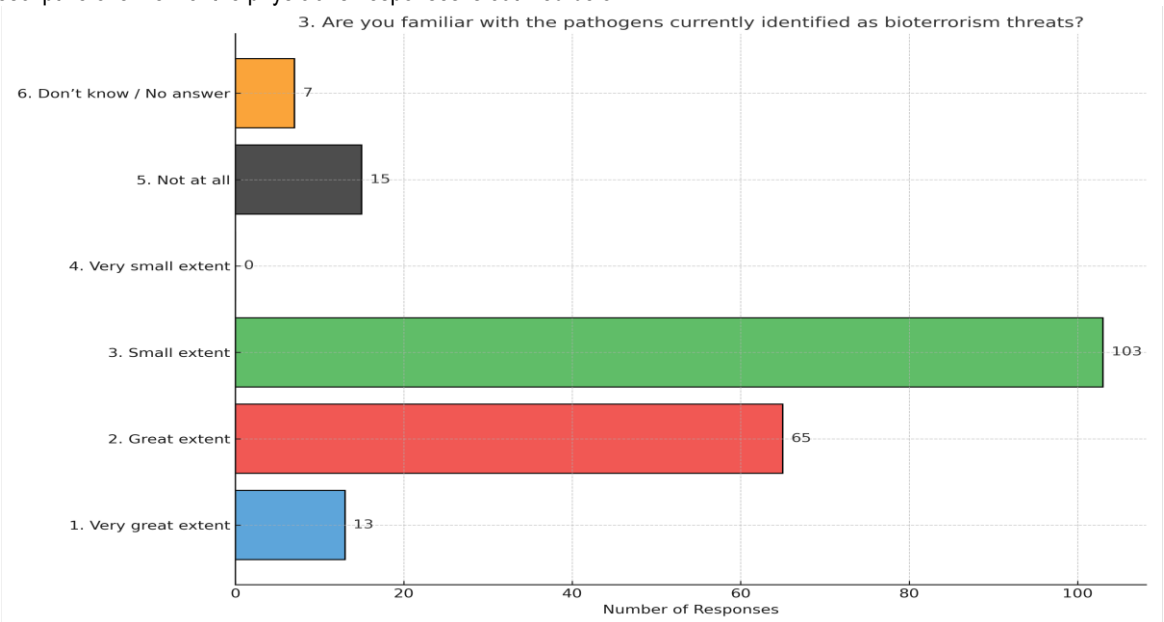
Given their direct and frequent contact with patients, senior physicians are often in a position to detect early signs of biological threats more readily than those in more technical specialties. This highlights the importance of ensuring that they are well trained in the recognition and management of biological threats.

1.

Table 1. The distribution of the answers to the questionnaire, recorded using a Likert scale

Q. no.	1. To a very great extent	2. To a great extent	3. To a small extent	4. To a very small extent	5. Not at all	6. Don't know / Prefer not to answer
Q3	6.40%	32.02%	50.74%	0.00%	7.39%	3.45%
Q4	4.93%	36.95%	46.80%	0.00%	2.96%	8.37%
Q5	2.96%	23.15%	58.13%	0.00%	12.32%	3.45%
Q6	3.45%	10.84%	42.36%	0.00%	39.41%	3.94%
Q7	7.39%	50.74%	25.62%	0.00%	8.87%	7.39%
Q8	0.99%	23.15%	50.25%	0.00%	20.20%	5.42%
Q9	26.60%	59.61%	12.32%	0.00%	0.49%	0.99%
Q10	0.00%	3.94%	38.92%	0.00%	56.16%	0.99%

A descriptive overview of the physicians' responses is outlined below.



2.

Figure 1. Responses to the question 3: "Are you familiar with the pathogens currently identified as bioterrorism threats?" based on a 6-point Likert scale.

It is noteworthy that approximately half of the respondents do not feel sufficiently informed about bioterrorism-related pathogens. This raises serious concerns regarding their preparedness to respond to real biological threats. Such responses may reflect a wide range of awareness levels with an apparent divide among physicians. Some possess relevant knowledge though many, as our data shows are underprepared. This gap could result in ineffective responses during a bioterrorist attack and makes a strong case for the urgent need to have targeted training initiatives. Furthermore, physicians lacking sufficient knowledge may inadvertently pose a public health risk, as they might be unable to properly identify or respond to bioterrorism threats. This could lead to delayed crisis management and generate compounding systemic vulnerabilities.

To combat these vulnerabilities training should aim to inform physicians about not only the specific pathogens that may be used in bioterrorism scenarios but also the symptomatology and appropriate treatment protocols. Indeed, response protocols are crucial for healthcare professionals in order to correctly respond to a bioterrorist incident starting from the initial phase where the incident must be reported to the relevant authorities all the way through to local on-site immediate measures that must be taken.

Once specialized training courses that may include both theoretical knowledge and also simulations are rolled out as a comprehensive preparedness program, physicians should participate in periodic evaluations to ensure their knowledge remains up to date and aligned with current information on biological threats.

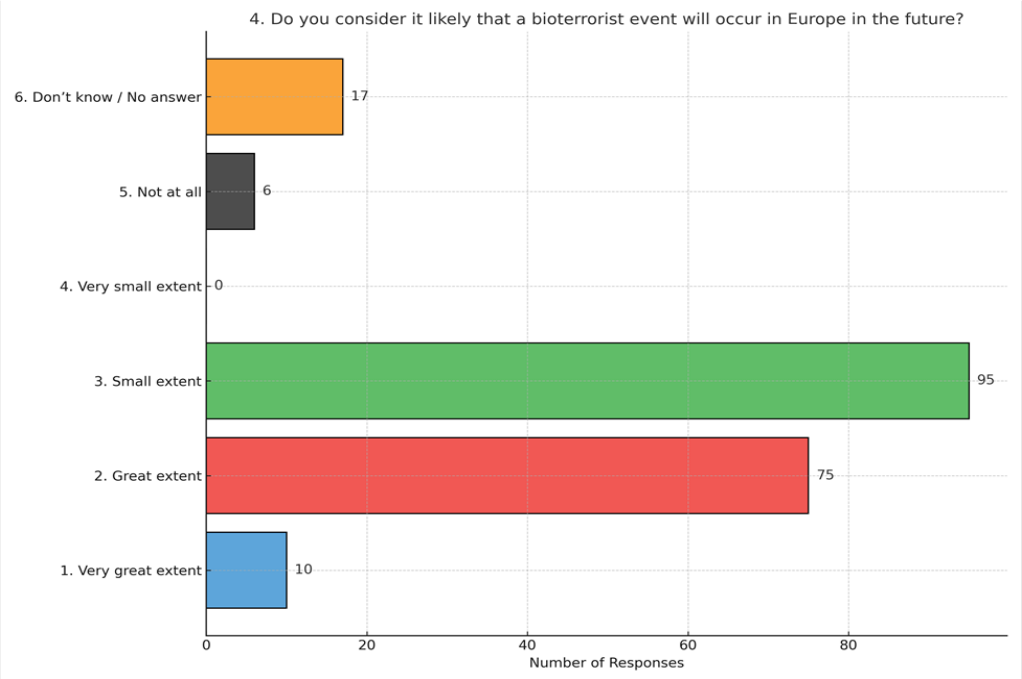
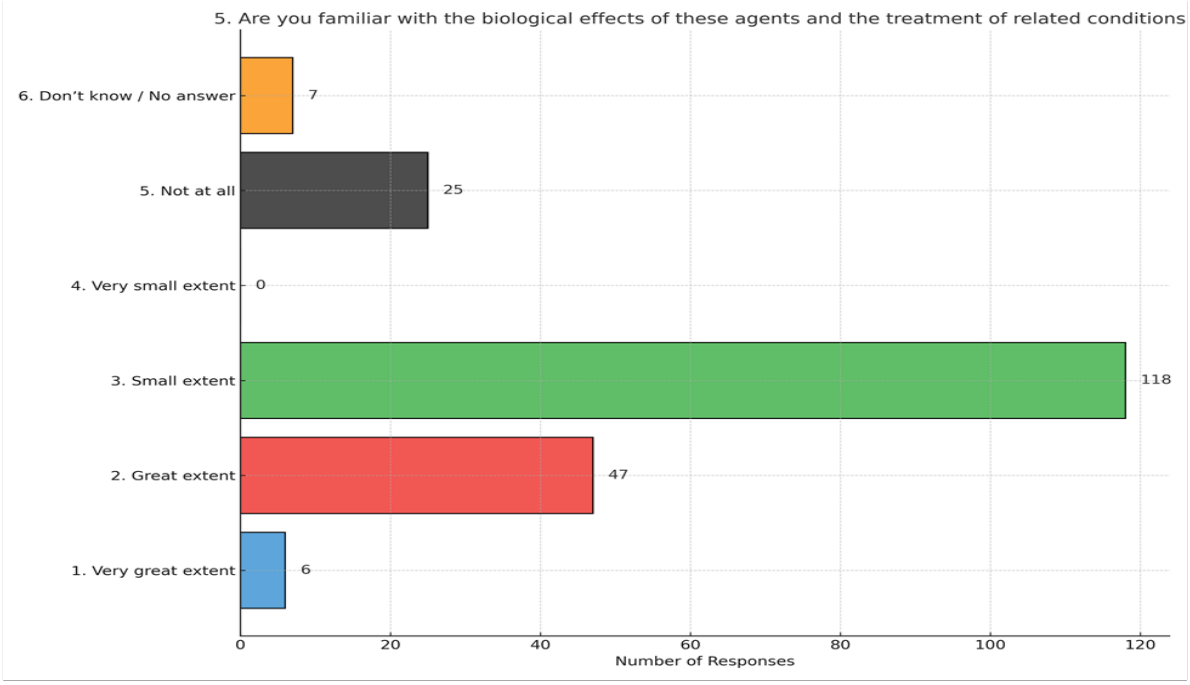


Figure 2. Perceived Likelihood of a Future Bioterrorist Event in Europe

The results suggests that a significant proportion of physicians (approximately 44% of the total) consider bioterrorism to be a real risk in Europe. These respondents may be aware of emerging threats and their potential impact on public health. However, the majority of physicians (around 56%) do not perceive the risks as immediate or significant. This may reflect either an underestimation of the threats or a lack of information regarding bioterrorism. The data reveals a clear divide among physicians, indicating inconsistency in the awareness of bioterrorism-related risks. Such a discrepancy could lead to an ineffective response from healthcare personnel in the event of a bioterrorist incident.



14.

Figure 3. Physicians' Knowledge of the Biological Effects and Treatment of Bioterrorism-Related Agents

We can observe that approximately 58% of respondents do not consider themselves sufficiently informed either about the clinical manifestations caused by agents classified as biological weapons or about the appropriate treatment once these agents are identified. The results point to a significant gap in medical education and training in this area. As we mentioned before the development and implementation of specific treatment protocols is recommended.

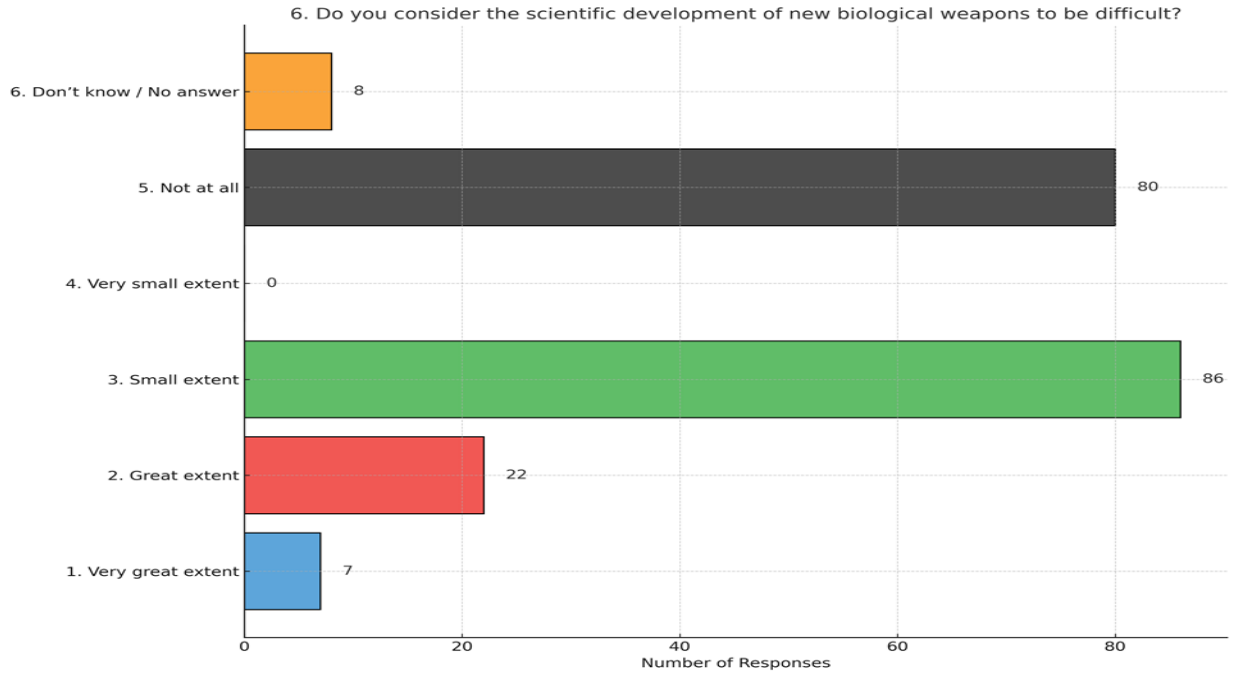


Figure 4. Perceived Scientific Difficulty in Developing New Biological Weapons

It is notable that approximately 42% of respondents consider the development of biological weapons to be only slightly difficult, while 39% do not consider it difficult at all.

Physicians, particularly those in medical specialties, may have a foundational understanding of the pathogens that could be used in bioterrorism. This includes knowledge of how such agents might be created or modified, as medical training curricula often include detailed information on microorganisms detailing such aspects such suitable conditions for multiplication, dissemination vectors as well as their biological effects on humans.

Physicians who understand the potential for the development of bioterrorism agents can take proactive steps in community education and in implementing safety protocols within hospitals and medical clinics as well as educating those in medical

training on the ethical concerns related to possessing specific knowledge on microorganisms.

Figure 5. Perceived Similarities Between Natural Pandemics and Bioterrorism

The large number of respondents who perceive strong similarities between natural pandemics and bioterrorist attacks reflects an awareness that both situations can have severe consequences for public health and may require similar intervention. In this case, regardless of the initial point of origin of the pathogenic microorganism or toxin, a rapid response would be essential as authorities would need to adjust quickly in order to make best use of that early temporal window in which the right actions may offer great benefits over time. To this effect risk communication would be something that needs to be mastered as communicating risks and offering accurate information to the public may prevent a widespread panic and ensure much needed cooperation.

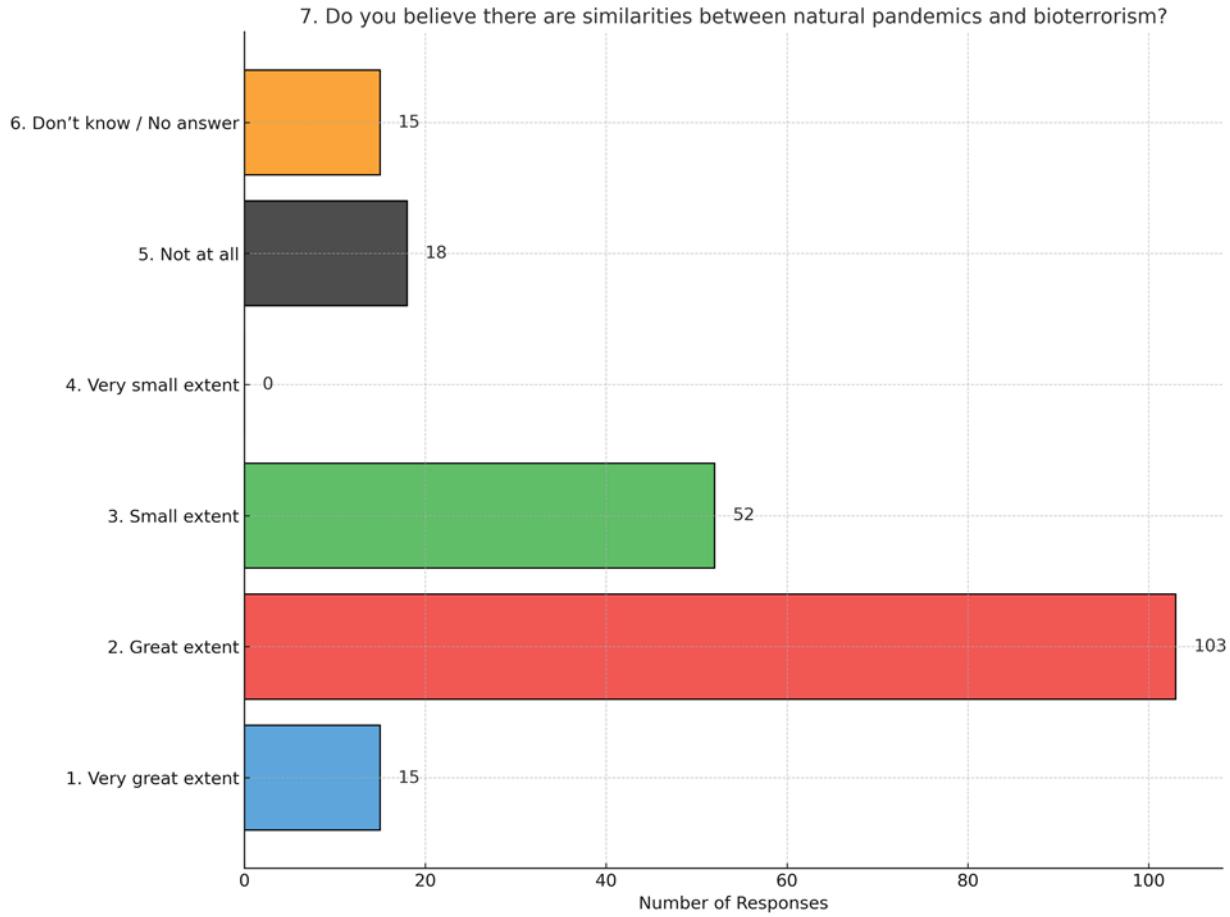
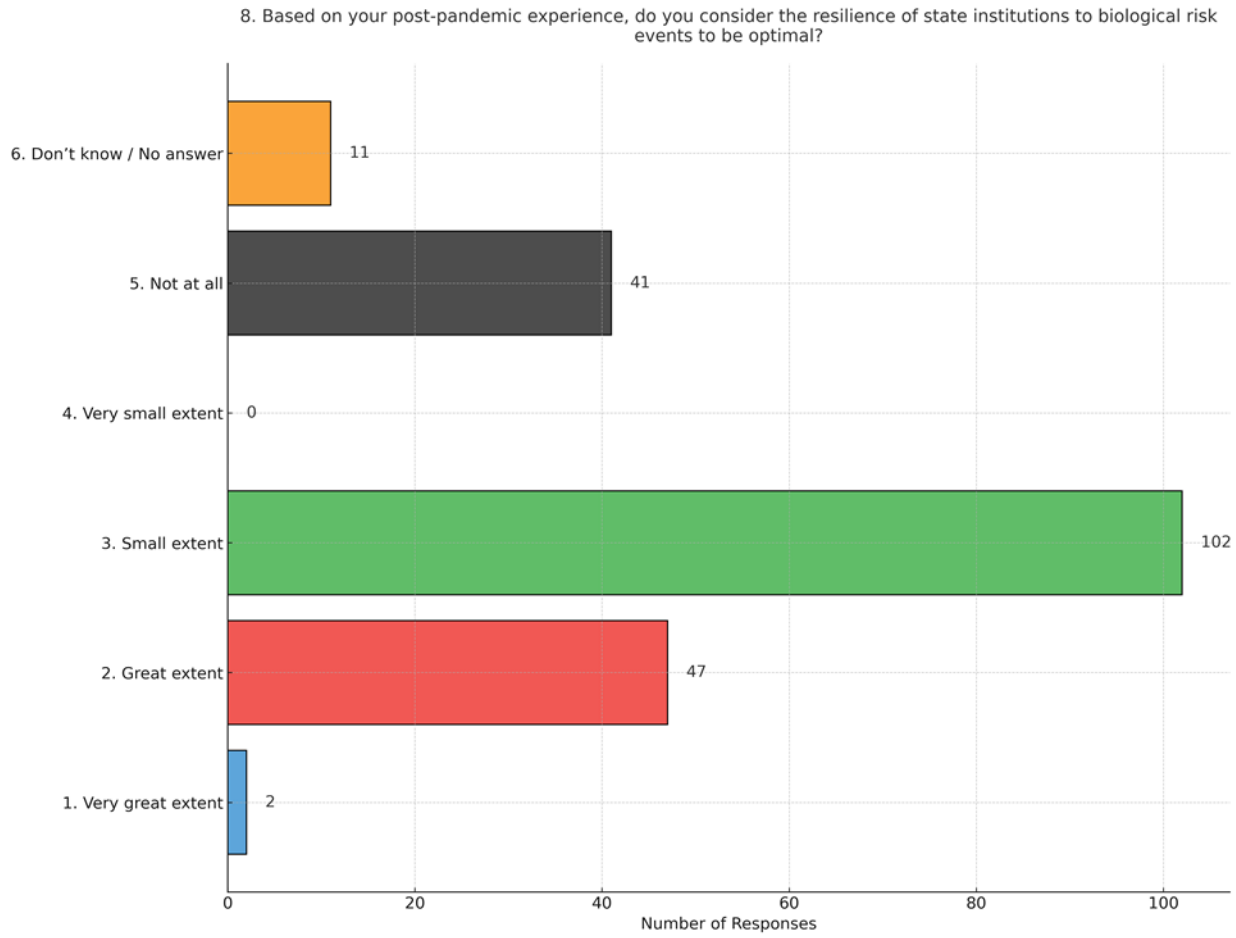


Figure 6. Assessed Resilience of State Institutions to Biological Risk

Over half of the respondents rated the resilience of state institutions to biological risk events as low, reflecting a limited level of confidence in the authorities' ability to effectively manage such crises.

This perception may be shaped by the public's previous experiences, particularly during the COVID-19 pandemic, when issues such as poor communication, logistical shortcomings, and perceived lack of coordination were frequently reported.

The results highlight not only institutional vulnerability, but also the urgent need to strengthen emergency response and communication mechanisms in the context of biological threats. A lack of public trust in authorities can further exacerbate risks during a crisis by undermining social cooperation and triggering chaotic or counterproductive behaviors.



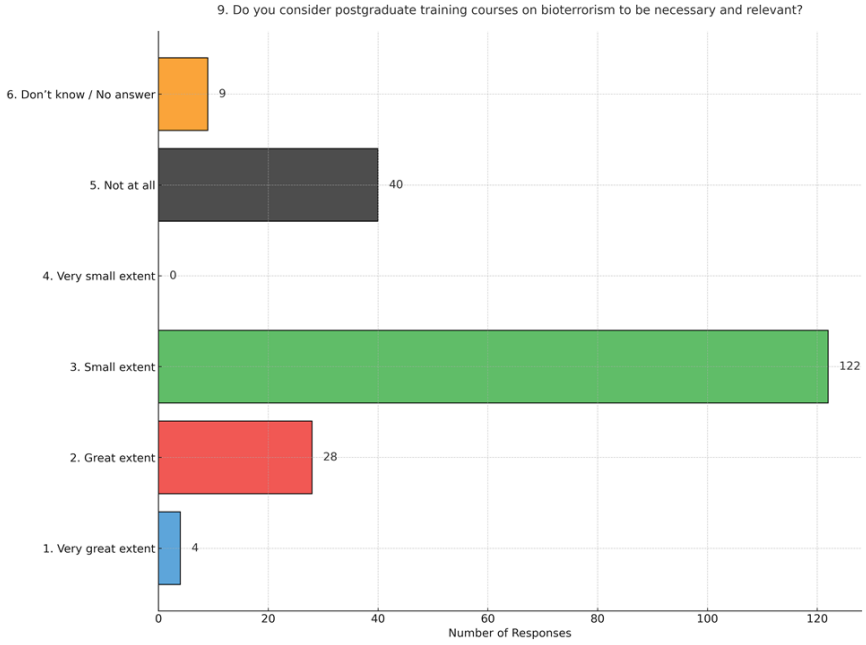


Figure 7. Reported Need for Postgraduate Training on Bioterrorism

The majority response reflects a strong desire for ongoing professional development and a willingness to take an active

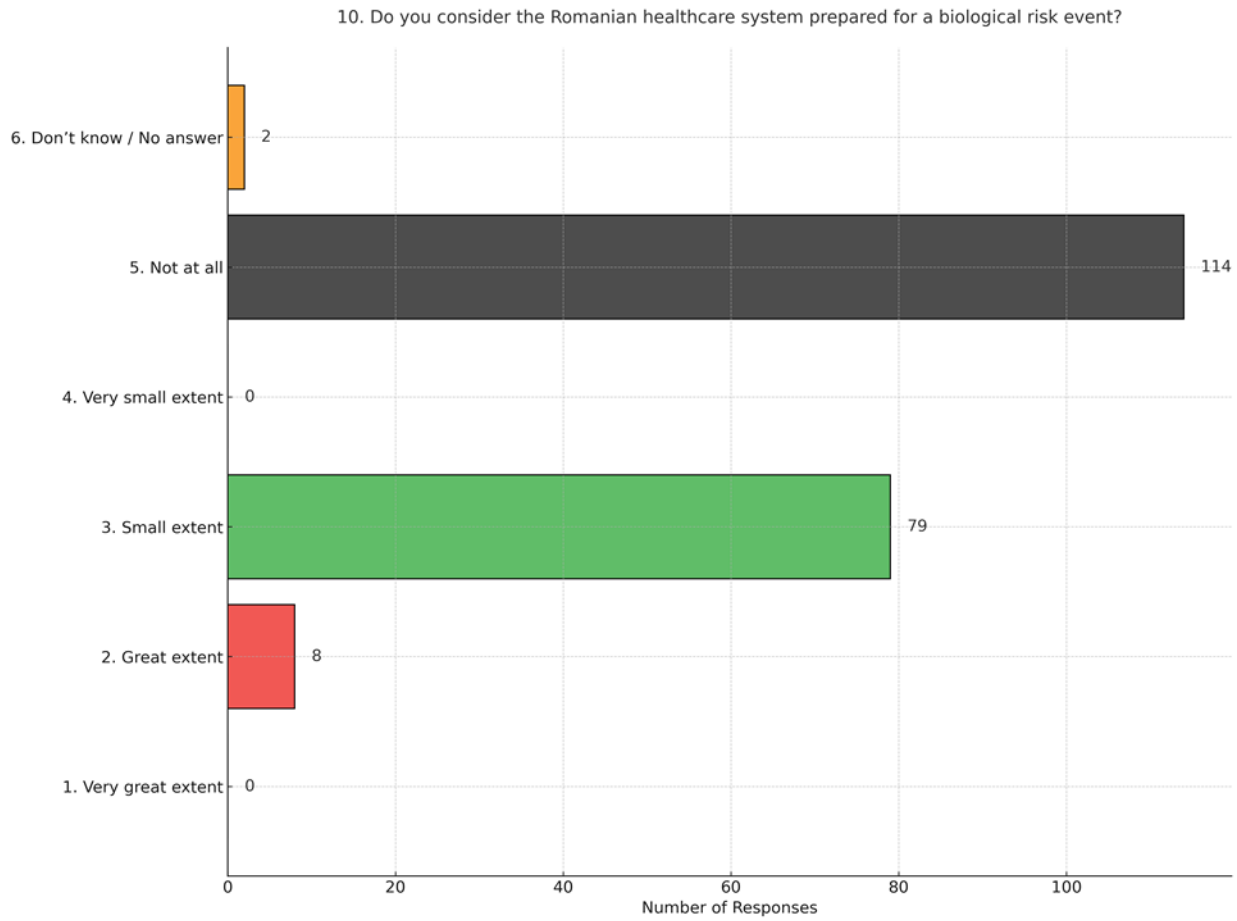
role in managing extreme biological risk situations. Physicians are therefore not only aware of the threat, but also receptive to educational initiatives that could enhance their capacity to respond in bioterrorism scenarios. The data presented here should serve as a signal to authorities and academic institutions, which could leverage this openness to develop specific, interprofessional postgraduate programs with immediate applicability in emergency healthcare and civil defense systems.

Figure 8. Assessment of the Romanian Healthcare System's Preparedness for Biological Risk

Approximately 95% of respondents consider the Romanian healthcare system to be either not at all or only minimally prepared for a biological risk event.

The results suggest that the healthcare system is perceived as not only lacking in logistical capacity but also as unprepared in terms of procedural and professional readiness when faced with unconventional biological threats.

This perception should be regarded as a strategic priority, as the trust of both the public and healthcare professionals is essential for the effective functioning of the system during a crisis. Strengthening response capacity, providing continuous training for medical personnel, and updating intervention plans are imperative steps forward.



Discussion and Conclusions

In the context of analyzing physicians' perceptions of biological risk events, it is highly relevant to foreground the complexity and evolving nature of modern biological threats. With the democratization of access to what used to be specific medical information, gated behind difficult accessibility in earlier decades and the emergence of tools based on artificial intelligence, the risk of artificial biological threats—difficult to detect and control using conventional methods—is increasing **[9,10,11].**

This technological perspective has direct implications for how physicians perceive and relate to biological risks. In the absence of clear tools for detection and attribution as well as a well put together systemic response by relevant authorities, uncertainty about the outcome of such an event regardless of origin of a pathogen - whether naturally occurring or laboratory-modified - can contribute not only to an increased perception of the healthcare system's vulnerability but also to real vulnerability. Moreover, physicians may feel additional pressure when confronted with events of uncertain etiology, which require not only a clinical response but also a technical understanding of the biotechnological potential involved. **[9,10,11].**

The capacity of medical systems to respond effectively to biological risk situations depends not only on institutional

protocols and logistical resources, but also on the perception and attitude of medical personnel directly involved as first responders to such a crisis.

In this regard, two key indicators should be noted: the operational approach within a military context, and the attitudinal perspective regarding the level of preparedness among healthcare professionals.

[12,13].

The Standard Operational Protocol - a 10-step sequential approach - is designed for situations involving biological uncertainty, where diagnosis is difficult and time is critical.

Although originally developed in a military context, this approach is highly relevant for civilian healthcare systems due to its applicability in scenarios involving biological attacks or emerging epidemics **[12,14,15].**

The 10 essential steps are:

1. Maintain a high level of clinical suspicion
2. Protect medical personnel
3. Stabilize the patient's vital functions
4. Perform decontamination when necessary
5. Diagnose through secondary evaluation
6. Administer prompt therapy
7. Infection control and patient isolation
8. Notify the appropriate authorities
9. Conduct epidemiological investigation and managing psychological effects
10. Ensure continuous preparedness **[12,14,15].**

Cieslak emphasizes that many high-risk biological diseases—such as anthrax, plague, botulism, and smallpox—can only be effectively treated if identified early. Therefore, protocols are effective but are insufficient without active clinical vigilance. **[12,14].**

A structured analysis of healthcare professionals' **Knowledge, Attitudes, and Practices (KAP)** regarding bioterrorism reveals significant disparities between what medical staff know and what they are actually prepared to do in practice. **[13,16,17].**

In this context knowledge refers to the theoretical knowledge about biological agents is often moderate and there are as we have seen notable gaps regarding reporting procedures, communication protocols and institutional roles.

Attitudes refers to the individual's response to such a context – and many medical professionals do feel a strong sense of moral responsibility but this is also coupled with a strong perception of high personal risk. Individual emotional responses can vary from mistrust and anxiety to calm acceptance.

Practices – in terms of participation to training courses attendance and participation is usually low. Only a small portion of healthcare professionals have developed robust personal and professional response plans. **[13,16,17].**

The situation as it stands reveals a form of structural tension: while clear protocols exist, there is no consistent institutional culture of preparedness. Many healthcare professionals feel that readiness for such risks is not prioritized by their institutions or by the state.

This disconnect significantly undermines response capacity. For instance, while structured models emphasize a rapid, pragmatic, and orderly response, the KAP model highlights indecision, lack of practical training, and a general mistrust in authorities.

It is therefore crucial that the concept of health security be understood not merely as a set of protocols, but as a professional and institutional philosophy.

The present study, based on a questionnaire administered to a sample of 203 Romanian physicians, clearly reveals a considerable gap between the awareness of biological risks and the actual preparedness to manage them.

Approximately 50% of respondents acknowledged insufficient knowledge of pathogens classified as bioterrorism threats, while over 58% reported lacking adequate information about the specific treatments for illnesses caused by such agents.

Furthermore, 42% of physicians did not consider the scientific development of biological agents to be particularly difficult, suggesting an awareness of technological advancements in synthetic biology and genetic engineering.

This perception should serve as a warning and also a call to action for authorities, as it points to the possibility that threats may also emerge from civilian or academic environments with access to these technologies and information base.

Another key question in the survey addressed the perceived similarities between pandemics and bioterrorism. The vast majority of respondents indicated that the two situations share numerous commonalities—both in terms of onset and spread, as well as their systemic impact. This observation supports the need for preparedness, response, and recovery systems to be aligned, regardless of the pathogen's origin.

At the same time, nearly 60% of participants believe that state institutions have not demonstrated optimal resilience in the face of biological threats. This figure reflects both direct experiences during the pandemic and a persistent lack of trust in public administration. Paradoxically, this distrust is accompanied by a genuine openness to professionalization: over 85% of physicians expressed their willingness to participate in postgraduate training courses focused on bioterrorism.

One of the most relevant findings of the study is the discrepancy between physicians' theoretical knowledge and their practical attitude toward biological risks. While many claim to understand the potential of bioterrorist threats, their actual level of preparedness is low, and participation in training programs is limited. This gap between knowledge and action is dangerous, as a real-life incident requires a prompt and well-coordinated response to avoid institutional chaos.

Healthcare professionals experience a range of emotions when facing such risks: anxiety, mistrust in authorities, and uncertainty about their role in the response chain are predominant. These findings suggest the absence of a clearly defined organizational culture around biosecurity preparedness.

The conclusions of this study highlight a complex and concerning reality. Although medical professionals are aware of biological risks, practical preparedness and response infrastructure remain inadequate. The crisis triggered by the COVID-19 pandemic has not been fully leveraged as an opportunity for reform, and confidence in public institutions continues to be undermined by perceived inefficiency and lack of coordination.

It is time for Romania to rethink its health security architecture through an integrated approach based on knowledge, training, and collaboration. Without a strong culture of biosecurity, future risks, whether natural or engineered may exceed the healthcare system's ability to respond. This study underscores the urgent need for coherence, professionalism, and investment in human capital - the only true safeguard against modern biological threats.

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PERCEPEREA BIOTERORISMULUI: INTERSECȚIA DINTRE CUNOȘTINȚELE MEDICALE ȘI CULTURA SECURITĂȚII PUBLICE

Rezumat: Criza COVID-19 a transformat percepția asupra biosecurității, evidențiind necesitatea unei abordări holistice, care să includă practici strategice de gestionare a riscurilor pentru a combate amenințările biologice. În același timp, a remodelat înțelegerea biosecurității, atrăgând atenția asupra capacității sistemului de sănătate de a răspunde eficient la situații de criză. Având în vedere similitudinile dintre izbucnirea unei boli infecțioase și un atac bioterorist, pregătirea pentru ambele tipuri de riscuri biologice necesită consolidarea sistemelor de sănătate, în special prin infrastructuri capabile să trateze simultan un număr mare de pacienți. Mai precis, un atac bioterorist necesită prezența personalului medical dotat cu cunoștințe specializate despre agenții patogeni și efectele acestora asupra organismului uman. Prezentul document are ca scop evaluarea percepțiilor medicilor cu privire la nivelul lor de pregătire, la reziliența sistemului de sănătate și la necesitatea reformelor și a formării profesionale suplimentare în cazul unui incident cu risc biologic. **Material și metodă:** A fost elaborat un chestionar structurat pentru a evalua percepțiile medicilor români cu privire la bioterorism, cunoștințele pe care aceștia le declară în acest domeniu și nivelul lor de încredere în sistemul național de sănătate. Chestionarul a fost distribuit unui eșantion de 350 de medici din toată România, fiind colectate 203 răspunsuri valide, ceea ce reprezintă un procent de răspuns de 58%. Chestionarul a fost elaborat utilizând platforma Google Forms și distribuit prin canale online. Prelucrarea datelor a fost realizată utilizând Microsoft Excel, iar pentru efectuarea testelor statistice complexe (testul Chi pătrat, calcularea coeficientului de corelație rho Spearman) a fost utilizat pachetul XLSTAT. **Rezultate și concluzii:** Aproximativ 50% dintre respondenți au recunoscut că nu au cunoștințe suficiente despre agenții patogeni clasificați ca amenințări bioteroriste, în timp ce peste 58% au declarat că nu dispun de informații adecvate despre tratamentele specifice pentru bolile cauzate de astfel de agenți. În plus, 42% dintre medici nu considerau că dezvoltarea agenților biologici este dificilă din punct de vedere științific, ceea ce sugerează o conștientizare a progreselor tehnologice în domeniile biologiei sintetice și ingineriei genetice. Una dintre cele mai relevante concluzii ale studiului evidențiază o discrepanță marcată între cunoștințele teoretice și pregătirea practică a medicilor în ceea ce privește amenințările biologice. Deși un număr considerabil de respondenți declară că sunt conștienți de riscurile potențiale asociate bioterorismului, nivelul lor real de pregătire rămâne scăzut, iar participarea la programe de formare specializată este limitată. Această discrepanță între cunoștințe și capacitatea operațională reprezintă o preocupare semnificativă, întrucât răspunsurile rapide și bine coordonate în timpul unui incident biologic real sunt esențiale pentru a evita perturbarea sistemică și pentru a asigura o gestionare eficientă a crizelor.

Cuvinte cheie: risc biologic, bioterorism, biosecuritate