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Editorial

This is the next book in the scientific book series entitled "Advances in biomedical research". This book series is the result of the meetings and work of scientists from various universities and institutes in Poland. In this monograph we present the topics in Biomedical Research from 2021. All presented articles have passed the peer-review process positively. The articles come from various fields of biomedicine from Cell-in-Cell phenomena throughout cancer research to skin disorders. Wishing you enjoyable and productive reading.

Editors,
Łukasz Biały MD, PhD
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Emperipolesis in neuroendocrine tumors of the thymus

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ABSTRACT

Emperipolesis is a biological phenomenon of rare origin and is characterized by a process in which a cell penetrates another living cell. In contrary to phagocytosis where the engulfed cell is killed or neutralized by lysosomal enzymes of the macrophage, in emperipolesis, the cell exists as a viable cell within another. Moreover, this cell can exit at any time without any structural or functional abnormalities for either of them. The process of emperipolesis is seen in many physiologic and pathophysiologic conditions. In this article we focus on the occurrence, pathogenesis and appearance of emperipolesis in the neuroendocrine tumors of the thymus. Moreover, we highlight the possible diagnostic and future therapeutic strategies in the treatment of thymic tumors.

INTRODUCTION

Neuroendocrine tumors of the thymus are classified according to World Health Organization (WHO) guidelines. Primary neuroendocrine tumors of the thymus (NETTs) are very uncommon and represent less than 5% of mediastinal and thymic neoplasms. They account for only 0,4% of all neuroendocrine tumors (Dinter, 2019). NETTs are classified according to WHO criteria into low-grade typical carcinoids, intermediate-grade atypical carcinoids (ACs), and two high-grade malignancies, large cell neuroendocrine carcinoma (LCNEC) and small cell carcinoma (SCC) (Dinter, 2019). To categorize tumors, morphology evaluation should be performed, with the assessment of parameters such as organoid nesting, rosette formation, peripheral palisading of tumor nests, and trabeculae (Dinter, 2019). This classification was made by determining the mitotic activity, cellular atypia and areas of necrosis (Moran, 2000). To classify a tumor as a typical carcinoid, it is identified to have no necrosis and a size of 0,5 cm or greater, AC is reported to have 2 to 10 mitoses per 2 mm with or without necrosis, whereas LCNEC and SCC have number of mitoses greater than 10 per 2 mm (Moran, 2000). According to this classification, AC and LCNEC are the most common subtypes in the thymus (Dinter, 2019).

The 3rd and 4th edition of the WHO Classification of thoracic tumors are considered to be most important. In accordance with the 2004 classification, WHO distinguishes A, AB, B1, B2 and B3 types of thymomas and thymic carcinomas and other seldom ones. (Marx, 2014; Petrini, 2014). The fourth edition is expanded to include an interdisciplinary perspective and improves histological and immunohistochemical diagnostic criteria in order to increase the diagnostic repeatability.

The nomenclature of the major thymoma types was retained in the 4th edition, as well as Masaoka-Koga system for the staging of thymomas (Marx, 2015). However, the term "combined NETTs" is no longer used, excluding type AB thymoma. Instead, there is a requirement to include all histologically diagnosed thymoma types, starting with the most important ones and quantified in 10% increments (Marx, 2015).

Primary neuroendocrine tumors of the thymus (NETTs), which include thymic neuroendocrine tumors, thymoma (TM) and thymic carcinoma (TC) are always considered to be malignant, and it is unrelated to subtype or histology of the tumor (Jeong, 2020; Marx, 2015).

In the past there was a problem with distinguishing between some thymoma subtypes and thymic carcinomas, because of morphological overlapping (Marx, 2014). Differences between thymomas and thymic carcinomas have been diagnosed by epigenetic and genetic methods and transcriptomic analyses, which showed different methylation patterns, expression profiles of antiapoptotic genes and specific mutations of epigenetic regulatory genes (Marx, 2015). Thus, interobserver reproducibility has been improved. Also point mutation in the GTF2I (general transcription factor 2-I) oncogene in all major thymoma subtypes and thymic carcinomas was observed, which indicates the common origin of the NETTs (Petrini, 2014).

The possible role of emperipolesis in neuroendocrine tumors of the thymus requires further clarification. In this review we discuss the previous findings in this area of expertise and the significance of this rare process, not much reported in the literature.

SEARCH STRATEGY AND SELECTION CRITERIA

The authors reviewed data published in 3 languages: English, German, and Polish between 1989 and 2020. Data were collected using keywords such as emperipolesis, entosis, and neuroendocrine tumors of the thymus. The following scientific databases such as PubMed, Google Scholar, Borgis, MEDLINE, and Cochrane Library were used to search for articles. The selected articles focused on determining the importance of emperipolesis in the pathogenesis, diagnosis and treatment of thymic neuroendocrine tumors. The number of articles selected was 54. In addition, this work was enriched with 7 manually selected materials that were related to the discussed topic. The strategy was aimed at presenting yet not entirely understood aspects of emperipolesis in the context of neuroendocrine tumors of the thymus as well as emperipolesis itself from various perspectives.

WHAT IS THE EMPERIPOLESIS?

Emperipolesis is characterized by the presence and movement of one cell within the cytoplasm of another. Emperipolesis is strictly related to cell-in-cell phenomenon, which can be associated with the prognosis of cancers (Wang, 2019). Histopathological screening shows an absorbed cell in a membrane-bound vacuole in the host cell. On occasion absorbed cells may continue to live for a short period of time after absorption. It is possible for an internalized cell to escape from the host cell, and it can survive after this process (Gupta, 2017).

The term "*emperipolesis*" originates from the Greek (*em*-inside; *peri*-around; *polemai*-wander about) and it was first reported and defined in 1950 as the active penetration of one cell by another (Humble, 1956). Wang and Li (2019) had discovered that emperipolesis can mediate natural killer cell-mediated tumor cell death, but requires membrane fluidity of the target cell, so that the interaction with natural killer cells could occur. The host tumor cell disintegration is preceded by lysosome-mediated

degradation pathway after the emperipolesis (Xia, 2008). According to Overholtzer's report, natural killer cells sometimes can undergo mitosis inside the host tumor cell after emperipolesis and that indicates the further fate of heterogeneous cells in killer cell-tumor cell emperipolesis (Overholtzer, 2007).

Rosai-Dorfman disease (RDD) is a pathological condition in which emperipolesis occurs. It was first observed by Juan Rosai and Ronald Dorfman in 1969, and has been diagnosed by cervical lymphadenopathy, lymph node sinuses and emperipolesis that occurred within histiocytes (Rosai, 1969). In RDD a dense histiocytic infiltrate with emperipolesis is present. The infiltrate contains associated lymphocytes, plasma cells, and neutrophils (Cangelosi, 2011). However, emperipolesis is a diagnostic feature only when S100 protein is expressed in histiocytes (Juskevicius, 2001). Yet, due to variable morphology characteristics in xanthogranulomatous diseases, emperipolesis is the most important histologic feature in distinguishing it from RDD disease (Cangelosi, 2011).

CHARACTERISTICS OF THYMIC NEUROENDOCRINE TUMORS

Primary neuroendocrine tumors of the thymus (NETTs) belong to the group of tumors with high aggressiveness (the ability to form metastases in more than 80% of patients) and a relatively low incidence (Chaer, 2002; Filosso, 2017). NETTs account for only about 0,4% of all carcinoids and less than 5% of all the anterior mediastinal neoplasms (Yao, 2007; Filosso, 2017). Primary neuroendocrine tumors of the thymus are found predominantly in males, with a male to female ratio of 3:1 (Moran, 2000). They are most common in white males and are typically seen in the fourth or fifth decades of life, with an average age of onset of 58 years (Gaur, 2010). NETTs likely arise from Kulchitsky cells and localize primarily to the anterior mediastinum (Berman, 2020).

According to the WHO (2015), primary thymus neuroendocrine tumors are classified into two main histopathological types: well-differentiated (typical and atypical carcinoids) and poorly differentiated (small cell and large-cell neuroendocrine crayfish) (Travi, 2015).

Clinically, NETTs may manifest as follows: 1. asymptomatic, coincidentally detected on chest radiography for other reasons; 2. with symptoms due to displacement/compression/invasion of mediastinal structures; 3. associated with endocrinopathies; or 4. with symptoms due to distant metastases, most commonly to the liver, brain, lung, or bone.

Primary neuroendocrine tumors of the thymus give many non-specific symptoms including chest pain, cough, dyspnea, superior vena cava syndrome, lingual nerve palsy, and diaphragmatic elevation due to damage to the phrenic nerve (Berman, 2020). In addition, half of the patients had lymph node involvement, but with no proven effect on reducing treatment efficacy (Filosso, 2017).

Approximately 50% of thymic neuroendocrine tumors are functionally active and have the ability to secrete hormones. Ectopic secretion of ACTH and serotonin can lead to paraneoplastic Cushing's syndrome and carcinoma, respectively. Less commonly, excessive secretion of somatoliberin (GHRH, growth hormone-releasing hormone) or growth hormone (GH) has been described, which can cause acromegaly (Melmed,

2009). People with hypertension, heart failure, diabetes, and arthropathies are primarily at risk of developing this condition (Bolanowski, 2014).

In addition, 25% of patients struggling with a thymic tumor have a coexistence of multiple endocrine neoplasia type 1 (MEN1) syndrome; one of the 8 contemporary multiple endocrine neoplasia syndromes (Gietka-Czernel, 2017). MEN1 is the leading cause of death among patients with thymic neuroendocrine tumors (Phan, 2010). Therefore, many authors recommend prophylactic thymic resection (parathyroidectomy) in patients with MEN1, which reduces the risk of cancer during life (Teh, 1998; Trump, 1996)

IMAGE OF EMPERIPOLESIS IN EPITHELIO-RETICULAR CELLS OF THYMIC TUMORS

Most thymic tumors have thymic epithelial cells that do not show cytological malignancy. Moreover, these cells are mixed with lymphocytes in different proportions (Verley, 1985; Lewis 1987). It appears, that the phenomenon of lymphatic emperipolesis, in which the intact cell is present in the cytoplasm of the larger cell, may occur in epithelioreticular cells. This issue however warrants further scientific evaluation. The subject of the thymoma in the context of emperipolesis is likewise not much reported in the literature.

In an ultrastructural study, Llombart-Bosch suggested that close contacts existed between the thymic lymphocytes and the epithelio-reticular cells. This appearance was suggestive of emperipolesis (Llombart-Bosch, 1975). In another research conducted by Izard, the cytoplasmic structures resembled the embryonic epithelio-reticular cells in the guinea pig thymus (Izard, 1966). Interestingly, Llombard-Bosch suggests that mitotic lymphocytes are found throughout the tumor near E-R cells (epithelio-reticular cells). Moreover, there is a morphological and lymphocytic death relationship, while the lymphocytes were in the cytoplasm of E-R cells. The onset of such necrosis is progressive nuclear pycnosis and secondary chromatolysis. By the time the cytoplasm was completely gone, the fatty degeneration and the mitochondrial vacuolization had started. The remaining monoliform reticular particles swallowed mesenchymal macrophages. Such cells were characterized by advanced degradation (Llombard-Bosch, 1975). Macrophages have the ability to phagocytose and to absorb what they phagocytize. They are classified as connective tissue and are associated with the body's defense mechanisms (Cichocki, 2002). In this case, the mesenchymal macrographs were randomized in the tumor stroma, but were more frequent near E-R cells. Moreover, phagocyte-ingested cell debris of lymphocytic origin were also present (Llombard-Bosch, 1975).

There are only very few publications on emperipolesis in the context of the thymus gland, and even less in relation to E-R cells. It seems that the topic of emperipolesis requires further attention and research. Similar observations to the two cases cited above were noted in epithelion-reticular cell thymoma in carp. Lymphocytes were taken up by E-R cells. It therefore seems logical that there is some kind of cytoplasmic communication system between lymphocytes and E-R cells. Such a phenomenon can take place in the human thymus, as indicated by Golditeinand MacKay (1969) (Romano, 2004).

It is important to properly distinguish between thymoma and T lymphoblastic lymphoma using needle biopsy as this has serious consequences in further treatment. Among diagnostic criteria, a factor that favors thymoma is the demonstration of increased numbers of keratin-positive epithelial cells using immunohistochemical staining. Loss of keratin expression in neoplastic epithelial cells could lead to detrimental misdiagnoses (Adam, 2014). Notably, false-positive or otherwise negative results of various tests may be related to the physiology of the cell itself, which may lose or gain certain properties under the influence of given factors or for unexplained reasons. Here the loss of keratin expression is observed. The research revealed that thymic epithelial tumors showed highly reduced expression of at least one keratin (Adam, 2014).

Moreover, emperipolesis in the form of thymocytes in the cytoplasm of epithelial cells was noticed in imprint cytology but was not noticed in a histological examination, which will be discussed in the next section (Nerurkar, 2000).

According to the research, emperipolesis was also noticed in an 83-year-old patient who underwent Chamberlain anterior mediastinotomy. The presumptive diagnosis was a thymic tumor versus lymphoma. It was suggested to consider the test sample as an atypical thymoma. Another suitable alternative might be a thymic carcinoma (Mackay, 1985).

Considering the aforementioned results, the image of emperipolesis in the thymus is rarely observed, and if it is noticed, it arouses curiosity. This phenomenon warrants further evaluation. The research on animals (guinea pig and carp) is aimed at highlighting the importance of a holistic approach to the issue. Similar studies in animals can possibly be done faster, easier and in a larger population. Results may emerge sooner, and the similarities between the human thymus and animal glands, which already have been demonstrated,

EMPERIPOLESIS AS A KEY FEATURE IN IMPRINT SMEARS OF THE THYMUS

Among diagnostic imaging of the thymus, imprint cytology has not received much attention, because the organ is rarely sampled in routine surgical practice.

It appears that emperipolesis may not be noticed on histology, but, surprisingly in imprint cytology. Based on the presented research, a fragment of the thymus was mistakenly sampled as a pre-tracheal lymph node in order to exclude metastasis. Interestingly, the presence of thymocytes in the cytoplasm of thymic epithelial cells (emperipolesis) was the most significant feature in the imprints (Nerurkar, 2000). Imprint cytodiagnostic is useful, for example, in examining breast tumors. Contrary to histopathology, which is more time-consuming, imprint smear can take less than an hour. Moreover, imprint smear can do amastigotes that take a short course without the need for a pathologist (Sousa, 2014). In the study of Nerurkar, the emperipolesis was based on the ingress of thymocytes into the TNC. TNCs are thymic nurse cells, which are epithelial cells in the thymic cortex, nourish the thymus and can surround the thymocytes to form lymphoepithelial complexes. Importantly, the thymocytes in the cytoplasm in this case did not show signs of nuclear degeneration. So, for example, pyknosis did not occur (Nerurkar, 2000). Pyknosis is the process of a cell in apoptosis

or necrosis and consists of irreversible chromatin condensation (Kroemer, 2009). Additionally, immunohistochemistry with keratin, which confirmed that thymocytes are double by TNC. The method also showed that thymocytes are alive but not proliferating. Such emperipolesis took place not only in the cortex, but also in the corticomedullary junctions (Nerurkar, 2000). Other scientists studying immunohistochemical characterization of nurse cells in normal human thymus had similar observations. Moreover, this study showed that internalized thymocytes retain their proliferative potential (Dispasquale, 1991). Imprint smear is a quick diagnostic method, e.g. for tumors, but the disadvantage is that it does not allow reliable results in the context of tumor infiltration (Mehar, 2014).

Among the available imaging techniques, observations with an electron microscope and phase contrast microscope are indispensable for distinguishing emperipolesis from phagocytosis (Shamoto, 1980). This can be more difficult to observe under a light microscope (Mackay, 1985).

Indisputably, a wide range of diagnostic methods is needed to fully diagnose and investigate a given tumor. Paradoxically, it appears that imprint cytology, being less advanced technique than fine needle aspiration (FNA) cytology or histology, enables demonstration of such rare phenomenon as emperipolesis. More studies are necessary for these findings to be placed in a proper perspective.

COMPARISON OF EMPERIPOLESIS AND ENTOSIS

Emperipolesis and entosis are very similar processes but differ in the pattern of action and the mechanisms involved. In the case of entosis, the predominant fate of internalized cells is lysosome-mediated degradation and non-apoptotic cell death (Peng Xia, 2008). Emperipolesis, on the other hand, is the process of entry and temporary 'storage' of one cell in the cytoplasm of another cell, but one that is histogenetically foreign. In emperipolesis, a cell exists as an intact living cell in the cytoplasm of another and can exit at any time without any structural or physiological abnormality for either (Amita K, 2011). Emperipolesis is thought to improve cell survival and help prevent cell apoptosis in the host cell. The engulfed cell can be destroyed and depending on its mode of death, there are different terms to describe this procedure. For example, non-apoptotic death can occur as a result of so-called "suicidal emperipolesis" (Benseler et al., 2011). Emperitosis (a combination of emperipolesis and apoptosis) can also occur. The host cell can also be destroyed; killing of lymphocyte-containing tumor cells has been observed (Wang et al., 2013).

Both emperipolesis and entosis require extracellular free calcium and adhesion molecules and an actin-based cytoskeleton (Peng Xia, 2008). To systematically define emperipolesis and entosis it is necessary to identify the key intercellular junction molecules involved in these processes.

Entosis – from the Greek "*entos*" – "within", involves the absorption of one cell by the vacuolar system of a neighboring cell of the same type, from the same population, due to the loss of linkages between the cell and the extracellular matrix. Non-apoptotic death of such a cell may then occur, in the absence of caspase-3, requiring autophagy by lysosomal enzymes, or it may divide and leave the parent cell by a transcytosis-like

movement. It is suggested that entotic cell death should be defined as a new type IV cell death.

Entosis can occur under physiological as well as pathological conditions. As a result of entosis of tumor cells, the tumor cell may undergo:

- incomplete heterophagocytosis with removal of the remaining cancer cell outside the phagocyte or complete heterophagocytosis;
- pseudo-cannibalism – no change in the tumor cell;
- disintegration into glandular bodies, which remain in the cytoplasm of the cell;
- malignant transformation, i.e. benign tumor cell becoming malignant;
- progression of the malignant tumor cell;
- suppression of the tumor process by repeated uptake of the malignant tumor cells.

PATHOGENESIS OF ENTOSIS

Entosis is caused by cell detachment from the extracellular matrix and also enhanced by an imbalance in actomyosin contraction between neighboring cells. Entosis is mediated by E-cadherins and P-cadherins increasing cell adhesion in the absence of integrin signaling. The process also requires Rho GTPase, Rho kinase ROCK and myosin-based contractile force.

Moreover, entosis is favored by the presence of the Kras oncogene and the expression of epithelial cadherins E and P. Oncogenic transformation and mechanical deformability of the cell promotes the ability to engulf other cells, which usually leads to non-apoptotic death of such cells, but may also increase the metastatic potential of the tumor and induce changes in cell ploidy, leading to the formation of binucleated cells in culture (Gupta N., 2017).

Recent studies have shown that entosis can occur even when cells are attached to the matrix. It is presumed that mitosis is then the inducer of entosis. Also, it is thought that the lack of glucose in the growth medium may induce it by increasing the activity of AMP protein kinase (AMPK) (Xinlong Wang, 2019).

PATHOGENESIS OF EMPERIPOLESIS

Emperipolesis can be physiological, pathological or a pathognomonic feature of certain diseases. It is thought to be a form of temporary cell protection against carcinogens and chemotherapeutics, as this process is often seen in some mesenchymal tumors (multiple myeloma, acute and chronic leukaemia, myeloproliferation) and also during the use of cytostatic drugs. In pathological states it also occurs in Rosai Dorfman disease, which is a histiocytic proliferative disorder in which emperipolesis can be observed in lymph nodes with inflammatory infiltration and in cerebrospinal fluid. Emperipoietic erythroblast activity in the liver has been found to increase during periods of high hepatic erythropoietic activity and relatively anemic fetal state.

Physiological findings include emperipolesis of erythroblasts by megakaryocytes in the fetal liver, emperipolesis of lymphocytes by human glial cells in the brain.

Free calcium molecules and adhesion molecules are important in emperipolesis, as well as the actin- and ezrin-based cytoskeleton (Xia et al., 2008). Emperipolesis has been shown to decrease by inhibiting actin polymerisation (Takeuchi et al., 2010). Abnormal P-selectin located in the demarcation membrane system of neutrophils and megakaryocytes has been proposed as a cause of emperipolesis in marrow fibrosis (Centurione et al., 2004). It is thought that also a lymphocyte function-related antigen-1 (LFA-1 or CD11a/CD18) that can mediate intercellular interactions between leukocytes and non-blood cells together with its ligand, intercellular adhesion molecules 1 (ICAM-1/CD54), may be associated with emperipolesis (Reina and Espel, 2017).

Emperipolesis and entosis are two different phenomena. The process of emperipolesis occurs with the involvement of Ezrin, LFA-1 and ICAM-1. The engulfed cell can escape from the host or be killed. The host cell can be destroyed by the engulfed cell. In contrast, entosis is homotypic, in which E-cadherins and P-cadherins, the Rho-ROCK-actin/myosin pathway and actomyosin contraction imbalance play important roles. The absorbed cell may be killed or survive (Xinlong Wang, 2019).

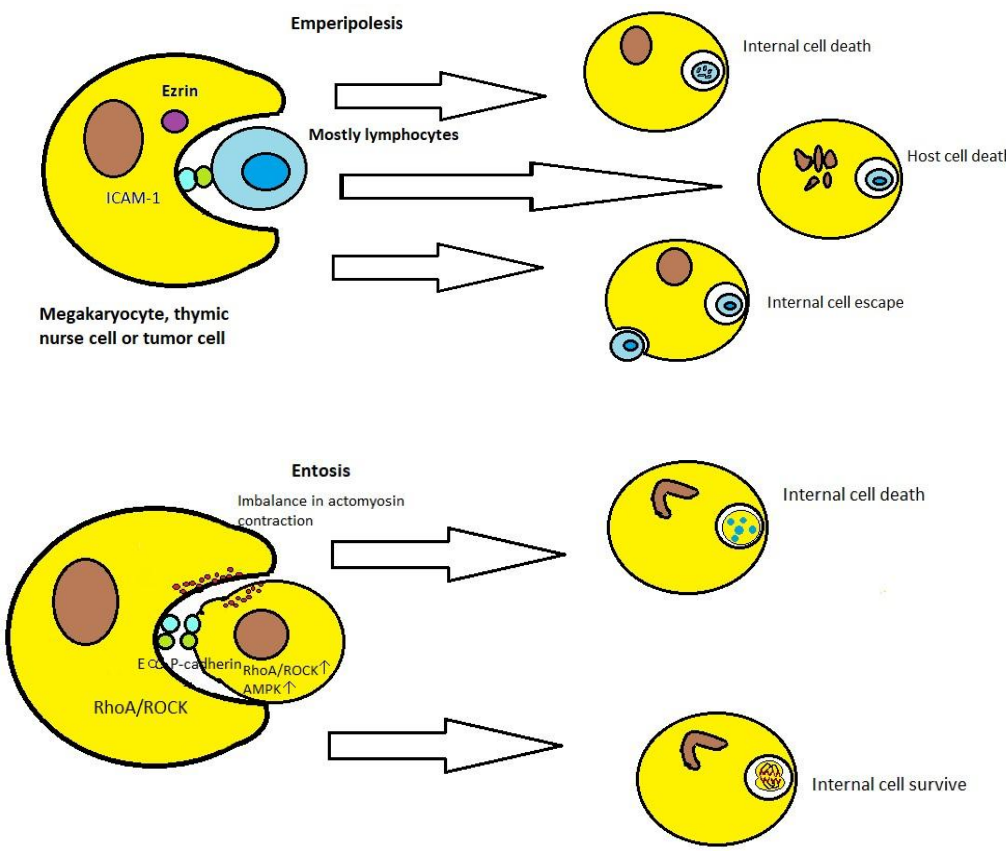

Figure 1. Emperipolesis and entosis – modified based on Wang et al., 2019

DIAGNOSIS OF NEUROENDOCRINE TUMORS

The standard procedure for the diagnosis of primary neuroendocrine tumors of the thymus is the combined use of anatomical and functional methods, since a single test technique has insufficient sensitivity and specificity (Ricke, 2000; Kaltsas, 2004).

The most commonly used diagnostic techniques for NETTs include anatomical examinations such as ultrasound, computed tomography (CT), magnetic resonance imaging (MRI) and endoscopic ultrasound (EUS) (Kaltsa, 2004).

The image of NETTs in CT is non-specific and takes the form of a large, clearly delimited tumor mass with a heterogeneous signal intensity. CT allows the identification of possible cystic lesions, necrosis, hemorrhage or hemorrhage within the tumor (Xiang, 2010). In an MRI scan, thymic tumors take the form of emerging tumor masses, which also show a heterogeneous signal intensity and allow the detection of cystic lesions. MRI scan is crucial in excluding possible tumor infestation into adjacent mediastinal structures (Berman, 2020; Xiang, 2010).

In turn, functional techniques, scintigraphic studies are used, which are based on a specific connection of synthetic somatostatin analogues labeled 111In or 99mTc with transmemphohelial receptor protein – scintigraphy of somatostatin receptor (SRS) (Krenning, 1989). The somatostatin receptors' presence in the neoplastic tissue justified the use of 111-Indium-diethylenetriamine pentaacetic acid-D-phenylalanine-octreotide (Octreoscan) scintigraphy, both in preoperative and in follow-up settings (Filosso, 2017).

Good quality imaging studies are the fundamental elements in establishing the starting point of primary neuroendocrine tumors of the thymus in assessing their stage. This is essential in determining surgical management, tracking response to therapy and prognosis (Plöckinge, 2005).

TREATMENT OF THE PRIMARY NEUROENDOCRINE TUMORS OF THE THYMUS

Primary neuroendocrine tumors of the thymus are rare yet very aggressive tumors, which grow relatively slowly. In almost 80% of the cases, they are malignant. NETTs very often infiltrate adjacent tissues. Local recurrence may occur many years later. They are more frequently diagnosed in men in the fourth and fifth decade of life. Nearly half of the cases are associated with endocrinology, such as Cushing's syndrome or acromegaly (Pier Luigi Filosso, 2017).

Completeness of resection is believed to be the strongest prognostic factor in the prognosis of this disease (Filosso, 2014). It has been found that patients in early stage of NETT survived longer and developed recurrences less frequently (Filosso, 2015). Furthermore, tumor size and metastatic development are also important in prognosis. According to previous studies, tumors with associated endocrinopathies also act more aggressively than tumors without them (Rabinowitcz, 2006). It was observed that patients with NETT and Cushing's syndrome or MEN-1 syndrome had a higher mortality rate than those without paraneoplastic syndromes (Wick, 1980).

Patients with NETT should be routinely referred to experienced centers and multidisciplinary facilities. For NETT, surgery to reduce the tumor mass is recommended to alleviate clinical symptoms resulting from the secretory activity of the tumor. These

tumors respond poorly to radiotherapy. The treatment of choice is surgery because almost 80% of thymic NETT cases behave malignantly (Moran, 2000). Complete resection of the tumor along with the involved mediastinal structures should always be sought. The preferred approach for NETT resection is through a median sternotomy. For advanced tumors, anterior thoracotomy, lateral thoracotomy, posterior-lateral thoracotomy, alone or in combination with sternotomy (combined access) can be used, which provide good exposure of the entire mediastinum and pleural space (Huang, 2008). Despite this, these tumors can often infiltrate adjacent structures and cause distant metastasis and recurrence, making their complete resection sometimes difficult and their prognosis poor (Pier Luigi Filosso, 2017).

NETT recurrences can be local, occurring in the anterior mediastinum, regional, present within the chest, or distant, occurring outside the chest or in the case of intrapleural nodules. An aggressive surgical approach if complete resection of the recurrence is possible and postoperative RT is thought to be effective in recurrent NETTS and to increase survival. (Sakuragi, 2002). For advanced NETT, induction chemotherapy (or CT + RT) has been used to reduce tumor size, increasing the likelihood of R0 resection (radical resection), although studies do not clearly define the effect of such a process. (Pier Luigi Filosso, 2017). Postoperative radiotherapy (or CT + RT) is also used for incomplete resections. Based on the reported cases, the medium-term survival in patients with NETT was quite good, especially in the case of complete surgical resection.

When surgical treatment is not possible, pharmacotherapy with somatostatin analogues, a hormone that inhibits secretory and cell proliferative processes, can be used. Somatostatin analogues are very well tolerated and usually relieve discomfort resulting from the secretory function of tumors (Dasari, 2017; Halperin, 2017; Davar, 2017).

A form of molecularly targeted therapy, peptide receptor radionuclide therapy (PRRT), appears to be very effective in the systemic treatment of metastatic thymic neuroendocrine tumors. PRRT is performed using a somatostatin analogue similar to octreotide, absorbed by the tumor, coupled to a radionuclide usually 177 lutetium and 90Itr emitting beta radiation that kills the tumor cells (Pier Luigi Filosso, 2017).

In order to reduce the tumor mass of metastases, thermoablation techniques are used, i.e. destroying cells with high temperatures obtained by laser or radiofrequency. In some patients with NET tumors, characterized by a high capacity for rapid cell division, classical chemotherapy is also used (Dasari, 2017; Halperin, 2017; Davar, 2017).

In MEN1 patients in whom NETT is a major cause of death, several prophylactic thymic resections at the time of parathyroidectomy using the same surgical access are suggested to reduce the risk of NETT (Teh,1998) (Trump, 1996).

As there is a high risk of recurrence or development of distant metastases in patients with NETT, close and lifelong follow-up of the patient is required. It is suggested to perform a chest CT every 6 months for the first 3 years (Pier Luigi Filosso, 2017).

THE IMPORTANCE OF EMPERIPOLESIS IN THE CONTEXT OF DEVELOPING FUTURE DIAGNOSTIC AND TREATMENT METHODS

In terms of diagnostics, it seems appropriate to conduct extensive research on a large population of neoplastic cells of neuroendocrine origin in terms of the occurrence of the phenomenon of emperipolesis. Based on the various studies and descriptions of clinical cases cited earlier, we conclude that there is a likelihood of a significant correlation between the number of cells in emperipolesis and a specific type of cancer. Furthermore, it is noteworthy that the proportions between different types of cells can serve as an indicator of a given tumor development and progression. It may be important to observe cells in the state of emperipolesis in a microscopic image and find the relationship between the occurrence of a specific image of cells and frequent detection of a specific tumor.

The use of lymphocytes in targeted therapy is very promising (Goswami, 2019). T lymphocytes tend to bind to antigens of cancer cells, which may be crucial for introducing therapeutic substances into cancerous cells, not into healthy ones. Targeted therapy can then only cover diseased cells, leaving healthy cells intact.

In biotechnology, great opportunities are attributed to the importance of liposomes as potential carriers of anti-cancer drugs (Temidayo, 2018). If the process of emperipolesis were to be explored even more and we would get an answer to the question of what induces emperipolesis, then one can try to construct a liposome that would resemble a lymphocyte externally, induce emperipolesis and thus deliver the drug to the inside of cancerous cells. Such a solution could be used locally or systemically if there is a risk of neoplastic metastases, since the outer surface of the liposome would have specific receptors targeting specific tumor epitopes distributed throughout the body.

A slightly different method could be to modify T lymphocytes by introducing specific drugs inside them and then using it in molecularly targeted therapy. This would save time and the biotechnological construction of the receptors would not be necessary, as we would use the receptors already present on the T lymphocytes.

Moreover, radioisotope therapy can be used in the treatment of neuroendocrine tumors of the thymus (Iskanderani, 2018). It is a molecularly targeted therapy in which a specially selected peptide, having the property of attaching to a cancer cell, is combined with a small amount of radioactive material to form together a drug (radiopharmaceutical) called a radiopeptide (Kolasińska-Ćwikła, 2018). After the injection into the patient's bloodstream, radiopeptide travels with the blood, reaches the tumor and attaches to the cancer cells, providing them directly with a therapeutic dose of radioisotopic radiation. The tumor absorbs both the drug and the radionuclide, and the emitted beta radiation particles kill cancer cells. The most effective radionuclides currently used are 177Lutet and 90Yttr.

SHORT CONCLUSION

Emperipolesis is a rare biological phenomenon, in which a cell penetrates another living cell. Emperipolesis is often described in relation to the thymus gland, however the precise mechanisms underlying this process are still elusive. In this publication we

have reviewed previous findings and determined the importance of emperipolesis in tumors formation and progression.

Lymphatic emperipolesis may occur in thymic epithelia-reticular cells. It is crucial to clarify the relationship between the presence of a particular cell image during emperipolesis and the detection of a particular type of cancer. Among available diagnosing techniques, imprint smear is an effective and quick method for detecting emperipolesis.

Thymic neuroendocrine tumors (NETTs) are rare tumors with high aggressiveness that present many non-specific symptoms. Diagnostic techniques that are most commonly used in neuroendocrine tumors assessment are ultrasound, computed tomography (CT), magnetic resonance imaging (MRI) and endoscopic ultrasound (EUS). The treatment of choice is surgery. The completeness of resection is the strongest prognostic factor, nevertheless PRRT appears to be very effective during therapy. Targeted therapy can cover only diseased cells, leaving healthy cells intact. The use of modified T lymphocytes in targeted therapy by introducing specific drugs inside them is an emerging and very promising method in treating cancer.

There is still a lot to uncover regarding emperipolesis, especially in terms of using this phenomenon in the therapy and treatment of cancer. An interesting approach would be to construct the liposome that delivers the drug to the inside of cancerous cells. The combination of the well-known treatment methods with not yet fully understood emperipolesis, may open up new possibilities especially in the treatment of neuroendocrine tumors of thymus.

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Insulinoma-associated protein 1 (INSM1) – new nuclear marker of neuroendocrine differentiation with high sensitivity and specificity in immunohistochemical diagnostics of neuroendocrine neoplasms – review

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ABSTRACT

Diagnostically difficult cases of neuroendocrine neoplasms require the use of markers of neuroendocrine differentiation. However, even the use of traditional neuroendocrine markers such as synaptophysin, chromogranin, and CD56 yields negative results in 10% to 25% of high-grade neuroendocrine tumors. Insulinoma-associated protein 1 (INSM1) is a novel nuclear marker of neuroendocrine differentiation. In terms of structure, INSM1 is a zinc-finger transcription factor. INSM1 (formerly IA-1) contains five zinc-finger motifs. INSM1 expresses transiently in embryonic neuroendocrine tissues. In adult tissues INSM1 has been identified in multiple tumors of neuroendocrine or neuroepithelial origin. INSM1 is a strong nuclear marker of neuroendocrine differentiation with high sensitivity and specificity. The results of the research analysed in this paper indicate that INSM1 can be very useful in the diagnostics of neuroendocrine neoplasms of the lung, gastrointestinal tract, pancreas, head and neck, uterine cervix, and Merkel cell carcinoma. In order to be included in the review, articles from PubMed (NCBI), Google Scholar, Web of Science and Scopus archive had to fit the following criteria:

- they had to be original articles, case studies and reviews connected with the following key words: neuroendocrine neoplasms, well-differentiated neuroendocrine tumors, poorly-differentiated neuroendocrine carcinomas, INSM1, traditional markers such as chromogranin, synaptophysin and CD56;
- they had to be written in English;
- they had to be published between 1992 and 2020, as the first article about insulinoma-associated protein 1 was written by Goto et al. in 1992.

INTRODUCTION

The term *neuroendocrine system* was introduced in the second half of the 20th century. The neuroendocrine system covers interactions between the nervous system and a variety of endocrine glands such as: the pituitary gland, the thyroid gland, the parathyroid gland, the adrenal gland, the ovaries and testes, the endocrine pancreas, the pineal gland, the gastrointestinal endocrine system, and the respiratory endocrine system. The endocrine/neuroendocrine cells found in these organs and systems synthesize and secrete a number of hormones that have key influence on the metabolism of the body through the interaction of these hormones with target tissues in response to stress and injury. These hormones are also involved in the control of a number of life processes such as growth, development, absorption of nutrients, energy, metabolism, water and electrolyte balance, reproduction, birth, and lactation. The endocrine/neuroendocrine cells appear in the early stages of development and are characterized by a unique

pathway of differentiation. According to Lan et al., abnormal differentiation and/or deregulation of these endocrine/neuroendocrine cells appearing in the pituitary gland, the thyroid gland, the parathyroid gland, the adrenal gland, the ovaries and testes, the endocrine pancreas, the pineal gland, the gastrointestinal endocrine system, and the respiratory endocrine system may lead to the development of neuroendocrine tumors that have a profound effect on the body′s metabolism (Lan, 2009). However, the term neuroendocrine neoplasms (NENs) includes not only tumors developing in the above mentioned organs and systems. Neuroendocrine neoplasms occur throughout the body, in all body organs, including paraganglia and soft tissue (Choi, 2018; Delaloge, 2000; Egashira, 2018; Fujino, 2015; Ramalingam, 2016; Weed, 2003). This prompted the participants of the 2017 WHO conference to accept the term neuroendocrine neoplasms for approval in relation to the classification of the types of tumors mentioned above. According to Rindi et al. the term "neuroendocrine neoplasms" is the best at "encompassing all tumor classes with predominant neuroendocrine differentiation, including both well and poorly differentiated forms" (Rindi, 2018). Moreover, the authors stated that the "key features defining these neoplasms at any specific anatomic site are, above all, multiple anatomic sources (neural structures, endocrine organs and/or neuroendocrine cells), morphology, and the expression of markers of neuroendocrine differentiation (general and specific)". The expression of neuroendocrine markers may fundamentally differ in different anatomic sites, and at the same time, expression depends on the degree of differentiation. Therefore, different general neuroendocrine markers to define neuroendocrine differentiation are currently applicable in different organs and systems (e.g. only chromogranin and synaptophysin in the gastrointestinal system and pancreas, while chromogranins, synaptophysin, and CD56 in the lung) (Rindi, 2018). According to the latest WHO classification from 2017, neuroendocrine neoplasms include well-differentiated neuroendocrine tumors (NETs) designated carcinoid tumors in some systems, as well as poorly-differentiated neuroendocrine carcinomas (NECs), including two separate morphologic types defined as small cell neuroendocrine carcinoma and large cell neuroendocrine carcinoma (Kim, 2016, Lloyd, 2017, Rindi, 2018). The above classification was accepted and adopted by the American Joint Committee on Cancer (8^{th} edition) and the current College of American Pathologists guidelines (Amin, 2017; Burgart, 2020; Shi, 2017; Shi, 2020). Moreover, it was proposed that well-differentiated neuroendocrine tumors be classified in three tiers as G1, G2 or G3 which reflects low-grade, intermediate-grade, and high-grade (Rindi, 2018). Unlike NETs, NECs are always high grade (G3). On the other hand, three grading parameters such as: mitotic count and/or Ki-67 cell labeling index, and/or the presence or absence of necrosis are prognostic (Rindi, 2018). For this reason, the above division of neuroendocrine neoplasms is based on genetic evidence at specific anatomic sites and differences in epidemiology, histology, clinical course and prognosis. NETs, belonging to the family of well-differentiated neoplasms, have potential to metastasize or invade the adjacent tissues depending on tumor site, type, and grade (Klimstra, 2010; Klöppel, 2017). In turn, NECs are characterized by a high degree of malignancy, a very rapid and aggressive course and a poor prognosis. NENs are among a relatively rare group of tumors in the population, ranging from 2.5 to 5 cases per 100,000 people per year (Rodriguez, 2018; Rosenbaum, 2015). However, in recent years, a gradual increase in

the incidence of neuroendocrine neoplasms has been observed both in the United States and in other countries (Dasari, 2015; Hallet, 2015; Hauso, 2008). The incidence of NENs has been increasing at all sites, stages and grades (Dasari, 2015), with the main sites of development in the human body affecting the gastrointestinal system and respiratory system (Oronsky, 2017). NENs occur in the population in all age groups, but the highest number of NENs is observed in patients aged 65 years and older (Dasari, 2015).

According to the latest WHO classification from 2017, neuroendocrine neoplasms have epithelial or neuronal/neuroectodermal origin, and share major morphological and protein expression signatures depending on differentiation, despite their different localization in the body (Rindi, 2018). While NENs are characterized by a diverse spectrum of proteins, many of these proteins are identical to proteins present in normal cells, organs or systems with different anatomical localization. Different localization in the body and origin make that NENs a heterogenous group of tumors, yet they share some common features, including presence of neurosecretory granules and typically showing a characteristic histology and immunoprofile (Rindi, 2018). The characteristic markers of general neuroendocrine differentiation occurring in NENs include chromogranin A, chromogranin B, and synaptophysin, as well as site specific markers such as hormones and transcription factors (Inzani, 2017). The following immunohistochemical markers of neuroendocrine differentiation are traditionally used in immunohistochemical diagnostics: synaptophysin, chromogranins and CD56. These immunohistochemical markers are characterized by a relatively low sensitivity and specificity. Research results indicate that synaptophysin shows expression in only 41% to 75% of small cell lung carcinoma (SCLC) and from 58% to 85% of large cell lung carcinoma (LCNEC) cases, chromogranin shows expression in only 23% to 58% of SCLC and 42% to 69% of LCNEC and CD56 showed expression from 72% to 99% of SCLC and 72% to 94% of LCNEC (Hamanaka, 2014; Jiang, 1998; Kaufmann, 1997). Since none of the above immunohistochemical markers are sufficiently sensitive and specific, they must be used in immunological diagnostics as a group. This creates a situation where diagnostics is overly complicated and expensive. Therefore, the search for a single immunohistochemical marker with high specificity and sensitivity that could be used in the diagnostics of neuroendocrine neoplasms has been going on for many years. Insulinoma-associated protein 1 (INSM1) may fulfill these expectations.

NEW INSIGHT

STRUCTURE AND FUNCTION OF INSULINOMA-ASSOCIATED PROTEIN 1

Insulinoma-associated protein 1 is a zinc-finger transcription factor. At the same time, the protein structure of INSM1 is highly conserved among homologues of different species. INSM1 (formerly IA-1) contains five zinc-finger motifs. Based on the deduced protein sequence, INSM1 can be divided into two major domains. The amino-terminal domain (aa 1-250) contains a high percentage of proline, glycine, and alanine residues. Proline-rich (20-30%) sequences occur in many mammalian transcription factors and serve as protein-protein interacting domains that mediate both transcriptional activation and/or repression (De Caestecker, 2000; Zilfou, 2001). The dibasic amino acids are cleavage recognition sites for processing peptide hormone precursors such as insulin,

glucagon, somatostatin and pancreatic polypeptide. An α-amide group is common to many bioactive neuroendocrine peptides. The carboxyl-terminal sequence (aa-251-510) contains five putative Cys2-His2-type zinc-finger motifs. These five zinc-finger motifs are symmetrically spaced at the carboxy terminus. Two tandem repeated zinc-finger motifs from either end are spaced by 45/46 aa from the middle zinc finger (Lan, 2009). The structural features of INSM1 indicates that INSM1 is a zinc-finger DNA-binding protein. INSM1 functions as a transcriptional repressor that simultaneously regulates entry into the cell cycle and controls expression of a neuroendocrine phenotype (Lan, 2009). Moreover, INSM1 is directly responsible for the transcription of synaptophysin and chromogranin (Fujino, 2015). In contrast, INSM1 is regulated by neurogenin 3 (Mellitzer, 2006).

INSM1 shows expression mainly in normal fetal neuroendocrine tissues and tumors of neuroendocrine origin regardless of age. In the fetal period, INSM1 is predominantly expressed in the nervous system in mammals, and plays an important role in early embryonic neurogenesis (Lan, 2009). Moreover, in the fetal period, INSM1 plays an important role in the development of normal neuroendocrine cells in various tissue throughout the body, mainly in the pancreas, digestive system and central nervous system (Gierl, 2006; Goto, 1992; Lan, 1993; Lan, 2009; Xie, 2002). It was found that INSM1 affects both terminal cellular differentiation and cellular proliferation in the pancreas (Gierl, 2006; Osipowich, 2014; Parent, 2008; Zhang, 2012; Zhu, 2002), enteroendocrine cells (Gierl, 2006), the autonomic nervous system (Widner, 2008), the central nervous system (Farkas, 2008; Jacob, 2009), olfactory epithelium (Rosenbaum, 2011), and the pituitary gland (Welcker, 2013). Moreover, INSM1 regulates downstream target genes and exhibits extranuclear activities associated with multiple signaling pathways, including Sonic Hedgehog, PI3K/AKT, MEK/ERK, ADK, p53, Wnt, histone acetylation, LSD1, cyclin D1, Asc1, and N-myc (Chen, 2018; Chen, 2019). However, a disadvantageous phenomenon is that INSM1 expression declines with age (Goto, 1992).

What is interesting, however, is that when it comes to tumors, the presence of INSM1 can be found in a number of tumors with neuroendocrine differentiation, such as pheochromocytoma (Sandgren, 2010), medullary thyroid carcinoma, pituitary adenoma (Goto, 1992), hypothalamic hamartoma (Parent, 2008), retinoblastoma, small cell lung carcinoma (Amelung, 2010; Goto, 1992; Lan, 1993; Taniwaki, 2006), and medulloblastoma (Breslin, 2002; De Smaele, 2008; Gilbertson, 2004; Pomeroy, 2002). INSM1 was found not only in human tumors but also in mice and rats (Farkas, 2008; Jacob, 2009; Kawaguchi, 2008; Xie, 2002). Initially, it was thought that INSM1 did not appear in the normal tissue of adults (Breslin, 2003; Duggan, 2008; Gierl, 2006; Goto, 1992; Welcker, 2013; Widner, 2008; Zhu, 2002). However, further research has shown INSM1 expression in normal adult cells such as neuroendocrine cells present in the gastrointestinal tract, pancreatic tract, bronchopulmonary system, adrenal medullary tissues, and in occasional individual cells in non-neoplastic prostate glands (Ames, 2018; Rosenbaum, 2015; Yoshida, 2018).

INSM1 is encoded by the insulinoma associated-1 (IA-1) gene of cDNA. This gene was first identified by Goto et al. in 1992 in human pancreatic insulinoma tissues and

murine insulinoma cell lines, which influenced its name (insulinoma associated protein 1) (Goto, 1992). However, the localization of the *INSM1* gene at the start arm of chromosome 20 was revealed by Lan et al. two years later (Lan, 1994). Research conducted on human lung cancer cell lines has shown that *INSM1* gene expression occurs in small cell lung carcinoma and carcinoid tumors, while expression of this gene does not occur in non-small cell lung carcinoma (Lan, 1993). Subsequent studies have shown that the expression of *INSM1* gene is not limited to small cell lung carcinoma but also occurs elsewhere of the body, including neuroendocrine tumors of the gastrointestinal tract, cervical cancer, prostate cancer, pheochromocytoma, medullary thyroid carcinoma, insulinoma, or pituitary tumors (De Smaele, 2008; Gilbertson, 2004; Goto, 1992; Parent, 2008; Pomeroy, 2002; Sandgren, 2010; Xin, 2018).

INSM1 AS IMMUNOHISTOCHEMICAL AND MOLECULAR MARKER

INSM1 shows high expression in tumors of neuroendocrine origin, with INSM1 expression significantly increased in neoplastic tissue compared to non-neoplastic tissue (Doxtader, 2018; Lan, 2009; Nakra, 2019; Rodriguez, 2018; Rosenbaum, 2015). Moreover, research conducted by many authors has confirmed that INSM1 is a strong nuclear, immunohistochemical marker of neuroendocrine differentiation in neoplastic human tissues (González, 2019; Rosenbaum, 2015; Roy, 2019; Staaf, 2020; Viswanathan, 2019). For this reason, INSM1, the only available nuclear neuroendocrine marker, is increasingly used in immunohistochemistry diagnostics (Ames, 2018; Kuji, 2017; Rooper, 2018; Rosenbaum, 2015; Xin, 2018). INSM1 in immunohistochemical staining gives a positive nuclear reaction, in contrast to synaptophysin and chromogranin, which show a granular cytoplasmic reaction. In turn, CD56 is both cytoplasmic or membrane positive.

REVIEW AND DISCUSSION

NEUROENDOCRINE NEOPLASMS IN THE LUNG

In one of the first large studies involving 111 primary thoracic neuroendocrine neoplasms (small cell carcinoma, large cell carcinoma, atypical carcinoid, typical carcinoid and mediastinal paraganglioma) and 156 non-neuroendocrine tumors (adenocarcinoma, and squamous cell carcinoma), the authors assessed immunohistochemistry sensitivity and specificity of INSM1 in surgical specimens and compared its performance to traditional neuroendocrine markers (synaptophysin, chromogranin and CD56) (Rooper, 2017). For this purpose, they used material from the surgical pathology archives from 1997-2017, but did not include thoracic mixed tumors in the study. In the presented study, the sensitivity of INSM1 for small cell lung carcinomas and large cell neuroendocrine carcinomas was significantly higher (94%, 91.3%) than the sensitivity of the panel of the three traditional neuroendocrine markers (74.4%, 78.3%). In addition, the authors found positive staining for INSM1 in all the atypical carcinoids, typical carcinoids and mediastinal paragangliomas. The sensitivity of INSM1 across all grades of thoracic neuroendocrine tumors was 96.4%, and significantly exceeded the sensitivity of the panel of traditional neuroendocrine markers (87.4%). However, in non-neuroendocrine tumors staining positive for INSM1, they observed only 3.3% of adenocarcinomas and 4.2% of squamous cell carcinomas.

In another large study, researchers examined a large series of whole-tissue sections of primary lung neoplasms (345), including 152 neuroendocrine tumors (64 small cell lung carcinomas, 24 large cell neuroendocrine carcinomas, 48 typical carcinoid tumors, 16 atypical carcinoid tumors), and 163 non-neuroendocrine tumors (130 adenocarcinomas, 33 squamous cell carcinomas) for sensitivity and specificity of INSM1 (Mukhopadhyay, 2019). The analyzed material also included mixed tumors. In this study, the sensitivity of INSM1 for neuroendocrine neoplasms *as a group* (95%) was similar to synaptophysin and CD56 (98%, 97%), but higher than chromogranin (84%). In contrast, the specificity of INSM1 and chromogranin (97%, 98%) was higher than the specificity of synaptophysin and CD56 (90%, 87%). The sensitivity of INSM1 in small cell carcinoma was similar to the sensitivity of synaptophysin and CD56 (98%, 100% and 95%), but was higher than the sensitivity of chromogranin (83%). For large cell neuroendocrine carcinomas, similar sensitivity for CD56 and synaptophysin (92%, 88%) was observed, while the sensitivity of INSM1 and chromogranin was unquestionably less (75%, 46%). Except for one case of atypical carcinoid tumor, all carcinoid tumors were positive for INSM1, chromogranin, synaptophysin and CD56.

A third study looked at surgically resected 54 primary lung neuroendocrine tumors (including 24 small cell lung carcinomas, 23 large cell lung carcinomas, 5 typical carcinoid tumors and 2 atypical carcinoid tumors) as well as 623 non-small cell lung carcinomas (Staaf, 2020). There were also mixed tumors in the material studied. Here, the authors determined the diagnostic value of INSM1 in comparison to the previously used traditional neuroendocrine markers (CD56, synaptophysin and chromogranin A). They observed positive INSM1 staining in 39 cases of 54 pulmonary neuroendocrine tumors (72%) and in 6 cases of 623 non-small cell lung carcinomas (1%). On the other hand, a positive CD56 staining for primary lung neuroendocrine tumors and non-small cell lung carcinomas were 47 of 54 (87%) and 14 of 626 (2%), for synaptophysin 46 of 54 (85%) and 49 of 630 (8%), and for chromogranin A 30 of 54 (56%) and 6 of 629 (1%).

Other authors tested for whether INSM1 could be used in cytology (Cellient) cell blocks and whether these results correlated with surgical pathology specimens (Doxtader, 2018). The aim was to compare the sensitivity and specificity of INSM1 with the sensitivity and specificity of synaptophysin, chromogranin and CD56. The study was conducted on seventy-four primary lung neoplasms, including 52 primary lung neuroendocrine neoplasms (41 small cell lung carcinomas, 1 large cell neuroendocrine carcinoma, 10 carcinoid tumors) and 22 non-neuroendocrine primary lung tumors (11 adenocarcinomas, 9 squamous cell carcinomas, 1 mesothelioma, and 1 poorly differentiated non-small cell lung carcinoma). In 20 cases, INSM1 staining was performed simultaneously on paired surgical pathology specimens (biopsy or resection). The specimens tested positive for INSM1 in all 20 paired surgical pathology cases. However, in cytology cell blocks, positive INSM1 results were found in 48 of 52 cases of primary lung neuroendocrine neoplasms (92%), including 38 of 41 small cell lung carcinomas (93%), in one case large cell neuroendocrine carcinoma (100%) and in 9 cases out of 10 carcinoid tumors (90%). The specificity of INSM1 for primary pulmonary neuro-

endocrine neoplasms *as a group* was identical to the specificity of chromogranin (100%), but was higher than the specificity of synaptophysin (95%) and CD56 (95%).

In another study, the authors compared the diagnostic utility of INSM1, CD56, synaptophysin and chromogranin in the largest cohort (143) of pulmonary cytology cell blocks (11 typical carcinoid tumors, 11 atypical carcinoid tumors, 9 small cell lung carcinomas, 8 large cell neuroendocrine carcinomas, 9 squamous cell carcinomas and 95 adenocarcinomas) and the largest available material (563) of surgical specimens including 17 typical carcinoid tumors, 14 atypical carcinoid tumors, 8 small cell lung carcinomas, 10 large cell neuroendocrine carcinomas, 58 squamous cell carcinomas, 415 adenocarcinomas, 17 large cell carcinomas and 24 other tumor types (Viswanathan, 2019). These authors obtained sensitivity and specificity for INSM1 of 92.3% and 100% in the cytology cell blocks, while the sensitivity and specificity for INSM1 in the surgical specimens was lower (89.8%, 98.1%). The sensitivity and specificity for CD56 were 97.4% and 93.3% in the cytology cell blocks and 93.9% and 93.6% in the surgical specimens. The sensitivity and specificity for synaptophysin and chromogranin were significantly lower in both the cytology cell blocks and the surgical specimens.

In the next study, the authors performed manual immunohistochemistry on small biopsies of INSM1 and immunocytochemistry on direct smears of INSM1 on archival material from 60 patients diagnosed with small cell lung carcinoma in order to check the suitability of each of these methods in the diagnostics of this tumor (Nakra, 2019). Of these 60 patients, 37 were tested for INSM1 immunohistochemistry on small biopsies and 36 were tested for INSM1 immunocytochemistry on direct smears. The sensitivity for INSM1 immunohistochemistry (small biopsies) was 97% (36 of 37 cases), while the sensitivity for INSM1 immunocytochemistry (direct smears) was lower, only 91% (30 of 33 cases). Moreover, INSM1 reactions were performed on 10 cases of non-small cell lung carcinoma on spare direct smears and on small biopsies, obtaining 100% specificity (all cases were negative for INSM1).

In yet another study, the authors performed immunohistochemistry staining for INSM1 on cytology samples from 32 patients with neuroendocrine tumors of the lung (8) and tumors with neuroendocrine differentiation of lung origin (22 lymph node, 1 chest wall mass, 1 thyroid) (Rodriguez, 2018). All the neuroendocrine tumors used in the study were small cell carcinomas. The material taken was from multiple aspirations. INSM1 was positively identified in 31 of 32 cases (97%). In the control group of non-neuroendocrine tumors all 13 cases were negative for INSM1. The sensitivity of CD56 in small cell carcinoma was 96%.

NEUROENDOCRINE NEOPLASMS IN THE GASTROINTESTINAL TRACT AND PANCREATICOBILIARY TRACT

In a retrospective study covering the archive material from 2013-2015, the authors examined 30 patients with primary gastroenteropancreatic neuroendocrine neoplasms and their metastatic diseases in the liver in terms of INSM1 sensitivity assessment and compared it with the sensitivity of chromogranin-A and synaptophysin (Gonzalez, 2019). Moreover, they assessed the changes in the expression of these markers in the material from primary and metastatic diseases. Most of the cases studied were small

intestine and neoplasms were present in ileum, duodenum, Meckel′s diverticulum, pancreas, stomach, rectum and caecum. All studied cases of primary gastroenteropancreatic neuroendocrine neoplasms were reactive for INSM1 and synaptophysin (100%), while the sensitivity of chromogranin-A was weaker (97%). In the material from metastatic neoplasms, sensitivity of INSM1 was weaker (94%) than the sensitivity of synaptophysin (100%) and chromogranin-A (97%). The specificity of INSM1 (96%) was comparable to the specificity of chromogranin-A (97%), and higher than that of synaptophysin (54%).

In turn, other authors compared the sensitivity and specificity of INSM1 with other neuroendocrine markers (synaptophysin, chromogranin and CD56) and the performance of the antibody according to site and differentiation of the tumor (Rodriguez, 2018). This study was performed using 134 specimens, including 91 neuroendocrine tumors with neuroendocrine features (taken from pancreas, liver, gastric and perigastric mass, abdomen, parotid gland, and other organs such as: lymph node, lung, soft tissue, vertebra, buttock, soft tissue of vagina, and pelvic wall). In this material, INSM1 showed a sensitivity of 99% and a specificity of 97%, while CD56 had a sensitivity only slightly lower (95.5%), but the specificity was very low (69.2%). In contrast, chromogranin had the weakest sensitivity (82.5%), while synaptophysin had the weakest specificity (66.7%). In contrast, among 10 cases diagnosed as non-neoplastic lesions, only two cases (pancreatic neuroendocrine islet cells and benign adrenal cells) were positive for INSM1.

In another study, the authors assessed the sensitivity and specificity of INSM1 in material covering 110 cases of primary neuroendocrine neoplasms of the gastrointestinal tract, appendix, and pancreas (McHugh, 2020). At the same time, they performed a sensitivity and specificity check of synaptophysin, chromogranin, CD56 and Ki67. INSM1 was positive in 89 of 110 (80.9%) primary gastrointestinal, appendiceal and pancreatic neuroendocrine neoplasms, while synaptophysin was positive 99.1%, chromogranin 88%, CD56 95.3%. In contrast, the specificity of INSM1 (95.7%) was higher than that of synaptophysin (86.0%), chromogranin (87.3%), and CD56 (86.0%).

Other authors studied INSM1 in conjunction with chromogranin, synaptophysin, and CD56 in 36 appendiceal adenocarcinoma ex-goblet carcinoid (21 primaries, 15 metastases) (Yang, 2019). In primary adenocarcinoma ex-goblet carcinoid, they obtained positive results for INSM1 62%, for chromogranin 86%, for synaptophysin 86% and for CD56 47%. In contrast, metastatic adenocarcinoma ex-goblet carcinoid showed staining for INSM1 53%, for chromogranin 73%, for synaptophysin 80% and for CD56 21%.

NEUROENDOCRINE NEOPLASMS OF PANCREAS

In a retrospective study, the authors examined the usefulness of INSM1 for identifying pancreatic neuroendocrine tumors in 26 cell blocks and 29 surgical resections (Kim, 2020). Additionally, they performed INSM1 staining in other primary pancreatic tumors such as solid pseudopapillary neoplasms (14 cases), 11 acinar cell carcinomas and 21 pancreatic ductal adenocarcinomas. They obtained in all 55 cases of pancreatic neuroendocrine tumors a positive nuclear test for INSM1, both in cell blocks and surgical

resections (100% sensitivity), while sensitivity of synaptophysin was 97%, chromogranin 92%, and CD56 85%.

In turn, other authors assessed the expression of INSM1 in 14 cytology specimens obtained from endoscopic ultrasound-guided fine needle aspiration cytology during diagnostics of pancreatic neuroendocrine tumors (Takase, 2018). These authors used cytological specimens from 15 cases diagnosed as pancreatic ductal adenocarcinoma as a control group. In all 14 pancreatic neuroendocrine tumor cases, INSM1 showed expression in the tumor cells (100% sensitivity). In the control group, these authors observed INSM1-expressing cells within the adenocarcinoma cell cluster, but found no expression of INSM1 in the pancreatic duct cells or acinar cells.

The presented study was aimed at detected INSM1, chromogranin, synaptophysin and neural cell adhesion molecule immunohistochemically, in 25 cases of pure pancreatic neuroendocrine tumors and 2 mixed adenoneuroendocrine carcinomas (Tanigawa, 2018). As a control group, they used 5 cases of solid-pseudopapillary neoplasm, 7 cases of acinar cell carcinoma, and 15 cases of pancreatic ductal adenocarcinoma. These authors found the nuclear expression of INSM1 in all pure pancreatic neuroendocrine tumors (100% sensitivity). In 2 cases of mixed tumor the neuroendocrine carcinoma component was positive for INSM1, while the adenocarcinoma component was negative for INSM1. All control cases were negative for INSM1, while they were positive for synaptophysin.

NEUROENDOCRINE NEOPLASMS OF SKIN

In one study, the authors assessed INSM1 staining on 56 cases of Merkel cell carcinoma (47 primary tumors and 9 nodal metastases) (Lilo, 2018). All 56 cases of Merkel cell carcinoma showed expression of INSM1 (100% sensitivity). In contrast, synaptophysin, cytokeratin and chromogranin in the same material had expressions of 96%, 92% and 32%. In the control group (50 cases included various non-Merkel cell carcinoma neoplasms), no positive staining for INSM1 was found in any case.

In turn, other researchers developed their own dual immunohistochemistry protocol for INSM1/cytokeratin 20 to detect dual expression of keratin and INSM1 on 15 small samples taken from Merkel cell carcinoma (Rush, 2018). They detected INSM1 in 14 of 15 specimens carrying a diagnosis of Merkel cell carcinoma (93% sensitivity). On the other hand, one specimen that was negative for INSM1 was also negative for cytokeratin and chromogranin, with only focal positivity for synaptophysin. Moreover, they checked the sensitivity of INSM1 in three other specimens of cutaneous neuroendocrine carcinoma (non-Merkel cell carcinoma) and obtained 100% sensitivity for INSM1. However, of the 8 cutaneous non-neuroendocrine neoplasms tested, only one tested positive for INSM1.

NEUROENDOCRINE NEOPLASMS OF THE HEAD AND NECK

Researchers performed INSM1 immunohistochemistry on 97 neuroendocrine tumors and 626 non-neuroendocrine tumors across all histologic grades and anatomic subsites of the head and neck (Rooper, 2018). These authors obtained the sensitivity of INSM1 99.0%, with a positive result for INSM1 they observed in all types of head and neck

neuroendocrine tumors (middle ear adenoma, pituitary adenoma, paraganglioma, medullary thyroid carcinoma, olfactory neuroblastoma, small cell carcinoma, large cell neuroendocrine carcinoma, and sinonasal teratocarcinosarcoma). These authors obtained 97.6% specificity for INSM1 in almost all non-neuroendocrine tumors.

NEUROENDOCRINE NEOPLASMS OF THE UTERINE CERVIX

In one study, the authors made an immunohistochemical assessment of conventional neuroendocrine markers (chromogranin, synaptophysin and neural cell adhesion molecule) and INSM1 by analyzing 37 cases of high-grade neuroendocrine carcinoma of the uterine cervix (Kuji, 2017). These authors obtained the highest sensitivity (95%) for INSM1, while sensitivity for both chromogranin and synaptophysin was 86% and for neural cell adhesion molecules only 68%.

In turn, other authors, examining malignant tumors with neuroendocrine differentiation from the gynecologic organs, assessed the expression of INSM1, synaptophysin, chromogranin, CD56, orthopedia homeobox and achaete-scute homolog 1 in 2 cases in the uterine cervix (Roy, 2019). They obtained 100% sensitivity for INSM1, 100% for synaptophysin, 100% for CD56, 50% each for chromogranin and achaete-scute homolog 1, and negative for orthopedia homeobox.

NEUROENDOCRINE TUMORS OF THE PROSTATE

In this study, the authors checked the expediency of the use of INSM1 in the diagnostics of neuroendocrine tumors of the prostate (Xin, 2018). They performed immunohistochemical tests on 13 needle biopsies of primary small cell carcinoma of the prostate, 5 samples of mixed small cell neuroendocrine carcinoma-acinar adenocarcinoma obtained from prostatectomy and 2 cases of metastatic small cell carcinoma. These authors obtained positive results for INSM1 in 12 cases of primary small cell carcinoma (92.3%), while the reactions for synaptophysin (84.6%) and chromogranin (53.8%) were weaker. In the remaining 5 cases of mixed tumors and 2 metastatic tumors sensitivity of INSM1 was 100%, similarly for synaptophysin, while the sensitivity of chromogranin (80%) was weaker. The test of the specificity of INSM1 was performed on the material including benign prostatic hyperplasia and prostate adenocarcinoma, in most cases they did not find nuclear reactivity for INSM1.

In turn, Roy et al. assessed the usefulness of INSM1 in immunohistochemical diagnostics – 32 cases included malignant tumors with neuroendocrine differentiation from the gynecologic organs, including prostate gland (n = 6) (Roy, 2019). Out of 4 examined cases of prostate adenocarcinoma with neuroendocrine differentiation, they obtained a positive result for INSM1 in 25%. However, for synaptophysin and Cd56 they obtained a positive result in 50%, and chromogranin was negative in all cases.

OTHER RARE LOCALIZATION OF NEUROENDOCRINE TUMORS

NEUROENDOCRINE NEOPLASMS OF THE URINARY BLADDER

In the presented study, the authors assessed the immunohistochemical expression of INSM1 on 32 whole sections of small cell neuroendocrine carcinoma of the urinary bladder and compared INSM1 expression with synaptophysin, chromogranin and

CD56 (Kim Jr, 2020). In 28 cases these authors obtained a positive result for INSM1, in 24 cases for CD56, in 19 cases for synaptophysin, and in 14 cases for chromogranins.

NEUROENDOCRINE NEOPLASMS OF THE BREAST

In another study, the authors compared the expression of INSM1, orthopedia homeobox, chromogranin, synaptophysin, CD56 and achaete-scute homolog 1 in invasive mammary carcinoma (Roy, 2019). In the material studied, they found the strongest expression for achaete-scute homolog 1 and synaptophysin (85.7%) and weaker for INSM1, chromogranin, and CD56 (71.4%). In contrast, the expression of orthopedia homeobox was negative.

PERIPHERAL NEUROBLASTIC TUMORS

In another study, the authors assessed the immunohistochemical profile of INSM1 in cases of peripheral neuroblastic tumors and compared INSM1 expression in these tumors to that seen in other embryonal neoplasms (non-neuroblastic tumors) (Wang, 2019). Nuclear expression of INSM1 was 78% in peripheral neuroblastic tumors, including in neuroblastomas 84%, in ganglioneuroblastomas 100%, and in ganglioneuromas 33%. In the non-neuroblastic tumors control group, these authors found INSM1 expression in rhabdomyosarcomas (50%), in nephroblastomas (32%), and in Ewing sarcomas (20%).

PRIMARY CENTRAL NERVOUS SYSTEM NEOPLASMS

Other authors checked INSM1 expression in primary central nervous system neoplasms (Ames, 2018). They obtained nuclear immunostaining for INSM1 in medulloblastomas (87%), while diffuse nuclear INSM1 immunostaining was observed in all central neurocytomas and pituitary adenomas. However, they found rare staining with INSM1 in other high-grade embryonal tumors and high-grade gliomas. These authors observed nuclear INSM1 staining in normal brain tissue only in early neuronal development, while they did not find nuclear INSM1 staining in adult normal brains, including areas of gliosis.

In typical cases, when the diagnostics of neuroendocrine neoplasms is not difficult, it is based on standard histologic and cytologic stains and there is no need to perform immunohistochemical testing (Mukhopadhyay, 2019). In diagnostically difficult cases, when the clinical picture of the disease and the histologic features of the examined tumor are not typical and differ from the accepted norm, immunohistochemical reactions are performed, thanks to which it is possible to identify the neuroendocrine differentiation, enabling the classification of neuroendocrine tumors. Currently, three conventional markers of neuroendocrine differentiation (synaptophysin, chromogranin, and CD56) are used in the histopathological diagnostics of neuroendocrine neoplasms, but the test result does not always give an explicit answer to the type of tumor present. This is due to the fact that synaptophysin is sensitive, but not specific enough, chromogranin is highly specific, while its sensitivity is very weak, and CD56 is highly sensitive, but due to its limited specificity it may stain a variety of non-neuroendocrine tumors. Even the use of a combination of these markers on surgical specimens or

cytology specimens gives negative results in 10% to 25% of high-grade neuroendocrine tumors (Hamanaka, 2014, Maleki, 2012, Nicholson, 2002, Travis, 2015, Zheng, 2013). There is therefore a need to find a new neuroendocrine marker that would demonstrate both high sensitivity and specificity. The use of INSM1 in histopathological diagnostics of neuroendocrine neoplasms, which is the only nuclear neuroendocrine marker with high sensitivity and specificity so far, gives hope for a more accurate diagnosis in diagnostically difficult cases. However, the results of studies on the usefulness of INSM1 in the diagnostics of neuroendocrine neoplasms are not conclusive.

Neuroendocrine neoplasms are mainly located in the respiratory system and digestive system, with 25% of primary lung neoplasms being neuroendocrine tumors, 75% of which are mixed neuroendocrine tumors containing also a non-neuroendocrine component (Gustafsson, 2008). These tumors are characterized by very high mortality (Friedberg, 1997, Travis, 1998). Investigating all primary lung neuroendocrine neoplasms on surgical specimens Rooper et al. and Mukhopadhyay et al. obtained high sensitivity of INSM1 (96.4% and 95%) (Mukhopadhyay, 2019, Rooper, 2017). Similar results were also obtained by Doxtader et al. and Viswanathan et al. comparing the sensitivity of INSM1 of primary lung neuroendocrine neoplasms in cytology cell blocks (92%), (92.3%) with surgical specimens (100%), (89.8%) (Doxtader, 2018, Viswanathan, 2019). In contrast, the study completed by Staaf et al. on surgical specimens found much weaker sensitivity of INSM1 (72%), which may be due to the fact that they had a much smaller number of cases than the other authors (Doxtader, 2018, Mukhopadhyay, 2019, Rooper, 2017, Staaf, 2020, Viswanathan, 2019). In turn, Rooper et al. found a significantly higher sensitivity of INSM1 for neuroendocrine lung neoplasms *as a group* compared to each individual neuroendocrine marker (synaptophysin, chromogranin and CD56) (Rooper, 2017). None of the other authors observed statistically significant differences when comparing the sensitivity of INSM1 with the sensitivity of individual neuroendocrine markers (Doxtader, 2018, Mukhopadhyay, 2019, Staaf, 2020, Viswanathan, 2019). In their research, both on cytology specimens and surgical specimens, sensitivity of INSM1 for neuroendocrine lung neoplasms *as a group* was similar to synaptophysin and CD56, and statistically higher than chromogranin (Doxtader, 2018, Mukhopadhyay, 2019, Staaf, 2020. Viswanathan, 2019). Rooper et al. also found a significantly higher sensitivity of INSM1 compared to all three markers (synaptophysin, chromogranin and CD56) treated *as a group* (Rooper, 2017), while in the study by Mukhopadhyay et al. and Kriegsmann et al. sensitivity of INSM1 (95%, 76%) was weaker than the sensitivity of the traditional three neuroendocrine markers treated *as a group* (100%, 97%) (Kriegsmann, 2020, Mukhopadhyay, 2019). The observed differences may be due to the fact that the study by Rooper et al. excluded mixed neuroendocrine tumors from their material, while the other authors also examined mixed primary lung neoplasms with non-neuroendocrine component (Doxtader, 2018, Kriegsmann, 2020, Mukhopadhyay, 2019, Rooper, 2017, Staaf, 2020. Viswanathan, 2019).

Results obtained by Mukhopadhyay et al. regarding sensitivity of INSM1 for small cell lung carcinoma (98%) are comparable to the data reported by Rooper et al. (94.9%),

Rosenbaum et al. (100%) and Fujino et al. in surgical specimens (100%), Doxtader et al. on cytology cell blocks (93%), Nakra et al. and Rodriguez et al. on small biopsies (97%) and on cytology specimens (91%) (Fujino, 2015; Mukhopadhyay, 2019; Nakra, 2019; Rodriguez, 2018; Rooper, 2017; Rosenbaum, 2015). In material derived from carcinoid tumors Mukhopadhyay et al. observed a sensitivity of INSM1 of 98% (Mukhopadhyay, 2019). This result is similar to the result obtained by Fujino et al., Rooper et.al. and Rosenbaum et al. (100%) (Fujino, 2015; Rooper, 2017, Rosenbaum, 2015). In contrast, the sensitivity of INSM1 in relation to large cell neuroendocrine carcinoma described by Mukhopadhyay et al. (75%) was significantly lower than the results obtained by Rooper et al. (91.3%) (Mukhopadhyay, 2019; Rooper, 2017). It is difficult to explain the reason for such a large disparity between the two studies, as both authors used the same clone (A8) from the same company (Santa Cruz). Perhaps the reason may be the slight difference in methodology. The dilution of INSM1 (1:250) used by Mukhopadhyay et al. was weaker than in the Rooper study (1:200). In Mukhopadhyay's study, the antibody was dispensed manually. Mukhopadhyay et al. used Ventana's Optiview detection kit with the optional amplifer, while Rooper et al. used Ventana's UltraView detection kit.

Doxtader et al. observed that the specificity of INSM1 for pulmonary neuroendocrine neoplasms in cytology cell blocks was similar to the specificity of chromogranin (100%) and higher than the specificity of synaptophysin (95%) and CD56 (95%) (Doxtader, 2018). Similarly, Viswanathan et al. in cytology specimens found the same specificity for INSM1, synaptophysin and chromogranin (100%) and weaker specificity for CD56 (Viswanathan, 2019). In surgical specimens, Rooper et al. and Mukhopadhyay et al. observed high values of the specificity of INSM1 (96.2%), (97%), synaptophysin (96.8%), (90%) chromogranin (99.4%), (98%), and CD56 (93.7%), (87%) (Mukhopadhyay, 2019; Rooper, 2017). On the other hand, the specificity of INSM1 (87%) for primary neuroendocrine neoplasms was significantly higher compared to the three traditional neuroendocrine markers *as a group* (61%) (Mukhopadhyay, 2019). In addition, Rooper et al. found an upward trend in the specificity of INSM1 compared to the traditional panel of neuroendocrine markers, but it was not a statistically significant difference (Rooper, 2017).

The second most common site of neuroendocrine neoplasms is the digestive system, with primary neuroendocrine neoplasms having a different digestive tract localization that strongly influences the expression of INSM1. Gonzalez et al. found 100% sensitivity of INSM1 in primary gastroenteropancreatic neuroendocrine neoplasms and 94% sensitivity of INSM1 in metastatic gastroenteropancreatic neuroendocrine neoplasms (Gonzalez, 2019). Similarly, Rodriguez et al. observed 99% sensitivity of INSM1 in neuroendocrine tumors with neuroendocrine features in the digestive tract (Rodriguez, 2018). On the other hand, Rosenbaum et al. found significantly higher expression of INSM1 of midgut gastrointestinal neuroendocrine neoplasms with known metastases compared to those that had not yet metastasized (Rosenbaum, 2015). In turn, McHugh et al. who included the material from the appendix in the primary gastroenteropancreatic neuroendocrine neoplasms, obtained much weaker sensitivity of INSM1 (80.9%) compared to the results of Gonzalez et al. and Rodriguez et al. (Gonzalez, 2019; McHugh, 2020; Rodriguez, 2018). Also, Yang et al. who tested only

primary appendiceal adenocarcinoma of ex-goblet cells obtained very poor sensitivity of INSM1 (62%) showing no differences compared to chromogranin, synaptophysin, and CD56 (Yang, 2019).

The results of tests by three independent teams on pure pancreatic neuroendocrine neoplasms showed 100% sensitivity of INSM1 (Kim, 2020, Takase, 2018, Tanigawa, 2018). Such high sensitivity concerned both cell blocks and surgical specimens, and it was higher than the 3 traditional neuroendocrine markers (synaptophysin 97%, chromogranin 92%, and CD56 85%). However, the disadvantage of INSM1 was the fact that in the case of pancreatic non-neuroendocrine tumors Kim et al. obtained positive staining in pancreatic solid pseudopapillary neoplasms, while Tanigawa et al. in the same tumor type observed no positive staining for INSM1 (Kim, 2020, Tanigawa, 2018). On the other hand, Takase et al. demonstrated the presence of INSM1 in pancreatic non-neuroendocrine tumors (pancreatic ductal adenocarcinoma within adenocarcinoma cell clusters) (Takase, 2018).

A rarer localization of neuroendocrine neoplasms is skin. When studying Merkel cell carcinoma, Lilo et al. observed 100% sensitivity and specificity for INSM1 (Lilo, 2018). Similarly, high sensitivity for INSM1 (93%) in Merkel cell carcinoma was obtained by Rush et al. (Rush, 2018). Also, neuroendocrine neoplasms of the head and neck showed high sensitivity and specificity for INSM1 (99.0%) (97.6%) (Rooper, 2018). Similarly, in the uterine cervix Kuji et al. observed sensitivity for INSM1 (95%), and Roy et al. obtained 100% sensitivity for INSM1 (Kuji, 2017, Roy, 2019).

On the other hand, the results of research on the usefulness of INSM1 in the diagnostics of neuroendocrine neoplasm of the prostate, the urinary bladder, the breast, peripheral neuroblastic tumors or primary central nervous system neoplasms require further study, as they were based on single scientific reports.

CONCLUSION

The results of the analysed studies indicate that INSM1 is a strong nuclear marker of neuroendocrine differentiation with high sensitivity and specificity. In addition, the great advantage of nuclear staining with INSM1 is that it can be performed even on very small material samples containing a few cells, and at the same time it is easy to interpret the results both in surgical specimens and cytology specimens. INSM1 can be very useful in the diagnostics of neuroendocrine lung neoplasms as the first-line marker of neuroendocrine differentiation or in combination with synaptophysin or CD56. INSM1 also appears to be very useful in the diagnostics of pure pancreatic neuroendocrine neoplasms, neuroendocrine neoplasms of the digestive system (excluding tumors from the appendix), Merkel cell carcinomas, neuroendocrine neoplasms of the head and neck and the uterine cervix. The remaining locations of neuroendocrine neoplasms, due to the very small number of cases studied, require further research. Finally, INSM1 cannot be used to differentiate neuroendocrine neoplasms, because it stains both tumor cells in small cell lung carcinoma, large cell neuroendocrine carcinoma, typical carcinoid, atypical carcinoid and mediastinal paraganglioma.

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Analysis of brain tumor emission spectra in patients treated surgically

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ABSTRACT

Spectrophotometry is an instrumental technique that uses energy transitions in molecules for analytical purposes, caused by the absorption of electromagnetic radiation in the ultraviolet, visible or near-infrared range. Cancer cells, during their life or as a result of decay, produce their characteristic metabolites, which are able to absorb electromagnetic radiation in the ultraviolet or infrared range in various ways. The subject of this study was the spectrophotometric analysis of brain tumors that were removed during surgery and the determination of the correlation between the obtained results and the histopathological diagnosis obtained after the surgery. The study involved 50 adult patients, both sexes, treated surgically at the Department of Neurosurgery, Neurotraumatology and Pediatric Neurosurgery, Collegium Medicum im. Ludwik Rydygier in Bydgoszcz, Nicolaus Copernicus University in Toruń, due to a brain tumor. The research confirmed the truth of the assumption that the spectrophotometric evaluation is of clinical importance, which is also consistent with other results of research on spectrophotometry. Spectroscopic examination of the lesions (carried out in parallel with the histopathological examination) may also contribute to a more accurate diagnosis, and further treatment of lesions of a less advanced stage. The results obtained from these studies are expected to be a preliminary step towards the precise determination of the biology of brain tumors and an attempt to use fluorescent techniques in the early diagnosis of neoplastic lesions of the central nervous system.

INTRODUCTION

Brain tumors constitute about 2% of all cancers occurring in the world's population. Every year in Poland, a brain tumor is found in about 4,000 people. Clinical symptoms in patients can be divided into non-specific (associated with the increase in intracranial pressure) and specific (depending on the location of the tumor). Complementing the diagnosis in patients who are suspected of having a brain tumor are imaging examinations such as a computer tomograph (CT) and magnetic resonance imaging (MR). So far, scientific research on brain tumors did not give a full answer to the changes taking place under the influence of the carcinogenesis process. Histopathological examination of the tumor removed during surgery determines the biology of change.

We are currently looking for new methods that are able to dispel any diagnostic doubts. One of these methods may be spectrophotometric analysis of brain tumors. Fluorescence spectroscopy (fluorimetry, spectrofluorimetry) is a kind of electromagnetic radiation spectroscopy where the sample is analyzed using the fluorescence phenomenon induced by light in the visible or ultraviolet radiation range (Dowling, 2001; Saraswathy, 2009). Fluorescent methods of medical diagnosis are based on optical differences in the properties of healthy tissues and tissues changed in the process of cancer. Currently, spectroscopy is used, among others in biochemistry (in studies of the dynamics of enzymatic processes, the mechanism of vision and the course of photosynthesis), in crystallography, forensics (non-invasive examination of evidence, can be helpful in determining the authenticity of artistic works), in medicine (non-invasive *ex vivo* and

en vivo studies on living tissues, for measuring blood glucose, diagnosing tissues, cellular tests, diagnosing cancerous changes and identifying the distribution of pigments in the skin). Over recent years, there has been significant progress in the biomedical sciences. Thanks to scientists, clinicians are provided with newer therapeutic methods and better and better diagnostic tools. Until now, doctors could obtain information about the patient's health status from an interview and a clinical trial – today they have many modern tools at their disposal. Modern diagnostics includes research at various biological levels: cells, tissues, organs and the entire organism. The most complicated apparatus allows you to look at numerous processes at the molecular level. Fluorescence spectroscopy is an example of such techniques. There are many reports in the literature on the use of photoluminescence for testing various tissues.

The apparatus for fluorescence measurements is called fluorimeters or spectrofluorimeters. Cancer cells, during their lifetime or as a result of breakdown, produce their own characteristic metabolites, which in various ways are able to absorb electromagnetic radiation in the ultraviolet or infrared range. This method it can allow to assess the completeness of the surgical procedure and create conditions for the diagnosis of possible recurrence. The subject of this study is the spectrophotometric analysis of brain tumors removed during surgery and the determination of the correlation between the results obtained and the histopathological diagnosis obtained after the surgery. The results will be able to be compared with other studies on a given topic and will contribute to the broadening of knowledge about spectrophotometry, which in the future may allow better planning and more effective treatment and monitoring of the activity of these cancers. It is expected that the results obtained from these studies will be a preliminary step towards the exact determination of brain tumor biology and an attempt to apply fluorescent techniques in the early diagnosis of neoplastic lesions of the CNS.

ASSUMPTIONS AND AIM OF RESEARCH

Despite the high interest in spectrophotometry, there are still not many reports on the use of spectrophotometric methods in the case of brain tumors in the available world literature (Lin, 2001; Kast, 2014; Milad, 2013).

Main thesis: Spectrophotometric analysis of central nervous system tumors is of great clinical significance in determining prognosis, planning treatment and monitoring the activity of these tumors.

In order to verify the main assumption made, specific hypotheses were also put forward: In patients with central nervous system cancers, there is a relationship between emission spectrum and histopathological diagnosis.

MATERIAL AND METHODS

The study involved 50 adult patients, both sexes (27 females, 23 males), treated surgically in the Department of Neurosurgery, Neurotraumatology and Children's Neurosurgery, University Hospital No. 1 in Bydgoszcz due to solid brain cancer, from January 2013 to March 2015. A total of 57 fragments of brain tumors were recovered. The patient's qualifying diagnosis for the study was based on the interview, subject

examination and neuroimaging. After surgery, histopathological diagnosis of brain tumor was established. Patients with impaired consciousness, making it impossible to express informed consent to participate in the research, were excluded from the study.

All persons qualified for the study (after having provided information about him) gave their written consent to participate in it. The research included spectrophotometric analysis of a brain tumor fragment taken during surgery. The material to be tested after sampling was placed in special containers with 0.9% NaCl, and then subjected to deep-freezing (temp about -30 degrees Celsius) (Richter, 2011; Moritz, 2012). Next, the research material was defrosted in accordance with GLP (Good Laboratory Practice), transferred to cuvettes and subjected to spectrophotometric analysis. In spectroscopic studies, measurements of fluorescence emission spectra were made. Excitation and fluorescence emission spectra were measured on an F-7000 spectrofluorimeter (Hitachi, Japan). Measurements of stationary autofluorescence spectra of brain tumor sections were made using a spectrofluorimeter for wavelengths of excitation 210 nm to 390 nm (measured every 20 nm – emission spectrum) and for light observations of 330 nm to 390 nm (measured at 20 nm – spectra excitation). The results in the form of diagrams were developed in the Origin 8. The statistical analysis of the collected material was carried out using the Statistica 12.5 package. Descriptive statistics and distribution characteristics were used to describe the variables. The intraoperatively collected material from the brain tumor was subjected to histopathological examination (histological type and tumor grade) in the Department of Clinical Pathomorphology of Collegium Medicum. Ludwika Rydygiera in Bydgoszcz of the Nicolaus Copernicus University in Toruń.

RESULTS – ANALYSIS OF THE EMISSION

In the examined group of respondents, the most prevalent were those with CNS metastasis and with grade IV malignancy according to WHO.

The division in terms of the degree of malignancy was also made in a general way: malignant tumor (WHO III or IV), which was diagnosed in 25 tumor fragments, non-malignant (WHO I or II) in 13 tumor fragments, in 17 patients were found metastasis to the CNS.

The type of cancer was estimated in four general groups of intracranial tumors: gliomas, meningiomas, metastatic tumors and others.

The results of measurements of stationary spectroscopy were systematized depending on the histopathological diagnosis. The study material was divided into 3 groups: benign and intermediate grade malignant tumors, primary malignant tumors and metastatic tumors. Emission spectra were tested at light excitation in the wavelength range 250 nm, 270 nm, 290 nm, 310 nm, 330 nm, 350 nm, 370 nm, 390 nm.

4.2.1. EVALUATION OF BENINGN(WHO I) AND INTERMEDIATE (WHO II AND III) EMISSION LEVELS OF CNS TUMORS, MALIGNANT CNS TUMORS AND CNS METASTASES

4.2.1.1. EMISSION SPECTRUM WHEN EXCITED WITH 250 NM LIGHT OF CNS TUMORS

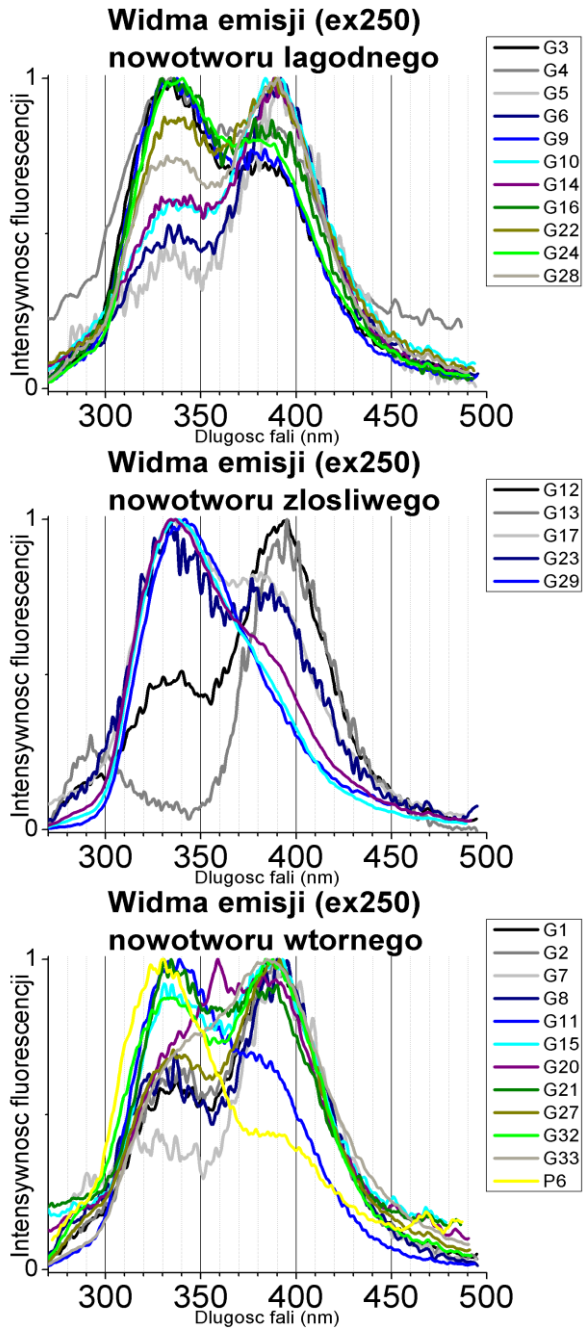

Figure 1. Normalized emission spectra at 250 nm excitation of benign (WHO I) and intermediate grade of malignancy (WHO II and III) of CNS tumors, CNS malignant tumors and CNS metastases

4.2.1.2 EMISSION SPECTRUM WHEN EXCITED WITH THE 270 NM WORLD OF CNS TUMORS

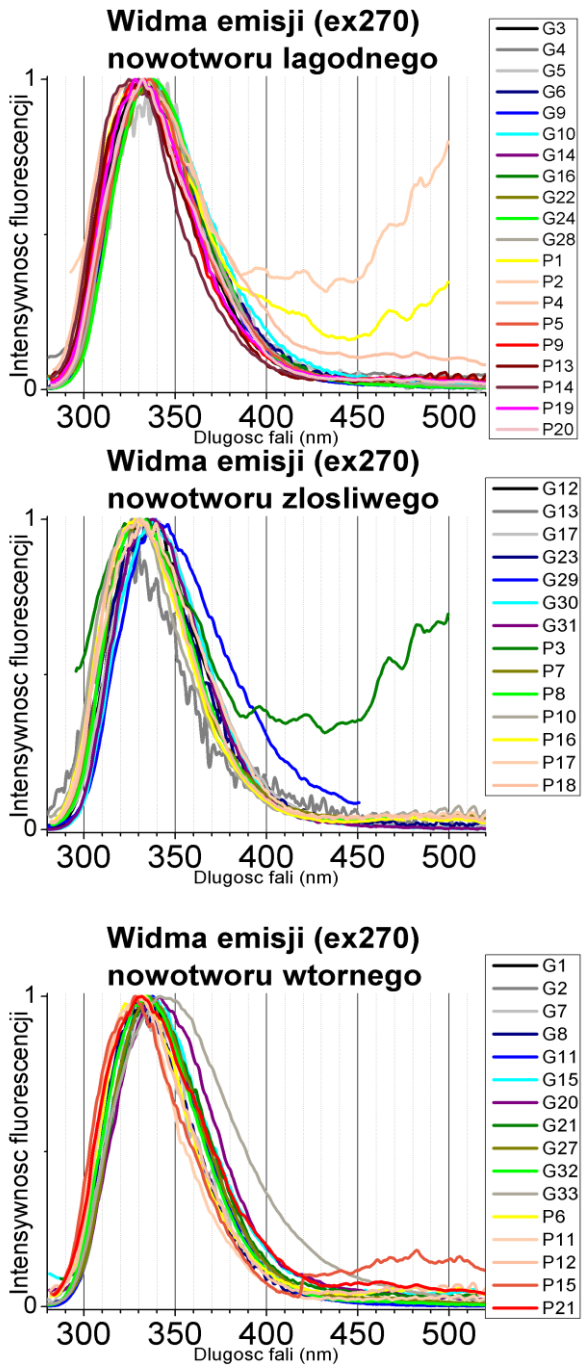

Figure 2. Normalized emission spectra at 270 nm excitation of benign (WHO I) and intermediate grade of malignancy (WHO II and III) of CNS tumors, CNS malignant tumors and CNS metastatic tumors

4.2.1.3 EMISSION SPECTRUM WHEN EXCITED WITH 290 NM LIGHT OF CNS TUMORS

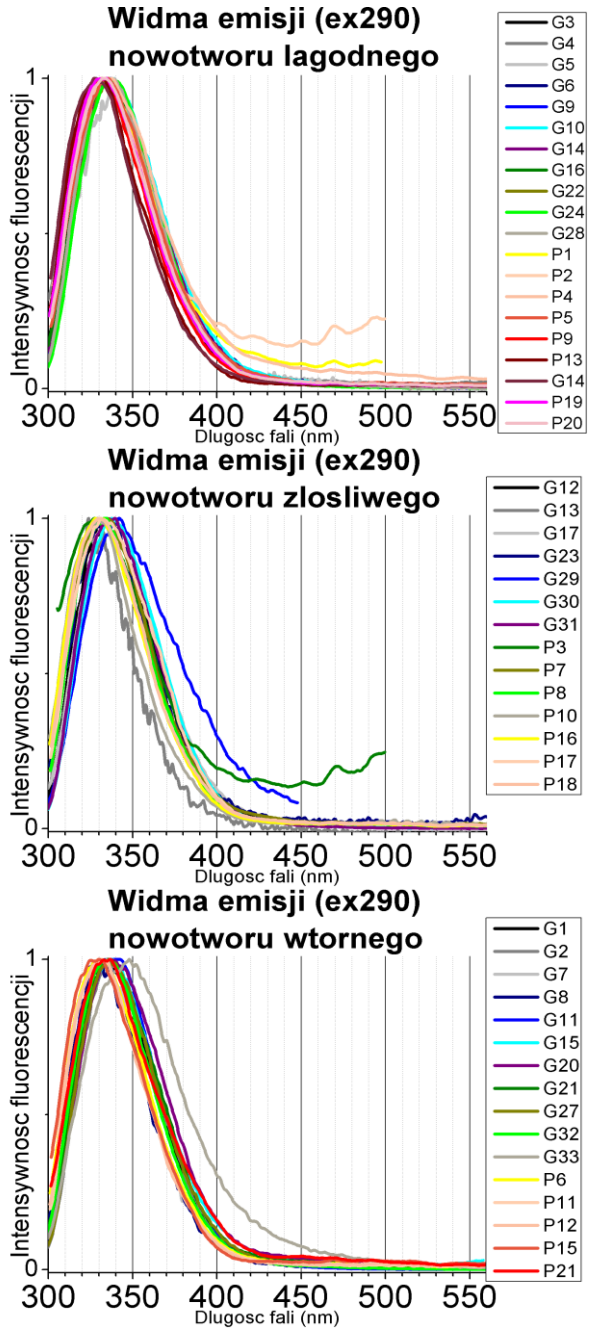

Figure 3. Normalized emission spectra at 290 nm induction of benign (WHO I) and intermediate grade of malignancy (WHO II and III) of CNS tumors, CNS malignant tumors and CNS metastatic tumors

4.2.1.4 EMISSION SPECTRUM WHEN EXCITED WITH 310 NM LIGHT OF CNS TUMORS

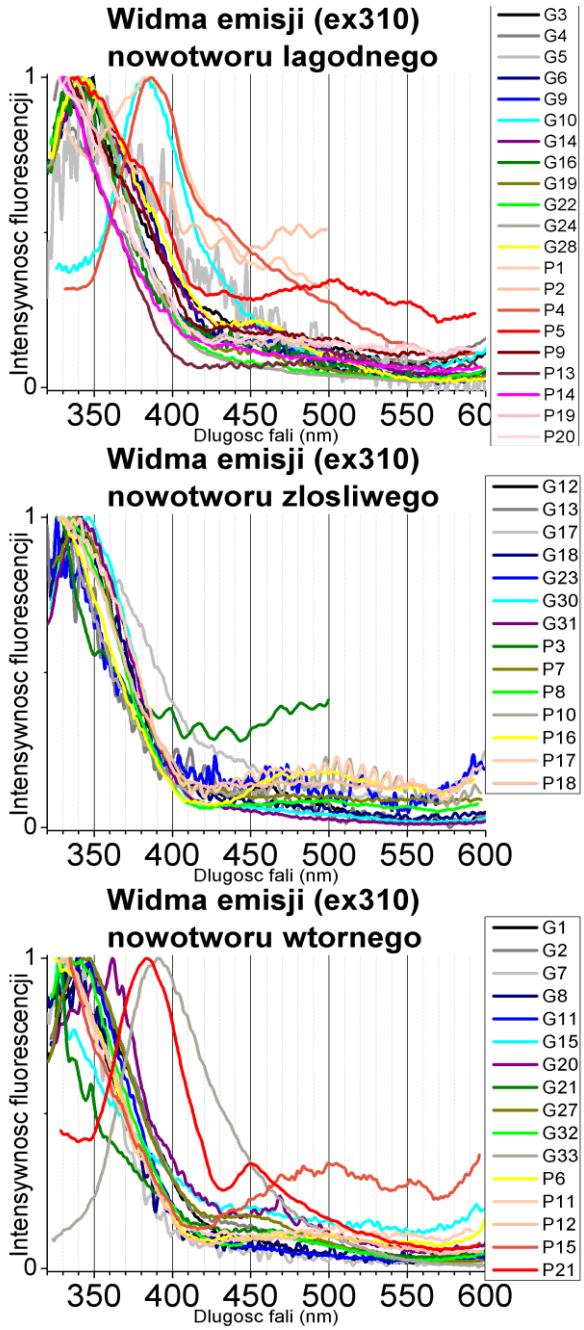

Figure 4. Normalized emission spectra at 310 nm induction of benign (WHO I) and intermediate grade of malignancy (WHO II and III) of CNS tumors, CNS malignant tumors and CNS metastatic tumors

4.2.1.5 EMISSION SPECTRUM WHEN EXCITED WITH 330 NM LIGHT OF CNS TUMORS

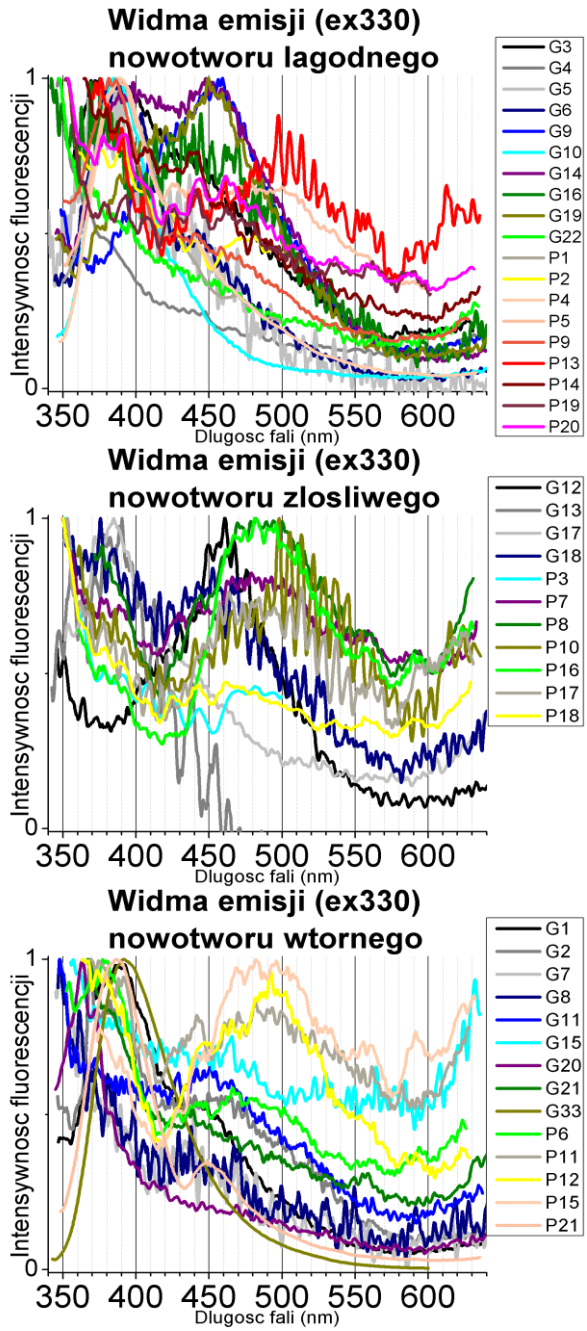

Figure 5. Normalized emission spectra at 330 nm induction of benign (WHO I) and intermediate grade of malignancy (WHO II and III) of CNS tumors, CNS malignant tumors and CNS metastatic tumors

4.2.1.6 EMISSION SPECTRUM WHEN EXCITED WITH 350 NM LIGHT OF CNS TUMORS

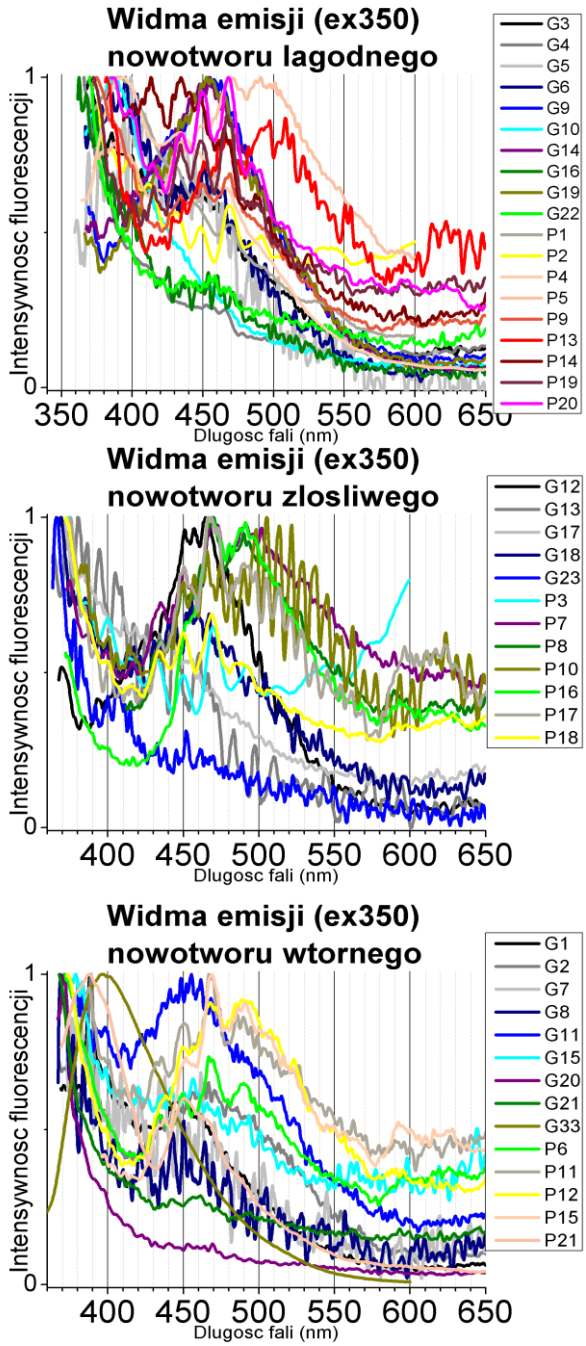

Figure 6. Normalized emission spectra at 350 nm induction of benign (WHO I) and intermediate grade of malignancy (WHO II and III) of CNS tumors, CNS malignant tumors and CNS metastatic tumors

4.2.1.7 EMISSION SPECTRUM WHEN EXCITED WITH 370 NM LIGHT OF CNS TUMORS

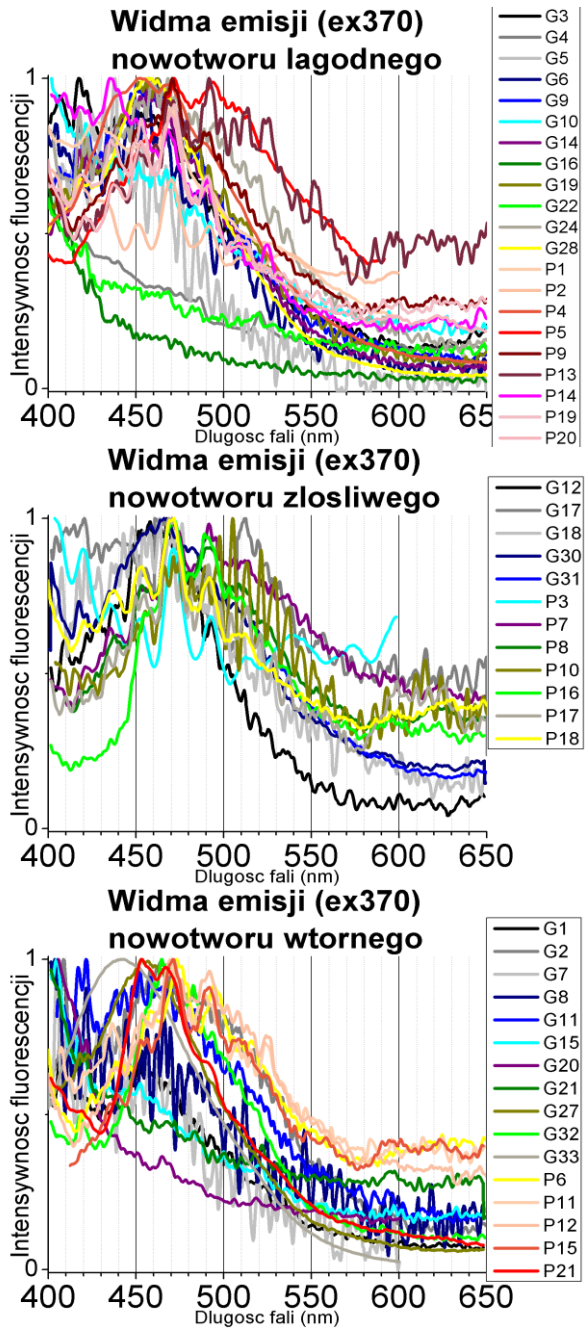

Figure 7. Normalized emission spectra at 370 nm induction of benign (WHO I) and intermediate grade of malignancy (WHO II and III) of CNS tumors, CNS malignant tumors and CNS metastatic tumors

4.2.1.8 EMISSION SPECTRUM WHEN EXCITED WITH 390 NM LIGHT OF CNS TUMORS

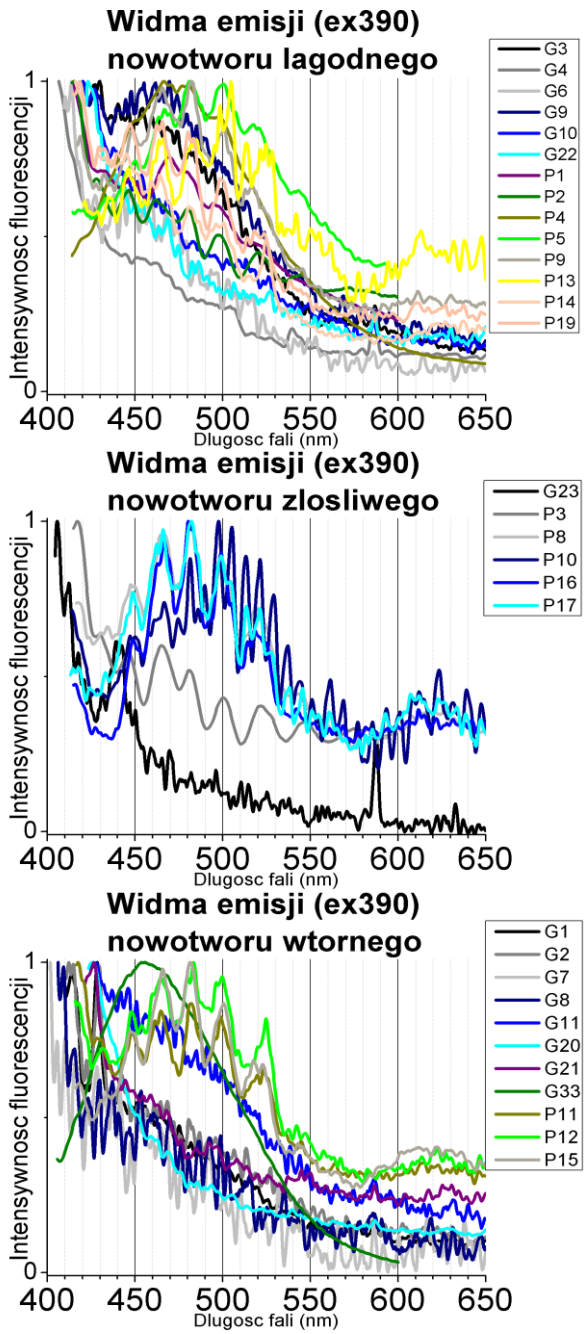

Figure 8. Normalized emission spectra at 390 nm induction of benign (WHO I) and intermediate grade of malignancy (WHO II and III) of CNS tumors, CNS malignant tumors and CNS metastatic tumors

Fluorescence emission spectra recorded at 250 nm light excitation are shown in figure 1. For all CNS tumor types, two major maxima can be distinguished – at 335 nm and 390 nm.

In contrast, in figure 2, showing emission spectra for excitation with 270 nm light, we only see one maximum at 335 nm.

Similar results can be seen in figure 3 showing the emission spectrum when excited with 290 nm light.

All results of the Pearson correlation analysis were at the p-level close to zero ($p < 0.05$), i.e. all results are statistically significant at $\alpha = 0.05$.

The emission spectra were statistically analyzed at excitation with 290 nm (ex290). The length of ex290 was chosen because from the correlation analysis it can be concluded that there are large differences between benign and malignant tumor and between malignant and secondary cancer. Such a combination would allow to show differences between all types of tumors (see fig. 3)

For the statistical analysis of the emission spectra at excitation, the length at which the band reaches the maximum fluorescence was chosen because the spectra are characterized by a single maximum – in relation to which the maxima ratio can not be compared. The lengths at maximum fluorescence, broken down by tumor group, are listed in the table (tab. 1).

Table 1. Table containing list of length at maximum fluorescence for emission spectra at excitation 290 nm with division into tumor groups: 1 – secondary cancer, 2 – malignant tumor, 3 – benign tumor

Patient	ex290 max	
	group	length at max fluorescence
G1	1	335,6
G2	1	335,2
G7	1	333,8
G8	1	330,8
G11	1	342,8
G15	1	338,2
G20	1	336,4
G21	1	335,6
G27	1	336,6
G32	1	336,4
G33	1	348
P6	1	331,6

P11	1	331,4
P12	1	329,6
P15	1	330,2
P21	1	336,6
G12	2	333,6
G13	2	323,8
G17	2	337,4
G23	2	332,6
G29	2	342,2
G30	2	337,6
G31	2	339,4
P3	2	328
P7	2	334,8
P8	2	333,2
P10	2	329,4
P16	2	328
P17	2	331,2
P18	2	330,2
G3	3	331
G4	3	328,2
G5	3	339,4
G6	3	334
G9	3	337,6
G10	3	338,4
G14	3	337,8
G16	3	334,4
G22	3	337,8
G24	3	335,2
G28	3	334,6

P1	3	331,2
P2	3	330,8
P4	3	338
P5	3	333,8
P9	3	332,2
P13	3	332,6
P14	3	327,2
P19	3	334,4
P20	3	332,8

Next, the consistency of the distributions in the three groups with the normal distribution were compared (table 2, 3, 4). It was assumed that $\alpha = 0.05$ and for assumed hypotheses H0 – the distribution is with normal distribution and H1 – the distribution is not a normal distribution

Table 2. Compatibility of distribution in group 1 – secondary cancer (metastasis)

	group = 1(ex290 max)		
Variable	N	W	P
length at max fluorescence	16	0,886237	0,048586

Table 3. Compatibility of the distribution in group 2 – malignant tumor

	group = 1 (ex290 max)		
Variable	N	W	P
length at max fluorescence	16	0,886237	0,048586

Table 4. Compatibility of distribution in group 3 – benign tumor

	group = 3 (ex290 max)		
Variable	N	W	P
length at max fluorescence	20	0,956355	0,473848

STATISTICAL ANALYSIS OF MAXIMUM SPECTRUM FLUORESCENCE

Only for the first group – neoplastic secondary distribution is not a normal distribution, because $p < 0.05$. Therefore, the nonparametric Kruskal-Wallis test was chosen for further analysis. For the test, the significance level $\alpha = 0.05$ and H0 were assumed – the distributions are the same, and the H1-distributions differ. The results are shown in the table below (tab. 5).

Table 5. Kruskal-Wallis nonparametric test result table for the length at which the maximum spectrum fluorescence is found for the groups: 1 – secondary cancer, 2 – malignant tumor, 3 – benign tumor

	ANOVA rang Kruskala-Wallisa; length at max fluorescence (ex290 max) Independent (grouping) variable: group Test Kruskala-Wallisa: H (2, N = 50) = 1,984965 p = 0,3707			
Dependent: · length at max fluorescence	Kod	N ważnych	Suma Rang	Średnia Ranga
1	1	16	461,0000	28,81250
2	2	14	298,5000	21,32143
3	3	20	515,5000	25,77500

The critical value of the Kruskal-Wallis test for two degrees of freedom is 5,991,464. The result of the Kruskal-Wallis test H = 1.984965 and p = 0.3707 show that the groups do not differ statistically with each other because the test result H < Critical value and p > 0.05.

DISCUSSION

Investigations of autofluorescence spectroscopy are a method that exploits the ability of endogenous fluorophores to fluoresce light of a different length than an excitation light. There are numerous molecules capable of autofluorescence in the tissues of living organisms.

In addition to the maximum at a length of about 335 nm, we observe for some cases (regardless of the type of cancer), an additional band with a maximum at 450 nm and/or 500 nm. It is intriguing that for cases G10, P2 and P4 with benign tumor and G33 and P21 with secondary cancer (metastasis), there was a shift in the maximum from 335 nm to about 385 nm. In addition, one can observe a certain tendency (although this is not the rule) – this applies to the second band, where the maximum is at 450 nm and/or 500 nm. For cases with benign tumors, most spectra have a maximum at 450 nm, and with malignant tumors, most spectra have a maximum at 500 nm. In graph 5 a shift in the maximum (for most spectra) from 335 nm to 385 nm was observed. In addition, an increase in fluorescence was recorded within the 450-550 nm band in relation to the maximum at 385 nm. Also here, there was a tendency to increase the maximum at 450 nm for benign tumors, and the maximum at 500 nm is more characteristic of malignant tumors.

Secondary tumors (metastases) are a mixture of spectra with a predominance of maxima at 450 nm or 500 nm. Unfortunately, also in this case it is not a rule. The samples marked as G10, P2 and P4 with benign tumor as well as G33 and P21 with secondary cancer still diverge from the others. When fluorescence was excited with 350 nm light in the 6,370 nm graph on the 7 and 390 nm graphs in Figure 8, the same trends were also observed – the maxima are the same as for the spectra shown on Figure 5. For benign malignancy tumors maxima are observed at the 450 nm, for malignant tumors at 500 nm, and for secondary cancers between 450 nm and 500 nm.

In the conducted stationary studies, numerous fluorescence spectra were obtained, indicating the presence of endogenous chromophores in the analyzed biological material. By excitation with light lengths of 250-290 nm, the amino acids contained in proteins are primarily induced. They are phenylalanine (Phe), tyrosine (Tyr), tryptophan (Trp). They emit fluorescence in the range of 250-450 nm with maxima found at 282 nm, 303 nm and 348 nm (Richards-Kortum, 1996). Therefore, it is likely that the spectra of light excitation excited at 250 nm, 270 nm, 290 nm and 310 nm show a band with a maximum of 330-340 nm corresponding to the fluorescence of amino acids (above all to tryptophan). The spectra also show a band with a maximum of about 390 nm. According to the data presented, it may be pyridoxine or/and collagen (Skoch, 2008). The fluorescence of these chromophores is probably excited by 330 nm light, as can be seen in graph 13 showing the excitation spectra at 390 nm light emission. The next significant maxima in the emission spectra are in the 450 nm and 500 nm positions. According to the data for fluorescence with a wavelength of 450 nm, nicotinamide, NADPH, which are excited by successive light with a length of 360 nm and 366 nm, may be responsible (Trehin, 2006). 330-340 nm. Fluorescences with a length of 500 nm are observed for elastin, which has maxima of excitations 350 nm, 410 nm and 450 nm (Senada, 2012; Teale, 1956; Pu, 2012).

The stationary spectra of individual cases differed among themselves, which suggests a just attempt to use this method for diagnostic purposes. It was suggested to divide the examined material into benign, malignant and metastatic CNS tumors. In the studied groups, there were often cases whose spectra differed significantly from the others. Unfortunately, the spectra obtained in each group were not completely consistent. Large differences in the shape of spectra within individual groups have been shown. These discrepancies may be due to the fact that in addition to the type of cancer, other factors such as medicines used, the exact location of biological material, and co-morbidity may also affect tissue fluorescence. Therefore, to make further divisions, which in a more systematic way could indicate differences in fluorescence between groups, it is necessary to increase the number of samples tested to be able to correctly distribute cases.

Researchers from China, Yan Zhou (Zhou, 2012), studied the brain and brain tumors unharmed. They had at their disposal three different types of tissues: normal, malignant tumor, benign tumor. Using fluorescence excitation of 300 nm, they distinguished malignant tumor from benign tumor and healthy tissue. Tissues covered by benign or malignant tumor lesions are characterized by a band with one maximum at a length of about 340 nm and a small fluorescence in the 400-550 nm range. These data correspond perfectly with those obtained in this work.

The obtained test results and their verification with the results of other scientists dealing with fluorescence spectroscopy allow to confirm the main assumption of this study that the assessment of excitation spectra, emission spectra and fluorescence decay time in patients with central nervous system tumors is of great clinical significance in determining prognosis, planning and monitoring the activity of these cancers (Fillipi, 2001; Geiger, 2011; Hayashida, 2006). Planning and appropriate treatment selection in case of intracranial tumors is very complex and often determines further prognosis.

The photodynamic method is distinguished by the relatively low invasiveness of the surgery itself and, at the same time, by high sensitivity and resolution in comparison to traditional diagnostic methods such as: nuclear magnetic resonance, computed tomography, ultrasound (Hongwei, 2014; Petrovsky, 2003; Toms, 2005). Hence, spectrophotometric analysis of brain tumors may be extremely useful. This will allow to make even more accurate diagnosis and to start the appropriate therapy (Yaroslavsky, 2002; Yinghua, 2010; Bergner, 2012).

CONCLUSIONS

Not without significance is the fact that the use of spectroscopic methods will allow very fast analysis of the material collected. The physician performing the examination will be informed in a short time whether the material collected by him shows the spectral characteristics of the changed cells and whether the material should be taken again from another place. The results of the study, limited by the small size of the group, do not allow for binding conclusions, but they confirm the usefulness of the method in neurooncological cases. Fluorescent techniques will not displace pathomorphological analysis in the near future.

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A new approach to *in vitro* micronucleus evaluation based on fluorescence staining

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ABSTRACT

When engineering a new biocompatible material, careful consideration should be paid to its biological evaluation. Therefore, proper *in vitro* tests should be performed prior to its implantation. Genotoxicity assay, one of the methods to assess the material's biocompatibility, can be distinguished herein. By this, one can easily investigate the material impact on genetic damage.

Genotoxicity, based on micronucleus (MN) evaluation, can detect both clastogenic and aneugenic effects, which is the main advantage of this method. However, well-established guidelines and protocols are not available up to date. To overcome this limitation, we propose a step-by-step, easy-to-handle procedure for mutagenic characterization. Moreover, a new visualization method for counting MN based on the fluorescence staining will also be presented. With F-actin and nuclei staining, it was possible to detect separate cells consisting of various extra-nuclear bodies formed due to impaired mitosis. Genotoxicity was evaluated on the cells incubated with extracts of the three-dimensionally (3D) printed scaffolds. By this, we intend to standardize the staining approach, which will consequently lead to data deviation elimination.

INTRODUCTION

Nowadays, a tissue engineering, introducing tissue-specific products, is widely used to prompt organ regeneration (Chlanda, 2021; Narayan, 2016). Clinical implementation of tailored scaffolds is growing in popularity, limiting the need to use xenografts, allografts, or autografts to replace the damaged tissue fragments (Walejewska, 2019). However, while shaping scaffolds to match the criteria of replaced tissue, several factors should be taken into consideration such as immunogenicity and genotoxicity. Polymeric-based scaffolds are known to degrade over the time to be replaced by newly formed tissue. Therefore, the potential impact of degradation by-products, and initial material components itself should be evaluated in terms of biocompatibility.

Several *in vitro* methods may be distinguished to assess biocompatibility of fabricated scaffolds, namely cytotoxicity, genotoxicity, and hemolysis test to test the interaction with human blood (Ramakrishna, 2015).

However, despite of above-stated approaches, genotoxicity represents a major concern when designing new biomaterials, as it focuses on detecting the effect of material composition on DNA damage. Thus, to ensure patients safety, the proper biological *in vitro* evaluations, with strict and well-established protocols need to be developed prior to its implantation (Bojar, 2015). Various *in vitro* and *in vivo* genotoxicity assays have been already comprehensively described in the literature (Hayashi, 2016), and the most

frequently used, approved by OECD are : (i) the Comet Assay (CA), (ii) the Ames one, and (iii) the micronucleus formation evaluation.

The Comet Assay enable to observe DNA fragmentation caused by electrophoretic separation. During the experiment, the cells are immobilized in agarose gel. However, it has nothing in common with traditional process of electrophoresis. In case of CA the agarose is placed on the microscope slide, which is later subjected to alkaline lysis. In such a matter, DNA is being released from the cell nucleus. The use of high ionic strength lysis buffer promotes the dissociation of proteins from DNA. After this stage, the electrophoresis is carried out under neutral conditions. In standard TBE buffer, or either in alkaline conditions. After the separation step, the DNA is subjected to fluorescent or silver salt staining, and consequently, the resulting image is analyzed under fluorescence microscope (Tice, 2000).

As an example, Seyedmajidi and co-workers utilized one of the genotoxicity tests to evaluate the DNA damage of nanohydroxyapatite/bioactive glass (HA/BG) and fluorapatite/bioactive glass (FA/BG) tissue scaffolds used in bone regeneration (Seyedmajidi, 2018).The comet assay results indicated that the tail elongation and proportionally DNA damage increased in a dose/time-dependent fashion with biomaterials extract exposure. In another study, comet assay was also used to measure the DNA damage in osteoblastic and fibroblastic cells *in vitro* in contact with Biosilicate scaffolds (Kido, 2013). The results revealed that DNA strand breaks in osteoblastic cells were not induced at any evaluation period. Although this test is compatible with many cell lines, is fast and inexpensive, it has some drawbacks, such as rapid repair of short primary DNA lesions often results in false-positive results. Moreover, the internal reference is also required to eliminate the experimental variation during the electrophoresis. Therefore, the use of the comet method in genotoxicity assays is criticised (Brendler-Schwaab, 2005).

Thus, to test the genotoxicity of tissue-engineered scaffolds, the most common Ames test might be also used (de Mello Silva Oliveira, 2016; Kohl, 2020). It utilizes several strains of the bacterium *Salmonella typhimurim* to detect the mutagenic potential of compounds causing genetic damage in their cells (Hsu, 2016). In order to perform the standard test, a mixture of so-called "surface agar" (top one), supplemented with trace amounts of histidine and biotin is prepared. The mixture consists of cells of the testes strain, refreshed after overnight incubation, the exanimated compound, and alternatively the microsomal fraction of rat's liver. The as prepared slurry is casted on the plates containing minimal agar, supplemented with mineral salts and glucose solution. Subsequently, all plates are incubated at 37°C up to 48 hours. The grown bacterial colonies on the "surface agar" are then calculated and compared to the control without mutagenic compound. The increase of the bacterial colony indicates the mutagenic potential of the surface (Mortelmans, 2000). However, the Ames test is often not suitable in the case of nanomaterials, as the bacterias' size is comparable with the nanoparticles of ceramic materials (i.e., hydroxyapatite, tricalcium phosphate). Additionally, the bacteria cell wall is different from the mammalian cell membrane, which may cause problems with gene mutation testing or leading to misunderstanding. The Ames test is well-established for the initial examination of the mutagenic potential of the material's

compounds but it is mainly limited by differences in the metabolic pathways of bacteria and mammalian cells. This may result in erroneous classification of the test compound (Nesslany, 2017). However, it is recommended to combine Ames test with a method, such as the micronucleus test (MN), that allows the measurement of particular structural chromosomal abnormalities (Lorge, 2006).

MN evaluation allows to detect activity that induces structural aberration of chromosomes (clastogenicity), as well as anaueginicity, which is defined as activity to induce numerical aberration of chromosome (Fenech, 1986). The MN-based approach involves the assessment of micronuclei formation during disturbed mitosis. Micronuclei are generally round or oval structures, containing chromatin surrounded by a nuclear membrane with no connections to the cell nucleus. They are formed from centric or acentric fragments of damaged chromosomes or even whole chromosomes that were not involved in the cell division spindle (Fenech, 2006, Richardson, 2016, Rudnicka, 2013).

Despite many protocols that can be found in the literature, very little effort has been made to establish proper guidelines for genotoxicity investigation of tissue-engineered scaffolds using micronucleus assay. Thus, here we aimed to develop/optimize a protocol for *in vitro* micronucleus assessment, combined with a simple visualization method, which allows for micronucleus counting.

MATERIALS AND METHODS

CHO CELL LINE GROWTH

The Chinese hamster ovary subclon K-1 (CHO K1) cell line was obtained from ATCC, as ISO 10993-3 and OECD 487 guidelines recommends to use it during genotoxicity evaluation (Aardema, 2006). Cells were grown in Ham's F-12 Nutrient Mix, GlutaMAX medium (Gibco) supplemented with 10% (v/v) Fetal Bovine Serum (Gibco) and 1% (v/v) Antibiotic Antimycotic Solution (Sigma Aldrich), in 5% CO_2 atmosphere at 37 ±1°C. Culture flasks were regularly inspected, and culture medium was changed every 2-3 days, depending on cell confluency. CHO K1 cells at 80%-90% confluency were washed with Dulbecco's Phosphate Buffered Saline (DPBS, calcium and magnesium free), and then harvested with Trypsin-EDTA (0.25%) (Gipco).

EXTRACT PREPARATION

3D-printed scaffolds, obtained from Faculty of Materials Science and Engineering, Warsaw University of Technology were used to prepare extracts. Extracting conditions were selected based on *ISO 10993-12 Biological evaluation of medical devices* (Standardization, 2012). In brief, extracts were prepared according to ISO standards – 0.2 g PCL-based scaffolds per 1 ml of eluate. Extraction was carried out in F-12 medium without FBS addition (0,9 ml medium for total 1 ml extract), for 24 h using orbital shaker at 37°C. After incubation, specimens were removed and FBS was subsequently added (0,1 ml FBS for total 1 ml extract).

CYTOKINESIS-BLOCK MICRONUCLEUS ASSAY (CBMN)

The genotoxic activity of scaffolding material was assessed by a reference micronucleus assay using CHO cells. A modified procedure was followed based on the *OECD* and *ISO 10993* guidelines (Co-operation, 2016) (ISO, 2012).

Our procedure was divided into 4 steps, which were carried out under the same conditions: 5% CO2 atmosphere, a temperature of 37°C and 95% humidity (tab.1).

1st Step

CHO cell line was seeded into 6 well culture plates at a concentration of 0.3×10^6 per well with a complete F-12 medium, and incubated for 24 hours. After the initial incubation, basal medium was exchanged with the treatment ones. The test was carried out parallel with and without metabolic activation.

2nd Step

The genotoxic properties of different chemical compounds were revealed after metabolic activation to imitate the conditions observed in higher organisms. In the experiment, tested extract and positive control requiring metabolic activation were preincubated (at 37 °C and 5% CO_2 atmosphere) for 1h, with S9 fraction (derived from rat liver) (Sigma-Aldrich), which was stored at -70°C prior to use.

As positive controls, two different clastrogens. Mitomycin C (MMC) from *Streptomyces caespitosus* (Sigma-Aldrich) (S9-), and cyclophosphamide (CP) (Sigma-Aldrich) requiring metabolic activation (S9+) were used. MMC and CP were dissolved in water and stored as stock solutions at a concentration of 1.0 mg/ml at -20°C.

3rd Step

The next step involved blocking the contractile microfilaments formation. Thus, medium was exchanged for a one that was supplemented with Cytochalasin B from Drechslera dematioidea (Cyt-B) (Sigma-Aldrich). The Cyt-B was previously dissolved in DMSO according to the manufacture's instruction, to achieve a concentration of 1 mg/ml in each stock, and stored at -20°C.

4th Step

After the last 24 h incubation, cells from four groups were detached by exposure to trypsin and resuspended to amount of 5×10^4 cells/ml. Fixation was provided in 10% Neutral buffered formalin (NBF) (Sigma Aldrich) mixed with F-12 medium (1:1 v/v) for 30 min. Subsequently, ActinGreen™ 488 ReadyProbes™ Reagent (Invitrogen) (2 drops/ml – 30 min) and NucBlue™ Fixed Cell ReadyProbes™ Reagent (2 drops/ml – 5 min) were added. The staining process was carried out in the dark, in shaking conditions. Once finished, cells were washed, and staining mixture was exchanged with PBS. For further analysis, samples were immobilised by centrifugation in CellSpin II. Cell suspension (100 µ) l was pipetted into each of the sample cytospine funnel, centrifuged at 1000 x g for 2 min, and then air-dried 2 h. Afterwards, 1 drop of ProLong® Gold Mountant (Invitrogen) was added onto glass slide, covered with coverslip, and leave for cure in the dark up to 4 h.

5th Step

As a last step, embedded cells were ready to examine under a fluorescence microscope. Nuclear position and its damaged forms were visualized within cytoplasmatic actin boundaries. To evaluate whether the material is genotoxic micronucleus in binucleated cells were counted. The result of the assessment is the percentage ratio of binucleated cells with micronuclei to binucleated cells without them (BN with MN in 1000 BN) x 100% (Fowler, 2010). If more cells than positive control have intracellular damages (generate micronuclei), this indicates a toxic effect of the examined material.

THE TYPES OF NUCLEAR FORMATIONS

During analysis of the cells, many variants of disrupted nuclei division was observed, but not all of them result from the test. It is therefore essential to verify cellular responses to mitogens. The correct micronuclei should not be linked or connected with any of the main nuclei. However, necrotic apoptotic structures can often be observed as with nuclear bundles and bridges (fig. 1).

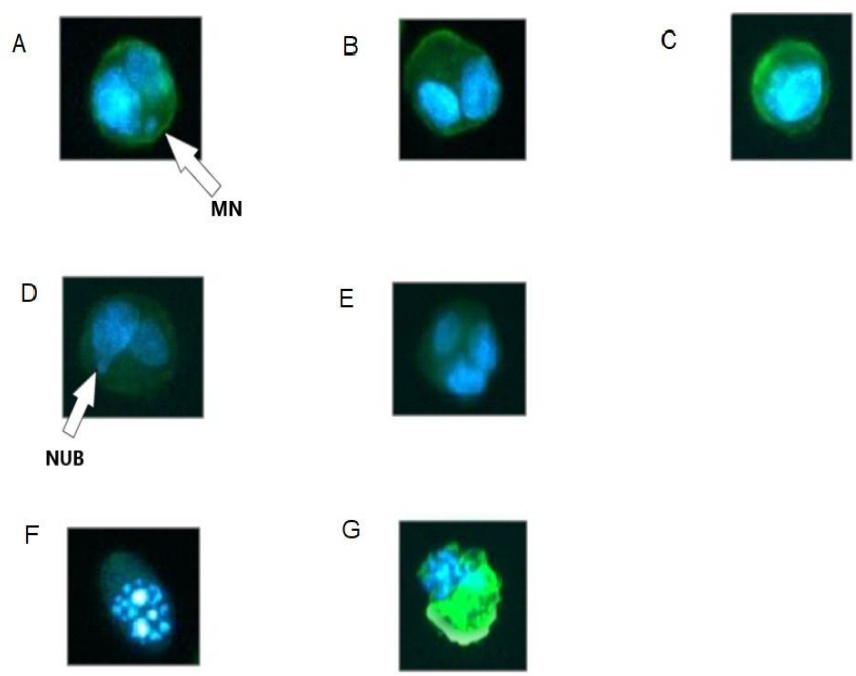

Figure 1. DNA damage in the CHO-K1 cells Cytokinesis-Block Micronucleus Assay (CBMN), nucleus and its damaged forms (blue) inside shaped by F-actin cells (green). A) micronucleus (MN) within binucleated (BN) cell, B) binucleated cell with two separate nuclei, C) Normal, single-nucleus cell (MONO), D) binucleated cell with nuclear bud (NUB)/bridge, E) three nuclei cell, F) apoptotic and G) necrotic cell can by scored to obtain total cell death and their types

CONCLUSION

The main advantage of the micronucleus test is the reduction of statistical error by counting a large number of cells in a single experiment. Thus, the micronucleus test has been constantly trying to be improved up to this day. Like any test it has many limitations related to the correct interpretation of nuclear forms. The use of a DNA specific stain can eliminate some of the artifacts associated with using a non-DNA specific stain. Flow cytometry experiments, or automated counting systems are proposed (Rodrigues, 2018; Schunck, 2004; Seager, 2014). Limitations may arise from strictly defined algorithms, cell suspension densities and fixatives used. Despite the high throughput of counted cells, false positive results often occur by up to 30-50% compared to the classical method (Decordier, 2008; Rossnerova, 2011). The presented protocol, divided into five steps give the clear situation about the testing conditions. From the preparation of extracts, to the selection of optimised clastrogens and a simple and relatively cheap method of micronucleus visualisation. Furthermore, a simple optical method allows direct visualization of all required events (e.g. MONO, BN and POLY nucleic cells, MN, nucleoplasmatic bridges, nuclears buds and apoptotic/necrotic cells) and define the causes and frequency of specific DNA damage occurring during the G1 phase of mitosis.

Table 1 Step by step protocol of Cytokinesis-Block Micronucleus Assay (CBMN)

		Without metabolic activation		Metabolic activation
Step I	Preincubation	24 h preincubated CHO cells 0.3 x 10^6 per well		23 h preincubated CHO cells 0.3 x 10^6 per well
Step II	Incubation with positive control and investigated extract	0,5 % Mitomycin C (v/v) in complete F-12 medium Investigated extract		1 h incubation with 2 % S9 (v/v) 6 % Cyclophosphamide (v/v) in complete F-12 medium investigated extract
		24 incubation		
Step III	Inhibition of actin polymerization	4% Cytochalasin B (v/v) in complete F-12 medium 24 h incubation		
Step IV	Fixation and staining	1. 2. 3. 4. 5. 6. 7.	30’ 30’ 5’ 2’ 2 h 4 h	4% Formalin: F-12 medium (1:1) ActinGreen™ 488 ReadyProbes™ Reagent 2 drops/ml NucBlue™ Fixed Cell ReadyProbes™ Reagent 2 drops/ml Wash cells with PBS 2-3 times Centrifuge 4x10^3 cells per funel in cytospine centrifuge at 1000 x g Air dry glass slides Add 1 drop of ProLong® Gold reagent onto glass slide, place coverslip onto slides, leave for cure in the dark
Step V	Visualization			

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Molecular basis of Marfan syndrome – short story about fibrillin-1

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ABSTRACT

Marfan syndrome (MFS) was firstly described in 1869, but this pathology existed for centuries. Probably MFS was observed in such people as Abraham Lincoln, Niccolo Paganini and Sergei Rachmaninov. MFS is an autosomal dominant connective tissue disorder. In this condition symptoms occurs mainly in cardiovascular, skeletal and ocular system. MFS is caused by mutations in gene encode extracellular matrix protein – fibrillin-1, which is major constituent of microfibrils and it plays role in local regulation of TGF-β signalling pathway. Impaired structure of fibrillin-1 may lead to disorganisation of tissue's integrity and function, it is observed in thoracic aortic aneurysm (TAA) and aortic wall dilatation. TAA is most life-threatening condition in MFS, and its propriety management may contribute to increase of mean-life expectancy in MFS. Nowadays the successful treatment of MFS does not exist. Mostly, drugs are used to prevent complications of the MFS, however the most common direction of scientific research today is to develop treatments for this condition by using inhibition of Angiotensin 2 pathway and enhancement of knowledge about induced pluripotent stem cells.

INTRODUCTION

Marfan Syndrome (MFS) is an autosomal dominant connective tissue disorder caused by mutations in fibrillin-1 gene (FBN1) (Sakai, 2016) Estimated incidence of MFS is 1 in 5000 individuals (Ho, 1981). MFS affected multiple organs, but three system are the most significantly exposed: cardiovascular, ocular and skeletal. The clinical spectrum of MFS is varying from mild (involve one or few systems) to severe and abruptly progressive neonatal type. Most life-threatening complications involve cardiovascular system, and occur as aortic aneurysms in the level of sinuses of Valsalva and aortic dissections, common with aortic valve regurgitation and mitral valve regurgitation, and in consequence congestive heart failure (Dean, 2008). In this review we also focus on historical point of view on MFS.

SEARCH STRATEGY AND SELECTION CRITERIA

The Medline database has been systematically searched from inception to April 2021. For the purpose of research were used such a keywords as "Marfan syndrome", "fibrillin1", "FBN1", "thoracic aortic aneurysms", "TAA".

CLINICAL FEATURES OF MARFAN SYNDROME

Diagnosis of MFS is difficult. This process is supported by clinical criteria known as Ghent revised criteria (tab. 1), also to complete the diagnosis the molecular testing of FBN1 mutations are used (Loeys, 2010).

The most noticeable and evident manifestation in MFS are consequences of dispro-portionate increase in linear bone growth such as remarkably high stature, especially on the background of their family, frequently associated with low BMI index and low fat tissue quantity. Too long extremities relative to trunk known as dolichostenomely can

be measure as increased upper segment to lower segment ratio and arm span to high ratio (Erkula, 2002).

Characteristic long fingers known as arachnodactyly, can be confirmed by two clinical signs: Walker-Murdoch's (wrists) sign and Steinberg's (thumb) sign (Dean, 2008). Outward or inward displacement of sternum caused by overgrowth of the ribs can resulted in pectus carinatum (known as pigeon) and pectus excavatum (when sternum is pushed in thorax and narrows anteroposterior diameter and which may lead to heart displacement). Scoliosis involve around 60% of MFS patients and may progress during intense growth leading to serious deformity, back pain and sometimes restricted ventilatory deficit. Widespread in MFS patient is also joint hypermobility (affecting 85% children and 56% adults) (Ho, 2005).

Ophthalmological examination of patient with MFS may reveal hallmark ocular feature – ectopia lentis. This condition is characterised by subluxation of the lens into upward and outward of normally pupil-centred lens may affected up to 56% of MFS patients. Myopia, second most frequent sign is present in 34-44% patients. Highly myopic patients have elongated globe and retina exposed to abnormal stretching. Detached of retina is serious condition, and require prompt specialist care. Cataract and Glaucoma is fairly common and can be early in onset in the course of Marfan's syndrome (Nemet, 2006).

As noted earlier major sources of morbidity and early mortality relate to the cardiovascular system. Approximately 75% of MFS patients have mitral valve prolapse, often associated with myxomatous changes in cusps of mitral valve and prolongation or thinning of its chordae tendinae. This changes may contributes to mitral valve regurgitation. Other cardiovascular complications of MFS are left ventricular dilatation, cardiac failure, dilatation of proximal pulmonary artery and at last thoracic aortic aneurysms (TAA) and dissections. TAA in MFS include dilatation at the level of sinuses of Valsalva, which tends to progress over time. The risk of aortic dissection increase when diameter of aortic root exceeds 5 cm, when aneurysm involve also another parts of thoracic aorta, when annual dilatation exceeds 1,5 mm, and when Family medical history is loaded with aortic dissections (Ho, 1981).

Patients with MFS may be discern also by long, narrow face, hypotrophic malar, high-arched palate known as gothic, small and placed backward mandible (micro- and retrognatia), posterior displacement of the eyeball (enophtalmos) and oblique eye position. Other conditions connected with MF are dural ectasia, different kind of haernias, spontaneous pneumothorax, skin stretch marks emerging transversely to the growth of axis (often on the back) (Ramirez, 2007).

FAMOUS PEOPLE WITH MFS

Due to early mortality caused by cardiovascular events it is important to broaden knowledge of MFS in community to ensure early diagnosis and complex assessment of aorta. Unfortunately, MFS was firstly described in 1869 only, by professor Antoine Marfan , and it took another 50 years to fully elucidate this syndrome and manage early mortality due to aortic dissection and rupture. Only when Bentall composite graft procedure was available in 1968, the outcome of operative techniques in MF was fully

satisfying and elective aortic root replacement safe. Only proven method to prevent risk of sudden death caused of mechanical failure of this main vessel is still prophylactic surgery (Gott, 1998).

Although MFS has just recently got recognized, it has been existing for centuries. Researches are still looking back and searching any characteristic signs of MFS in some historical figures like Abraham Lincoln, Niccolo Paganini, Sergei Rachmaninov.

Nobody can examine these patients anymore and confirm the diagnosis. Mostly only paintings, pictures, articles, books and letters remained. But there are some exceptions. Panzer et al. performed full-body computer tomography to the mummy from Capuchin Catacombs of Palermo from 19th century. They have found evidence for Stanford A aortic dissection (type common in MFS) and despite some technical problems they assessed systemic findings according to Ghent revised criteria (tab. 1). In result MFS was confirm in that case by signs such as pectus carinatum, enlarged sacral foramina (evidence of dural ectasia presence during life), protrusion acetabuli, reduced right elbow extension, dolichocephaly, down-slanting palpebral fissures and malar hypoplasia (Panzer, 2018).

Niccolo Paganini, the most brilliant violin virtuoso of all time may have also suffered from MFS. He was depict as tall, with narrow chest and abnormalously long extremities, with characteristic spider-like fingers, often in Lyser's drawings in unconvinient for most people positions. In some letter, Matteo de Ghetaldi describe another proof of the diagnosis of MFS. Supposedly Niccolo Paganini during examination performed by doctor Martecchini show positive thumb (Steinberg's) sign and hypermobility of joints (Pedrazzini, 2015).

Another famous musician – Sergei Rachmaninov, also equipped with large hands and tall, slender silhouette, exhibited some features of MFS. In the pictures is revealed his long, narrow face, with flat cheek and long, thin nose. It is possible that, on the basis of his letters, in which he complained about the deterioration of his eyesight and headaches, he suffered from severe myopia (Ramachandran, 2006).

At last – Abraham Lincoln. Two hypothesis exist – that he suffered from MFS or multiple endocrine neoplasms type 2B (MEN 2B). Both diagnoses indicate Lincoln's marfanoid body morphology. From descriptions we know, that as a child he was unusually tall compared to peers and in age of 17 his height was already 193 centimeters. Contemporary described him as greatly lean, with sunken breast and spider-like legs. In the photographs, he cannot help to not notice that his face was elongated and relatively small to the rest of his body. During the last two years of his life, he complained about cold extremities and increasing fatigue. Before his sudden death as a result of the assassination, he spent more and more time in bed and had difficulty getting up from it. When he was getting up rose, he sometimes fainted. Due to the marfanoid features, the symptoms could be a manifestation of heart failure caused by aortic valve insufficiency and aortic aneurysm, which would support the diagnosis of MFS (Schwartz, 1964).

FIBRILLIN-1 AS A KEY TO DIAGNOSIS OF MFS

Understanding of MFS and its symptoms would not have been possible without knowledge about fibrillin-1. Discovery of fibrillin-1 is closely connected with monoclonal antibody technology, which was developed by Köhler and Milstein in 1975. For their work, they received the Nobel Prize in 1984. In the one of large screens of antibody close, two of these clones, yielded patterns, which were characteristic to microfibrillar components of elastic fibres. There were isolated and used in experiments with medium of cultured human fibroblast. As a result of these research fibrillin was described in 1986. The name of that protein comes from electron microscopic immunolocalization, because it forms periodic labeling along the lengths of 10 nm diameter microfibrils (Sakai, 1986; Sakai, 2016). In 1990 the history of fibrillin-1 was forever merged with MFS by Hollister et al. Their pioneering work demonstrated deficiency of fibrillin-1 in patients with MFS tissue and prove, that synthesis and secretion of fibrillin-1 is abnormal in cultured cells (Dietz 2007; Hollister, 1991).

Fibrillin-1 is encodes via FBN1 gene, which is localized on chromosome 15q21.1 (Magenis, 1991). That is 130-kb gene. FBN1 is composed of 65 coding exons. As a result of translation of it 2 871 amino-acid long proprotein in produced. That product is named profibrillin. After that, it is proteolitycally cleaved in the C-terminus. In that process a furin convertase plays a key role. The finally products are fibrillin-1 and a 140-amino-acid protein – asprosin, which is the hormone (Romere, 2016). In the proper structure of fibrillin-1 there are three main domains. First, six-cysteine EGF-like domain, which is repeated 47 times. Second, eight-cysteine domains homologous with latent TGF-β binding protein and third, proline-rich region (Ramachandra, 2015).

Fibrillin-1 is a large protein – 350 000 MW. It is a part of extra-cellular matrix and builds 10-12nm microfibrils. These structures are not periodically cross-striated or banded. Furthermore, they have a characteristic morphology. In fibrillin-1 structure are light or dark or hollow areas, which looks like railroad tracks. Microfibrils composed of fibrillin-1 forms large bundles or short individual microfibrils, or peripheral microfibril mantle near the elastic fibers. The second form is mainly localized in the close of basement membrane. The fibrillin-1 microfibrils are organized to the best suit of the integrity of the tissue, for example in muscular arteries encircle the lumen (Sakai, 1986).

The crucial function of fibrillin-1 is providing a scaffold for elastic fiber formation. On the another hand, in the tissue which elastic fibres are not observed fibrillin-1 micorfibrils plays structural role especially near to basement membranes. That function is important in eyes and kidneys. The second function of fibrillin-1 is to regulation of TGF-β signalling pathway. That protein binds a latent TGF-β binding protein -1 and -4 which result in stabilizing the large latent TGF-β complex (Zilberberg, 2012).

Nearly 2,000 different FBN1 mutations have been described over many years. But in reality this number is probably much higher. The penetrance of FNB1 mutations is very high and age-dependent. There was not any example of nonpenetrance described yet. ¼ of cases are new or spontaneous mutations. Moreover, gonadal mosaicism was also described in Marfan syndrome. Mutation is spermatagonium which can be a frequent cause of sporadic Marfan syndrome due to advanced age of the father. There

are many types of mutations described in FBN1 gene. The most frequent is missense mutation, which occurs in over than 65% cases. About 10-15% of mutations are small insertions, deletions or duplications. These gene changes result in premature termination of FBN1 translation. Another 10-15% mutations result in inappropriate splicing. Large rearrangements like both deletions and insertions or entire gene deletions are rare (Sakai, 1986).

Mutations which occurs in FBN1 gene are firmly correlated with Marfan syndrome phenotype. For example cysteine substitutions in EGF-like domains results in high incidence of lentis ectopia and severe. On the another hand, premature codons termination is a cause of phenotype with large joint hypermobility, skin striae and aortic dissection but not ectopia of lentis. The is also representative FBN1 gene changes in incompleted Marfan syndrome. In these cases there are a lot of different mutations in 59-65 exons (Faivre, 2009). Changes in the middle regions of FBN1 (exons 24-32) result in neonatal cases of Marfan syndrome (Faivre, 2009).

THORACIC AORTIC ANEURYSM AS A MOST IMPORTANT COMPLICATION IN MFS

The next part of the work will discuss the pathogenesis of the most life-threatening complication of MFS – thoracic aortic aneurysm (TAA). The wall of unchanged aorta is consisted of three layers: most inward tunica intima, in the middle tunica media and at last tunica adventitia. The tunica intima first component is monocellular layer of endothelium attached to basal lamina built from extracellular matrix such as collagen IV, laminin and proteoglycans. Next is subendothelial matrix and most outwardly internal elastic lamina. The tunica media consist multiple of layers of fenestrated elastic lamellae and intercalated between vascular smooth muscle cells (VSMCs), fibroblasts and proteoglycans, glycosaminoglycans, collagen (I, III, IV) and reticular fibers. The external elastic lamina separates the media from adventitia, which play additional supportive structural function. It is consisted of fibroblasts and progenitor cells of VSMCs embedded in collagen-rich loose connective tissue. This is also a place, where are running vasa vasorum – providing supply for outer two-thirds of aortic wall (Creamer, 2008).

Aorta in MFS has a tendency to dilate due to degenerative process mainly affected tunica media. Histological effect of this many simultaneous processes is mistakenly called by Erdheim in 1929 "cystic medial necrosis", and it is still used in contemporary literature. That, what Erdheim recognized as cyst and necrosis was fragmentation and disarray of elastic lamellae, paucity of VSMCs with accumulation of glycosaminoglycans which create lacunar appearance of tunica media (Romaniello, 2014).

Mutations of FBN1 gene as aforementioned may impair fibrillin-1 ability to bind and sequester the latent TGF-β complex into the extracellular matrix and have influence on microfibrils functions, and its role as connection between elastic fibres to cellular integrins, which conduct to defective VSMCs – elastic lamellae connections. The latter initiates phenotypic changes in VSMCs (Creamer, 2008).

VSMCs play crucial role in regulation of vascular tone through their contractile function. In MFS and other TAA VSMCs phenotype can be changed by environmental cues from "contractile" to "synthetic". "Synthetic" VSMCs are characterized by enlarged

endoplasmic reticulum , decline amount of contractile proteins and amplify secretion of matrix degrading enzymes. Increased proteolytic activity can lead to degradation of extracellular matrix and in turn activate extracellular matrix dependent signalling pathways for example this regulate by TGF-β. TGF-β receptor type I activation lead to pro-fibrotic extracellular matrix remodelling with collagen, elastin, fibrillin, fibronectin, laminin and proteoglycans accumulation through Smad- dependent canonical route and via Smad-independent non-canonical pathway stimulate VSMCs to synthesis of metalloproteinases. This changes makes aorta more susceptible to influence of other factors like angiotensin II, insulin-like growth factor-1, platelet-derived factor and finally mechanical stress (Creamer, 2008; Robertson E., 2015; Romaniello, 2014).

METHODS OF TREATMENT TAA IN MFS

One of the most common and causing a large number of deaths in Marfan syndrome patient is thoracic aortic aneurysms which has already been exhaustively described earlier in this review. In this part we focus on potential methods which can protect and minimize risk of disruption TAA in MFS. The main role of these protection possess pharmacotherapy, but also some group of patient take a benefits form surgical procedure. The most used drugs are β-adrenolytics and ARBs. To assess the benefits of these treatment in MFS was performed by many studies. The most of all used to aortic root growth as a surrogate outcome for aneurysm rapture or dissection (van Dorst, 2020). The first drugs which were used in MFS to minimalize the dilatation of aorta are β-adrenolytics such as propranolol or atenolol. Shores et. Al. as the first showed in randomized clinical trial in small group of MFS patients that use of propranolol contribute to decreased the rate level of dilatation of the aortic root and reduce the development of aortic complications in MFS (Shores, 1994). Base on this and other studies β-adrenolytics constitute the primary form of therapy in treatment guidelines. The first clinical study which assess the benefits of another groups of drug – ARBs on TAA pathology in MFS were performed by Brooke et. Al. The authors showed that ARB usage associated with reduction aortic root growth, but this study was performed only on 18 MFS pediatric patients, so it was necessary to perform the randomized trials which asses the role of ARB in MFS patient (Brooke, 2008). In 2013 the results of randomized open-label controlled COMPARE study was showed and it confirmed the previous results about using ARB in MFS. Also in this study MFS patients have benefits from adding the ARB in standard therapy. Nowadays the guidelines suggest using only the β-adrenolytics in TAA in MFS (van Dorst, 2020).

A NEW AIM OF TREATMENT IN MFS

The new aim of the MFS treatment are concentrated on using the human embryonic stem cells (ESC) and induced pluripotent stem cells (iPSC) as a platform the asses the efficiency of new drugs. These type of cells have an unlimited capacity to proliferate, have the same genotype and phenotype of the donor, because of this they are a good model of disease in individual case of MFS patient. For example Granata et all. developed a vascular model of iPSC smooth muscle cells from dermal fibroblasts which were taken from the MFS patient and it showed the molecular disturbance TGF-β pathway. Also these scientists used the iPSC to test the drugs with potential to apply in treatment in MFS. To sum up, the study of the iPSC and ESC can contribute to the

process of extending the knowledge of molecular basics in MFS and fill the gap between the study of the animals model to patient specific strategies (Rurali, 2018; van Dorst, 2020).

SHORT CONCLUSION

The phenotype of patients with MFS is variable, but there are some characteristics features that Make it possible to establish the right diagnosis with high probability. This may be reflected in an attempt to diagnose this condition in historical figures from available historical sources. Unfortunately, without a detailed interview, physical and imaging and genetic examination, it is impossible to make a certain diagnosis. Detailed investigations of the molecular basis of this disease may contribute to better understanding of pathological processes as well as knowledge of possible new therapies, which in turn may significantly extend the life of patients with MFS and improve the quality of their life.

Table 1.

Revised Ghent criteria (Loeys, 2010)
In the absence of family history:
Ao* (Z ≥ 2) and ectopia lentis
Ao* (Z ≥ 2) and fibrillin-1 mutation
Ao* (Z ≥ 2) and ≥ 7 pts in systemic features
Ectopia lentis and fibrillin-1 mutation with known Ao*
In the presence of family history:
Ectopia lentis and family history of MFS
≥ 7 pts in systemic features and family history of MFS
Ao* (Z ≥ 2 above 20 years old, ≥ 3 below 20 years) and family history of MFS
Scoring of systemicfeatures**
Wrist AND thumb sign – 3 / Wrist or thumb sign - 1
Pectus carinatum deformity – 2 / Pectus excavatum or chest
asymmetry - 1
Hindfoot deformity - 2 / Plain pes planus - 1
Pneumothorax - 2
Dural ectasia - 2
Protrusioacetabuli
Reduced upper segment to lower segment and increased arm/height and no severe
scoliosis - 1
Scoliosisorthoracolumbarkyphosis - 1
Reducedelbowextension - 1
Facial features (3/5) e 1 (dolichocephaly, enophthalmos,

downslanting palpebral fissures, malar hypoplasia, retrognathia) - 1
Skin striae - 1
Myopia > 3 diopters - 1
Mitral valve prolapse (all types) - 1

* Aortic diameter at the sinuses of Valsalva above indicated Z-score or aortic root dissection
** Maximum total: 20 points; score ≥ 7 indicates systemic involvement.

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Pericyte-myofibroblast transition

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ABSTRACT

Pathological fibrosis may occur during chronic inflammatory reaction or as a response to the repeated activity of the destructive factors. This phenomenon can lead to the loss of organ function. The main cells responsible for the fibrosis are myofibroblasts. They are formed as a result of the differentiation of local fibroblasts as well as some other cells located within the organ in which this phenomenon occurs. One of such cells, pericytes, reside within microvessels wall where they play an important role in homeostasis and vessel's integrity. Furthermore, pericytes actively participate in angiogenesis, and in the pathological conditions in response to the signals from the environment, undergo transition to myofibroblasts and become collagen-producing cells. Numerous studies indicate the functional role of Notch1-PDGFRβ in pericytes proliferation and participation in the pathogenesis of fibrosis. The TGF-beta, Jagged1/Notch and PDGFBB/PDGFR signalling pathways initiate this latter phenomenon. It cannot be ruled out that by recognizing the details in signalling pathways leading to the differentiation of pericytes, it will be possible to effectively prevent this undesirable phenomenon.

INTRODUCTION

Post-injury tissue and organ healing can be divided into two main stages. The reparative phase, which precedes organ regeneration and during which damaged cells are substituted by cells of the same type and the stage of fibrosis – where connective tissue cells, named fibroblasts, migrate to the site of injury and replace normal parenchymal tissue. This process may lead to pathogenic changes in the organ if fibrosis prolongates. The fibrotic response can be divided into three phases: the initiating phase, the effective phase and amplificative phase (Ma, 2018). Fibroblasts are engaged in every stage of this process and undergo several changes in phenotype to finally convert into myofibroblasts (MFs) – their contractile form which largely participates in pathological fibrosis by excessive accumulation of extracellular matrix (ECM). Under normal conditions, after healing the wound by covering it with epithelium, MFs disappear in the process of apoptosis. However, during pathological events, activated MFs persist in the tissue finally leading to organ dysfunction. Taking all of the above into consideration, analyzing MFs origin appears to be an important step not only in understanding the pathogenesis of fibrosis, but also a promising therapeutic target.

There are various theories concerning MFs origin. It seems that their provenance depends on the organ, where fibrosis occurs but also on the type of disease. Often these data are contradictory and it is difficult to identify one cell population even within the same pathological condition. In kidney, local fibroblasts, including circulating fibrocytes, local pericytes (PCs) and resident epithelial cells, through epithelial-to-mesenchymal transition (EMT) have been suggested as main MFs progenitors (Grgic, 2012). In lungs – bone marrow progenitors and the lung epithelium have been proposed (Kramann, 2015). Also in other organs, such as the heart, the liver and the skin, different cell types

may be the source of MFs (El Agha, 2017). Almost all organs are vascularized and have an extensive network of capillaries, in the wall of which, apart from the endothelium, there are also PCs. The latter cells represent a heterogeneous cell population and participate in various processes occurring in damaged tissues. The aim of the presented work is to briefly summarize the knowledge on the role of pericytes in the process of fibrosis.

SEARCH STRATEGY AND SELECTION CRITERIA

All data presented in this review was collected based on the PubMed database, using keywords: pericytes, myofibroblasts, fibrosis, TGFβ, Notch.

STATE OF THE ART

PERIVASCULAR CELLS – PERICYTES

MAIN FEATURES

Perivascular cells, also known as pericytes, are branched, periendothelial mesenchymal cells residing within microvessels where they perform numerous functions of which vessel growth, permeability and contractility are only a few to mention (Cappellari, 2013). PCs are embedded within the basement membrane, in close contact to endothelial cells (ECs) and encircle them with their processes which is their hallmark. Initially, these cells were considered only as supportive to capillary walls, however lately PCs gain rising interest due to their participation in pathogenesis and regenerative processes in many organs (Bergers, 2005).

ORIGIN

Their provenance still remains unclear, varies across organs and depends on their ultimate function. It is postulated that PCs in the cephalic region and thymus are of neuroectodermal origin, while in lung, heart, liver and gut, the mesothelium is the main source of perivascular cells. Generally, in most other organs, PCs derive from the mesoderm; specifically from the sclerotomal compartment (Birbrair, 2015). According to some researches, classical microvascular PCs and adventitial pericyte-like progenitors might be considered stem cells due to their high proliferative and clonogenic potential and capability of multilineage differentiation in vitro similar to mesenchymal stem cells (MSCs) (Crisan, 2008). Recently, Guimarães-Camboa and co-authors in lineage-tracing experiments with Tbx18CreERT2 line showed that *in vivo* PCs do not have such properties and the plasticity observed in vitro is due to the influence of the cell cultured environment (Guimarães-Camboa, 2017).

SUBSETS

Some authors distinguish on the basis of the presence or absence of Nestin-GFP expression two subtypes of PCs: type-1 (Nestin-positive) and type-2 (Nestin-negative). It is postulated that type-1 PCs participate in aging by fibrous tissue formation in muscle (Birbrair, 2014). This type of PCs is also able to proliferate and migrate to the site of injury as it was observed in lung, kidney, heart, brain and spinal cord (Laredo, 2019).

CELL-CELL COMMUNICATION

The PC-PC and PC-EC communication is provided by peg-socket, gap and tight junctions or by release of several paracrine molecular factors. Regardless of the organ, Angiopoietin-1 (Ang1)/Tie2, transforming growth factor-β (TGF-β)/ALK-1/5, Vascular Endothelial Growth Factor A (VEGFA)/VEGFR2, PDGF-BB/PDGFR-β, N-Cadherin/ N-Cadherin, Jagged1/Notch are distinguished as main ligands/receptors regulating crosstalk between PCs and ECs (Avolio, 2011).

VESSEL'S INTEGRITY AND PERMEABILITY

PCs play a major role in homeostasis and vessel integrity in many organs, such as the brain, lung, kidney or heart. It has been noted that density of these cells varies depending on the organ. The vasculature of the central nervous system is considered to be the most abundant in PCs, with a 1:1 ratio of PCs to ECs. Here, the role of these cells is pivotal for maintaining functional integrity of the blood-brain barrier. In comparison, the ratio between PCs and ECs in the vasculature of striated skeletal muscle is just 1:100 (Armulik, 2011), however in the heart this ratio is about 1:2 to 1:3 (Su, 2021).

ANGIOGENESIS

PCs actively participate in angiogenesis and physical or paracrine interaction between ECs and PCs plays an undisputed role in this process. Under the specific conditions, these cells undergo the transition from a quiescent to the angiogenic state including multiple changes in PCs, following modifications in the contact with ECs: PCs migration and proliferation, cell maturation, coverage of ECs, presentation of growth factors and modulation of extracellular matrix (Ribatti, 2011).

MOLECULAR MARKERS

Various reviews try to provide molecular markers for these cells, however not all of them are useful from a practical point of view. As PCs are multi-functional cells, defining one, specific marker dedicated to these cells seems challenging. These cells may express different markers depending on the organ (for example NG2 (Neural/Glial antigen 2) or CSPG4 (chondroitin sulfate proteoglycan 4 in the lungs) (Yamaguchi, 2020) in central nervous system), development stage, tissue and cell conditions, provenance and finally their ultimate functions and direction of differentiation. Regardless of location, the markers of PCs are: PDGFRβ, NG2 (Ozerdem, 2001), CD13 (Cluster of Differentiation 13), αSMA (alpha Smooth Muscle Actin), desmin, CD146 (Murray, 2021).

PERICYTE TO MYOFIBROBLAST TRANSITION (PMT)

During tissue fibrosis, PCs undergo several changes in phenotype. Activated PCs detach from endothelial cells, change their shape, migrate and participate in extracellular matrix production. All these changes are defined as pericyte to myofibroblast transition (PMT) (fig.1).

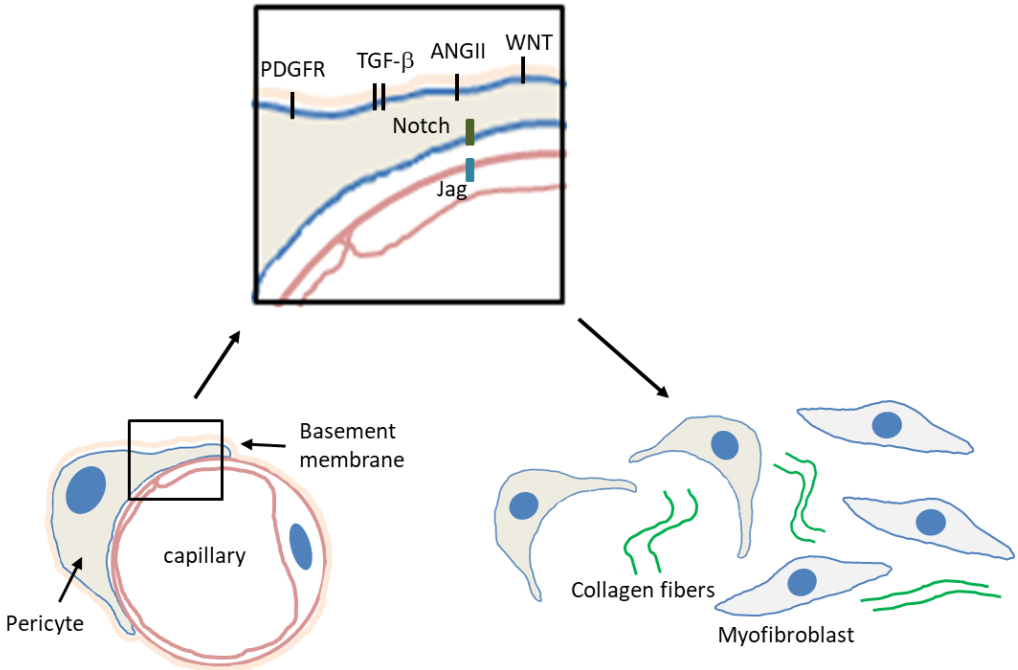

Figure 1. Pericyte to myofibroblast transition (PMT). Original elaboration

This phenomenon has been described in all organs in which loose connective tissue exists and where, under pathological conditions, fibrosis can occur (Wang, 2019). This phenomenon is involved in pathogenesis of idiopathic pulmonary fibrosis (IPF) (Sava, 2017), renal interstitial fibrosis (RIF) and unilateral ureteral obstruction (UUO) (Wu, 2013).

Some studies indicate that PMT might occur also during subretinal fibrosis as PCs residing within choroidal microvasculature start to occupy subretinal space between 3 and 7 days after injury. Subsequently, PCs gain stellate morphology and myofibroblastic markers. Hence, there exists a hypothesis that these cells may contribute to the fibrotic scarring (Luo, 2018).

CROSSTALK BETWEEN PERICYTES AND OTHER CELL TYPES

Inflammation leads to damage and/or activation of endothelial cells (ECs) in the organ which further causes expression of various genes and release of their products to the external environment, both into the lumen of the vessel and the abluminal part of the endothelium. These cells are the source of PDGF and transforming growth factor β (TGFβ), which stimulate PCs to differentiate into MFs.

In addition to endothelial cells, tubular epithelial cells and recruited inflammatory macrophages are the crucial PDGF sources (Chen, 2011). After UUO, an increased level of TGF-β1 was observed abundantly in injured epithelium. However, the TGF-β1 signaling pathway was increased in both epithelial cells and PCs (Wu, 2013).

Interesting results were obtained during transplantation of the bone marrow-derived putative endothelial progenitor cells (pEPCs) into mice with renal fibrosis induced by UUO. The injection of pEPCs instead of protection against organ fibrosis by promotion of the vascular repair alleviated renal fibrosis by reducing PMT. However, the mechanisms responsible for this remain unclear (Yang, 2019).

SIGNALING PATHWAYS LEADING TO PMT

Several mechanisms triggering PMT are known. PMT requires several, cooperating factors in damaged tissue.

NOTCH AND PDGFRB PATHWAYS

Numerous studies indicate the functional role of Notch1-PDGFRβ in PC proliferation and participation in the pathogenesis of fibrosis.

Notch, a receptor for membrane-bound ligands Jagged and Delta, is a central regulator of many essential cellular processes. One of them is regulation of MF differentiation in chronic fibrosis including the lung, kidney, heart, liver, skin (Hu, 2016). The interaction between the Notch extracellular domain with its ligand, located in the cell membrane of the neighboring cell, leads to enzymatic release of the Notch intracellular domain (NICD). NICD translocates to the nucleus to regulate transcriptional complexes containing the DNA-binding protein CSL. NICD converts CSL from a repressor to an activator of Notch downstream target genes such as Hes, Hey, PDGFR-β and αSMA (Kofler, 2015). PCs and perivascular fibroblasts are the primary source of collagen-producing cells in obstructive fibrosis of the kidney. Blockade of PDGFR-β attenuated fibrosis in transgenic mice with UUO (Lin, 2008). Likewise, the use of the soluble ectodomain of PDGFRβ delivered by adenovirus or anti-PDGFR antibodies can decrease the PMT and renal fibrosis (Sacks, 2018). PDGFRβ activates multiple downstream signaling pathways, including Ras, PI3K and PLC, the crucial effector of which is Rho-associated protein kinase 1 (ROCK1). The latter contribute to bleomycin-induced pulmonary fibrosis in mice (Knipe, 2018). In tissue samples obtained from patients with IPF Notch1, as well as PDGFRβ, and ROCK1 were upregulated, which suggests the overactivation of the Notch1/PDGFRβ/ROCK1 pathway in IPF patient lung tissues. Inhibition of the Notch signaling pathway decreases markers of the MFs: αSMA and collagen I, and concurrently increases the PCs markers: NG2 and desmin in a mouse pulmonary fibrosis model (Wang, 2019).

TGF-BETA PATHWAY

Transforming growth factor β (TGF-β) is well recognized as a cytokine responsible for organ fibrosis by regulating the expression of genes such as α-SMA, collagen and fibronectin. Both TGF-β and its receptor are expressed by most cell types. PCs isolated from normal human lungs are able to differentiate to myofibroblast-like cells under influence of TGF-β. In lung with IPF areas of active fibrosis known as fibroblastic foci are most likely to be derived from PCs (Sacks, 2018).

PMT is observed in many tissues where fibrosis occurs, specifically in kidney fibrotic disease. PCs are perceived as a significant source of MFs in this organ, however the exact mechanisms of the transition still remain undiscovered. TGF-β and PDGF

pathways are recognized as two prominent signaling pathways leading to PMT in RIF. Fucosylotransferase 8 (FUT 8) – the enzyme responsible for catalyzing core fucosylation, appears to modify key receptors in these two pathways. According to some studies, core fucosylation increased with the extent of RIF in patients with IgA nephropathy. Similar event was observed in PCs in the course of the UUO mouse model and in an *in vitro* model of PC transition. Both PC transition and RIF were significantly diminished by inhibition of core fucosylation by adenoviral-mediated FUT8 shRNA in vivo and FUT8 siRNA *in vitro*. Moreover, inhibition of core fucosylation resulted in blockage of the TGF-β/Smad and PDGF/ERK pathways. According to these findings, core fucosylation may be in control of PC transition in RIF by regulating the TGF-β/Smad as well as PDGF/ERK pathways (Wang, 2017).

Recently, a link between TGF-β activation and Amphiregulin-induced epidermal growth factor receptor (EGFR) signaling has been described. Amphiregulin, the EGFR ligand, has been associated with tissue fibrosis. It is well known that TGF-β is secreted as a latent inactive complex and binding to an αV integrin is required for its activation. During tissue repair and inflammation Amphiregulin, secreted by the macrophages, may induce the release of bioactive TGF-β by activating integrin-αV complexes on PCs and consequently lead to the TGFβ-dependent PMT (Minutti, 2019).

SHORT CONCLUSION

Pathological fibrosis ultimately leads to organ dysfunction. Improving our understanding of the cells that can convert into MFs and the knowledge of the signal pathways leading to PMT could contribute to the development of potential, effective treatment strategies to reduce the organ fibrosis. PMT is widely investigated in terms of pulmonary fibrosis, particularly in IPF. As this disease is unresponsive to typical anti-fibrotic therapies, modulation of PMT seems to be a promising target. The Notch pathway arouses interest in pathogenesis and progression of IPF as well as in other fibrotic diseases in other organs (such as liver or kidneys). This pathway is also known to be engaged in PC differentiation. For the above reasons, inhibition of the Notch pathway may constitute a potential treatment for tissue fibrosis. Some studies indicate that diminution of the Notch1 signal in mesenchymal tissues leads to alleviation of pulmonary fibrosis (Hu, 2015).

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Significance of extracellular matrix and TGF-β signalling pathway in heart pathologies

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ABSTRACT

The extracellular matrix (ECM) is non-cellular structure which mainly consists of water, proteins and polysaccharides. The proteins in ECM can be divided into three groups: 1) fibrillar collagen (type I and III), 2) non-fibrillar collagen (for example type IV, V, VI) and 3) specialized matrix proteins (for example fibrillin, fibronectin or EMILIN). There are many conditions linked with the dysfunction or dysregulation of ECM network. One of them is cardiac fibrosis where the accumulation of the pathological connective tissue occurs by inappropriate regulation of ECM production and degradation. The major factor regulating this process is transforming growth factor β (TGF-β) pathway which is also involved in regulation of the ECM expression. Another condition where ECM regulates the heart structure is pressure overload, where the ECM role is not limited only to providing stiffness of the heart wall, but most of all it regulates the activity of myocardial cells such as myocytes, fibroblasts or myofibroblasts and prevents from developing heart failure. Also, the ECM regulates and is necessary in the regeneration in infarcted heart, by participation in all phases of regeneration. In this review we are focusing on the role and changes in ECM in cardiac fibrosis and two pathological states of heart – pressure overload and cardiac infarct.

INTRODUCTION

The ECM is a three-dimensional, non-cellular structure, which mainly consists of water, proteins and polysaccharides. ECM proteins production begins from early embryonic stages and occurs in every organ. The main producent of the ECM in the heart, excluding cardiomyocytes or endothelium, are resident cells, like fibroblast, which regulates extracellular matrix synthesis and degradation. By many ways ECM can regulate the cardiac function and condition in such processes as fibrosis or regeneration (Song, 2020).

The cardiac fibrosis is a process caused by ~~uni~~imbalance in ECM production and degradation. The main factor regulating the process of fibrosis in the heart is transforming growth factor β (TGF-β) signalling pathway, which by activation of the Smad2/3 can increased expression genes of fibrotic proteins such as collagen type I and III or proteoglycan. The ECM in heart causes heart wall stiffness and protects disruption of the heart wall. Also, the ECM can impact cardiac cells in many ways by regulation of activation and migration of immune cells, fibroblast or myocyte (Khalil, 2017; Kong, 2014; Ma, 2018). In this review we are focusing on role of ECM in cardiac conditions.

CARDIAC FIBROSIS: CAUSES AND CONSEQUENCES

Cardiac fibrosis is a process caused by inappropriate regulation of extracellular matrix production and degradation. As a result, accumulation of pathological connective tissue is observed in many organs, such as the heart. It can be caused by many cardiological diseases as well as endocrine disorders or toxins like alcohol (Fernández-Solà J, 2020)

or anthracyclines (Bernaba, 2010; Ma, 2018). Human myocardium has got an inappreciable regenerative capacity, so acute ischemia and death of cardiomyocytes results in stimulation of collagen – based scar formation (Frangogiannis, 2012). On the other hand, other pathologic conditions besides occlusion of coronary vessels also induce perivascular and intestinal fibrosis. Pressure overload of heart caused by aortic stenosis or hypertension as well as volume overload underlay by valve regurgitation results in increased fibrosis of cardiac tissue (Kong, 2014). Moreover, aging is connected with cardiac progressive fibrosis, which may lead to development of heart failure of the elders (Meschiari, 2017). The most common endocrine disorders, inducing cardiac fibrosis are obesity (Packer, 2018) and diabetes (Russo, 2016).

Fibrosis in the cardiac tissue is a complicated process, but there are three crucial phases:1) initiation; 2) effective phase; and 3) amplification. Briefly, at the beginning, in the response to the stimuli, secretion of circulation and myocardial pro-fibrotic growth factors and cytokines is increased, causing fibrosis. In the second step, these molecules bind their receptors and activate signalling pathways like TGF-β pathway, and transcriptional factors ex. Smad, mitogen-activated protein kinases (MAPKs) or protein kinase B (PKB/AKT). As a result of it, cardiac fibroblasts transform into myofibroblasts, which start producing matrix metalloproteinases and it's inhibitors, which are crucial in regulation of homeostasis in ECM. Moreover, these transcriptional factors bear influence on cardiac fibroblast and control secretion of pro-fibrotic cytokines and growth factors. Also, another cells like endothelial, cardiomyocytes or inflammatory cells are stimulated by these molecules, which result in positive feedback to amplify pro-fibrotic response (Ma, 2018).

As a result of disturbances in collagen homeostasis structural and functional changes can be observed in the heart (Kong, 2014). Fibrotic area impair physiological coordination of myocardial excitation-contraction coupling in systole as well as in diastole, which is a crucial in worsening of an appropriate cardiac contraction (Janicki, 2002). Furthermore, deposits of interstitial collagen in perymysial space correlates with stiffness of ventricle wall and impaired diastolic function. Despite this, degradation of cardiac ECM matrix can be a background of developing ventricular dilatation and systolic dysfunction due to active fibrotic remodeling in cardiac interstitium (Iwanaga, 2002). Moreover abnormal collagen network in cardiac interstitium is crucial in impaired systolic function via another mechanisms. For example, uncoordinated conduction in cardiomyocytes bundles is caused by degradation of fibrillar collagen (Baicu, 2003). Also fibrotic area in the heart are a place to generated re-entry circuits which promotes arrhythmias (Khan, 2006). In the other hand, fibrosis can result in sliding displacement of cardiomyocytes. In that mechanism ventricular wall is getting thinner and dilates (Beltrami, 1994).

Extracellular matrix as a source of the molecules regulating tissue remodeling processes.

ECM is composed of two crucial elements. First, is a many types of fibries. In the cardiac ECM mostly collagen type I, III, IV, V and VI are observed. Fibrillar collagen types like I and III are crucial proteins, that forms extracellular framework. The most common in the heart are collagen I and V fibrils (70-85% of all collages types). Their main function is transmission of contractive force. Morevoer, 1/5 of cardiac ECM

plays role in composition of basement membranes. The most common proteins, which are present there are collagen type V and laminin, agrin, perlecan, and nidogen (Chute, 2019). Second, it is ground substance, which has got an amorphous and gel-like structure. It is cmposed of large molecules, like glycoproteins, proteoglycans and glycosamino-glycans and water. Components building it are tissue-specific. In the heart typically proteoglycans as biglycan and decorin and fibrous glycoproteins – fibrillin 1 are observed (Li, 2018; Packer, 2018; Song, 2020).

Fibrillin 1 is encoded by three genes – FBN 1, FBN 2 and FBN 3. Is expressed during development and in postnatal tissues. On the other hand, fibrillin 2 and fibrillin 3 are mainly observed in foetus. In immunohistochemistry fibrillin 1 is especially localized in the papillary muscle. The present of it in these areas suggest, that fibrillin 1 plays a crucial role in transmitting contractive forces from ECM to cardiac myocytes. In rat's heart fibrillin is highly expressed in ventricular myocardium (Salvi, 2018). Furthermore, that protein synthesis is increased in cardiac fibrosis states, which suggest, that it is also important in cardiac tissue repair. Fibrillins are the important elements of the 10-12 nm diameter microfibrils, which are typically connected with elastin in elastic fibres. The most characteristic FBN 1 mutation is Marfan syndrome (Bouzeghrane, 2005; Sakai, 2016; Steijns, 2018).

In the cardiac ECM proteins as fibronectin, fibulin 5, dermatopontin, emilin 1, lumican, prolargin, periostin and thrombospondin 2 are also present (Johnson, Hill, 2016). The most interesting are fibronectin and emilin.

Fibronectin gene is localized on chromosome 2q34 and it is composed of 46 exons. Fibronectin typically is a dimer of proteins which are connected via pair of disulphide bonds. Monomers are composed of three types of repeating units. There are two main forms of fibronectin – plasmatic form and cellular. First one is a circulatory form, mainly produced by hepatocytes. Second form, cellular fibronectin is larger and more heterogeneous. Expression of this form is tissue specific. Fibronectin interact with many another proteins of ECM like for example collagen, fibrins or proteoglycans or integrins. Regulation of cell adhesion, migration and proliferation are the crucial processes in which fibronectin is involved (Pankov, 2002; Valiente-Alandi, 2018; Zhang, 2006).

Elastin microfibril interface – located protein (EMILIN) is another glycoprotein, which is present in ECM and is connected to elastin. There are known five isoforms of this protein. Especially one – EMILIN 1 is an interesting one. Main function of EMILIN 1 is to interact with integrins like α4β1 and α9β1. It is important in regulation of cell adhesion, migration, proliferation and cellular differentiation of many types of tissues (Frangogiannis, 2012). Moreover, EMILIN 1 expression is observed in endocardium, of the right ventricle. That protein also plays role as a chaperone protein in the elastin producing cells. It helps in a proper formation of elastic fibres. Moreover, EMILIN 1 is crucial in regulation of TGF-β signalling pathway (Randell, 2017; Spessotto, 2003).

TGF-B SIGNALLING PATHWAY IN CARDIAC FIBROSIS

The major cytokine involved in activation of fibrotic processes in the heart is TGF-β. This protein is released when the cardiac tissues are injured and in many pathological conditions such as heart failure, diabetes or autoimmune rheumatic disease and

promotes the activation of the cells like fibroblasts, myofibroblast or pericytes. These cells are directly involved in synthesis of the ECM proteins and progression of fibrosis. Also, TGF-β is involved in regulation of proliferation, differentiation, apoptosis and activation of immune cells such as T and B lymphocytes or neutrophils and dendritic cells. There are three isoforms of TGF-β (TGF-β1, TGF-β2, TGF-β3), but the most important in fibrogenesis is TGF-β1, which is expressed in endothelial, hematopoietic and connective tissue cells. TGF-β binds a heterodimeric receptor complex, composed of structurally related type I (TβRI) and II (TβRII) receptors and various co-receptors. The TGF-β receptors are single transmembrane spanning protein with an extracellular ligand binding domain and an intracellular specificity kinase domain (referred to as serine-threonine kinase domain). There are seven types of TβRI (ALK-1 through ALK-7) and five types of TβRII (TβRII, BMPRII, ActRIIA, ActRIIB, and Müllerian inhibiting substance RII). The activation of transcription by TGF-β can occur in two ways. Firstly, TGF-β binds to a heterodimeric receptor in the plasma membrane consisting of the TβRI and TβRII which together induce phosphorylation of the Smad2 and Smad3 transcription factors. This pathway is referred to canonical TGF-β signalling. It is important that Smads can regulate TGF-β family signalling by Smad6 and Smad7 so called inhibitory Smads. Secondly, TGF-β family members can also activate non-Smad signalling pathways (referred to noncanonical) initiating activation of the MAPK cascade, which culminates in p38, c-Jun N-terminal kinase 1/2 (JNK1/2), and extracellular signal-regulated kinase 1/2 (ERK 1/2) signalling. To sum up the TGF-β/Smad signalling is a major pathway for fibrogenesis and in consequence leads to activation of various profibrotic genes expression for example collagen type I and III, proteoglycans or other ECM protein (Khalil, 2017; Ma, 2018).

ECM CHANGE IN PATHOLOGICAL HEART CONDITION

Some pathological heart conditions such as pressure overload, diabetic or volume overload, genetic disorder or ischemia can modulate proteins building the heart ECM.

Myocardial response to pressure overload is based on hypertrophic growth of the cardiomyocytes in a concentric manner to reduce the wall tension and preserve left ventricular function. This pathological condition is characteristic to hypertension and conditions associated with left ventricular outflow obstruction such as aortic stenosis and eventually contribute to heart failure development. When this pathological state takes a long time usually intensive interstitial and perivascular fibrosis occurs. Also, pressure overload impairs function of the cardiomyocyte relaxation and contribute to increasing level of myocardial stiffness. In consequence this condition causes the diastolic and in late-stage systolic heart dysfunction. Pressure overload impacts myocardial ECM reconstruction in many ways, not only does it contribute to increase of passive stiffness of heart wall, but also play an important role in regulating inflammatory, fibrotic and hypertrophic cellular response (Messerli, 2017; Moore-Morris, 2014; Perrucci, 2018). It was shown that in cardiomyocytes and fibroblast in a model of left ventricular pressure overload the TGF-β signalling pathway is upregulated and promotes the fibrosis. Also, the major role of the regulation of ECM changes and fibrosis process in the condition play the activation of the local and systemic renin-angiotensin-aldosterone system (RAAS). The levels of the ECM components are

increased in the pressure overloaded heart. Changes in ECM structure consists of both reconstruction of the fibrillar collagen structure, mostly collagen types I and III and matrix proteins such as fibronectin or proteoglycans. The pressure overloaded heart increases significantly the expression of the collagen types I and III. It was shown that in the early stage the amount of the collagen III increases more than of collagen I, but over time, when pathological condition increases, in both compensatory and decompensatory stages the amount of the collagen I increases more than collagen III to decreased myocardial distensibility, by increasing the myocardial stiffness. To suppress the pathological changes in collagens expression mediated by activation of the RAAS many different drugs (such as angiotensin converting enzyme inhibitors (ACEI), angiotensin II receptor type 1 blockers (ARB) and mineralocorticoid antagonists) are used, but in the literature there are many conflicting findings which ambiguously define the impact of the changes in collagen network in pathogenesis of heart diastolic dysfunction (Dobaczewski, 2010; Frangogiannis, 2019; Pauschinger, 1998). Also pressure overload contributes to reorganization and increased expression of the non-fibrillar collagens. It was shown that collagen VI can stimulate the myofibroblast conversion, whereas collagen VIII promotes ECM expansion by regulation of the fibroblast migration and increase of TGF-β synthesis. Additionally *in vivo*, loss of the collagen VIII contributes to reduction of infiltration of myofibroblasts to pressure overloaded heart and decreases fibrosis processes. The role of non-fibrillar collagens is not limited to regulation of the fibroblast activation, but may also be involved in cardiomyocyte survival, inflammatory cell activation or vascular cell function. Furthermore, some of the non-fibrillar collagens can be cleaved during the injuries and generate the important bioactive fragments. For example, canstatin which is a collagen IV-derived peptide can regulate the cardiomyocyte survival, fibroblast migration or angiogenesis in worsening heart failure. Another peptide, endostatin which is a fragment of the collagen XVIII inhibits the angiogenesis that may play an important role in regulation of cellular responses in heart failure. Moreover, changes in the pressure overloaded myocardium are associated with the secretion and deposition of specialized ECM proteins. These proteins in normal heart condition are not expressed and do not play a crucial role in a regulation of the heart function, but in pathological conditions can impact cardiomyocytes and interstitial cells, modulating important cellular responses. It was shown that in pressure overload heart the expression of the fibronectin, matricellular proteins and extracellular proteoglycans is modulated. For example, fibronectin in pressure overload heart contain an extra domain A (ED-AFn) and B (ED-BFn). ED-AFn together with the TGF-β stimulates myofibroblast conversion, however role of ED-BFn is still unknown. On the other hand, pressure overloaded heart is enriched with a wide range of small leucine rich proteoglycans (SLRP) such as biglycan, decorin, fibromodulin, lumican and osteoglycin. These SLRP expression is stimulated by such factors as TGF-β, angiotensin II or proinflammatory cytokines. These proteoglycans play significant role in regulation of fibrotic process by binding the collagen fibrils and organizing the structural ECM or interacting with growth factors and cell surface receptors to transduce or modulate signalling responses. For example, bigylcan mediate fibrosis and cardiomyocyte hypertrophy in mouse models of pressure overload heart. Some of the SLRP such as decortin or osteoglycin protect from development of heart dysfunction. Decortin gene therapy attenuated fibrosis and hypertrophy of the heart by

improved function by regulation of the TGF-β signalling pathway, while osteoglycin protected the pressure overloaded heart from diastolic dysfunction by attenuating inflammatory activation of immune cells and inhibition of the TGF-β dependent fibrosis (Bradshaw, 2009; Frangogiannis, 2019; Shamhart, 2010).

Myocardial infarct and ischemia modulate the ECM structure in the heart. The regeneration of the heart caused by these factor occurs in three phases: 1) inflammatory 2) proliferative and 3) maturation. The biochemical profile changes and composition of ECM plays a significant role in regulation of key cellular events in all of three phases of infarct healing. Over the first phases of heart regeneration the ECM is degraded and generates signals to activate the inflammatory and reparative program. Also, extravasated fibrinogen and plasma fibronectin form the provisional matrix network serving as a highly plastic conduit for infiltrating inflammatory cells. The immune cells clear the infract zone of the heart from dead cells and matrix debris and induct anti-inflammatory mediators which suppress inflammation and contribute to transition to second phase of regeneration – proliferative. In this stage of the regeneration the myofibroblast deposit structural and matricellular ECM proteins, preserving the structural integrity of the heart. In the last stage of the heart regeneration – maturation, the collagenous ECM is crosslinked and the fibroblasts undergo apoptosis. The matrix-dependent molecular steps that regulate the reparative response following infarct of the heart are necessary to protect the infarcted place of the heart from rupture and form the pathogenesis of heart failure. Moreover, dysregulation of ECM genes expression which are involved in regeneration of the infract zone has a major impact on the extent of adverse post-infarction remodelling and in consequence contribute to developing a post-infarction heart failure (Bornstein, 2009; Frangogiannis, 2017).

CONCLUSION

The ECM network is not only the structural support for the tissue, but contributes to regulation of crucial for heart function and condition such as force transmission, transduction of molecular signals or generation of the bioactive molecules which play a crucial role in regulation of the reparative, fibrotic or angiogenic processes in the heart. A thorough understanding of the role the ECM in cardiac normal and pathological condition can contribute to develop new potential therapeutic drugs and methods. Also, investigation of the molecular basis of the regulatory role of ECM on cardiac cells can contribute to better understanding of the pathogenesis and consequences of such disease as cardiac fibrosis or heart failure (Frangogiannis, 2019).

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The role of thrombospondin-1 in the pathogenesis of diseases associated with angiogenesis disorders

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ABSTRACT

Cancer and cardiovascular disorders are the leading cause of death in developed countries. A key element of the pathophysiology of these diseases is disturbed angiogenesis which involves numerous cells, cytokines and the extracellular matrix. Due to the existence of pro- and antiangiogenic molecules, this multistage process is precisely regulated.

Currently, research on modulation of signaling pathways underlying vascular formation is underway to develop innovative therapies dedicated to disorders associated with abnormal angiogenesis. Thrombospondin-1 (TSP-1) is an endogenous extracellular matrix protein which, due to its strong antiangiogenic properties, is a potential therapeutic target. Its structure includes seven modular domains that allow interaction with various ligands, including components of the extracellular matrix, cell-surface receptors, growth factors, cytokines and other biomolecules. For this reason, TSP-1 is involved in complex biochemical pathways, proliferation modulation, migration and adhesion of cells, as well as stimulation of their death through apoptosis. Moreover, it also plays a key role in the reorganization of the cytoskeleton and mediates the interaction of cells with the extracellular matrix.

Previous reports indicate that this multifunctional glycoprotein affects intracellular processes via the integrins and CD36, CD47, TGFβR receptors, and by modulating the release of the ECM components.

Although the positive effects of TSP-1 have already been confirmed in preclinical and clinical studies of phase I and II, there is still little evidence regarding the holistic effect of this molecule.

The purpose of this review is to summarize the current knowledge on thrombospondin-1 in the context of angiogenesis disorders. As in the literature there are extensive reports analyzing the issue, this study focuses primarily on presenting the role of TSP- 1 in cardiovascular diseases and cancer.

Keywords: angiogenesis, cardiovascular disease, cancer, thrombospondin-1 (TSP-1)

List of abbreviations: **aFGF** = acid fibroblast growth factor **Akt** = serine-threonine Akt kinase (protein kinase B); **AMD** = age-related macular degeneration; **Ang-1, Ang-2** = angiopoietin-1,2; **α3β1/β1/αvβ3/αvβ5** = integrins: α3β1/β1/αvβ3/αvβ5; **BAI-1** = vasculostatin; **Bax** = Bcl-2 associated X pro-apoptotic factor, **Bcl-2** = B cell lymphoma 2 protein; **bFGF** = basic fibroblast growth factor; **CD36** = receptor, platelet glycoprotein 4; **CD47** = receptor, integrin associated protein (IAP); **cGK-1** = cGMP dependent protein kinase I; **cGMP** = cyclic guanosine monophosphate; **ECM** = extracellular matrix; **ECs** = endothelial cells; **EGF**=epidermal growth factor; **eNOS** = nitric oxide synthase; **ERK½** = MAP kinases; **FAK** = kinase of the focal adhesion process; **FGF** = fibroblast growth factor; **HGF** = hepatocyte growth factor; **HIF-1α** = hypoxia induced factor; **HL-60** = human leukemia cells; **IAR** = intussusceptive arborization; **IBR** = intussusceptive branching remodeling; **IGF-1** = insulin-like growth factor; **Il-4, Il-10, Il-12** = interleukins-4, -10, -12; **IMG** = intussusceptive microvascular growth; **LVH** = left ventricular hypertrophy; **LSKL** = synthetic peptide, thrombospondin-1 inhibitor; **MAPK** = mitogen-activated protein kinases; **Meg-01** = chronic myeloid leukemia cells; **MMPs** = matrix metalloproteinases, **MMP-9** = metalloproteinase 9; **NO** = nitrogen oxide; **PDGF** = Platelet Derived Growth Factor; **sGC** = soluble guanylate cyclase; **TGFβ** = transforming growth factor beta; **TGFβR** = TGFβ receptor; **Tie 1/Tie 2** = tyrosine kinase with immunoglobulin-like and EGF-like domains 1/2, **TIMPs** = tissue matrix metalloproteinase inhibitors; **TIMP-2** = MMP-2 inhibitor; **TNFα** = tumor necrosis factor; **tPA** = tissue plasminogen activator; **TSP-1** = thrombospondin 1; **TSR (1,2,3)** = thrombospondin type repeats; **VEGF** = vascular endothelial growth factor; **VEGFR2** = VEGF receptor; **VLDLR** = very low density lipoprotein receptor; **VSMC** = vascular smooth muscle cells; **VWc** = Von Willebrand factor type C (Von Willebrand factor type C) repeat, homologous region with type I procollagen; **WISP-1** = wispostatin-1

INTRODUCTION

Cancer and cardiovascular disorders remain the leading cause of death in developed countries. Although there are many drugs available nowadays, new solutions are still being sought to improve the therapeutic effect and the quality of life of patients. Impaired angiogenesis is a key element observed in the course of these diseases. The proper organization and functioning of the body is based on a multi-stage process of vessel formation involving many cells, cytokines and the extracellular matrix. Due to the presence of molecular factors of pro- and antiangiogenic nature, it is subject to precise regulation, which gives a wide range of possibilities in designing new therapeutic concepts.

The anti-angiogenic molecule that deserves special attention is thrombospondin-1 (TSP-1). It is an extracellular matrix protein that interacts with integrins as well as other key surface receptors such as CD36 and CD47. It also influences migration, proliferation and apoptosis of endothelial cells, fibroblasts and inflammatory cells.

Numerous scientific reports have shown that thrombospondin-1, as a strong angiogenesis inhibitor, significantly hinders neoplastic processes, reduces the risk of metastasis, prevents myocardial fibrosis, and serves as an important protective factor preventing left ventricular hypertrophy. Additionally, it plays a key role in activating the cytokine TGF-β, transmitting angiostatic signals, modulating the function of the extracellular matrix and inhibiting the inflammatory response through the CD47 receptor.

While the multiplicity of functions possible through the activation of many receptors make thrombospondin-1 a promising therapeutic target, it is still unknown what is the holistic effect that this molecule exerts on the body. It seems that the continuation of research on TSP-1 in the context of angiogenesis disorders may contribute to the development of innovative therapies based on protein signaling.

ANGIOGENESIS

VESSEL FORMATION MECHANISMS

The formation of blood vessels is a complex process that determines the growth and development of the organism throughout its ontogenesis. A functional vascular network is the foundation for the proper development of the embryo. It also plays an important role in adulthood, for example in tissue regeneration (Saman, 2020; Senger, 2011).

New blood vessels are mainly formed in the fetal period through differentiation and proliferation of angioblasts (precursor endothelial cells), a process known as vasculogenesis. Another equally important process is angiogenesis (neovascularization) involving reorganization and expansion of the existing vascular network, and the advantage of this process is observed after birth (Saravanan, 2020; Sobocinska, 2016).

The mechanism of angiogenesis requires an interaction of many molecules, cells, cytokines and the extracellular matrix (ECM) which both plays a structural role for multicellular aggregates and releases the relevant components involved in signal transduction (Skora, 2006; Wang, 2021). In physiological conditions, two main models

of blood vessel formation may be distinguished (Mehes, 2019; Muhleder, 2021; Ribatti, 2012; Saravanan, 2020):

- endothelial sprouting;
- intussusceptive angiogenesis.

The mechanism of endothelial sprouting is branching of existing blood vessels by creating characteristic structures called vascular sprouts, which consist of three types of endothelial cells. At the head of this structure there is a dedifferentiated tip cell that emerges from other ECs as a result of VEGF activation (De Spiegelaere, 2012). It has filopodia facilitating migration in accordance with the gradient of proangiogenic factors secreted during this process (Muhleder, 2021). Another group are stalk cells (without filopodia) characterized by a high multiplication rate and involved in the formation of the basal membrane. Due to the Notch pathway, stalk cells show reduced expression of VEGF2 and VEGF3 receptors, therefore they are unable to transform into apical cells (De Spiegelaere, 2012; Mentzer, 2014). The last type are phalanx cells characterized by a slower division (Chen, 2019). They constitute an ordered monolayer, and their role is to create the basal membrane and tight intercellular connections.

In contrast to the first model, intussusceptive angiogenesis involves lower metabolic expenditure as well as limited migration and proliferation of cells. The process is faster and occurs in the existing vascular network only (Lugano, 2020). The mechanism was first described in the 1980s (Caduff, 1986). Intussusceptive angiogenesis begins with a recess of connective tissue in the lumen of the vessel, resulting in the formation of an interstitial column from opposite ECs. It leads to reorganization of intercellular connections and formation of central perforation between endothelial cells where interstitial tissue is deposited (Lugano, 2020; Saravanan, 2020). Then the matrix is transformed with the participation of myofibroblasts and pericytes. The formation of the interstitial column may take place symmetrically ("kissing contacts"), asymmetrically ("peglike contacts"), transversely ("mesolike contacts"), by fusing adjacent vessels (Burri, 2004; Saravanan, 2020). As a result of the process, two new capillaries are formed from the original vessel. Thus, intussusceptive angiogenesis leads to four possible forms:

- intussusceptive microvascular growth (IMG) – when a simple network of vessels of similar size is formed;
- intussusceptive arborization (IAR) – as a result of which is a hierarchical vascular structure ("tree") composed of major and secondary vessels is created;
- intussusceptive branching remodeling (IBR) – changing the branching pattern of the vessel;
- intussusceptive pruning – optimizing the number of blood vessels by trimming and removing redundant sections (Makanya, 2009; Mentzer, 2014).

PROCESS REGULATION

Proper functioning of angiogenesis requires a precise regulation provided by pro and anti-angiogenic molecules. Usually, the balance between them shifts towards molecules that inhibit angiogenesis, which leads to angiostasis (Saman, 2020).

As a result of the local predominance of stimulating factors, a number of events occur such as stimulation of endothelial cells, increased vascular permeability, activation of essential proteolytic enzymes and signaling pathways, culminating in angiogenesis (Lopes-Coelho, 2021).

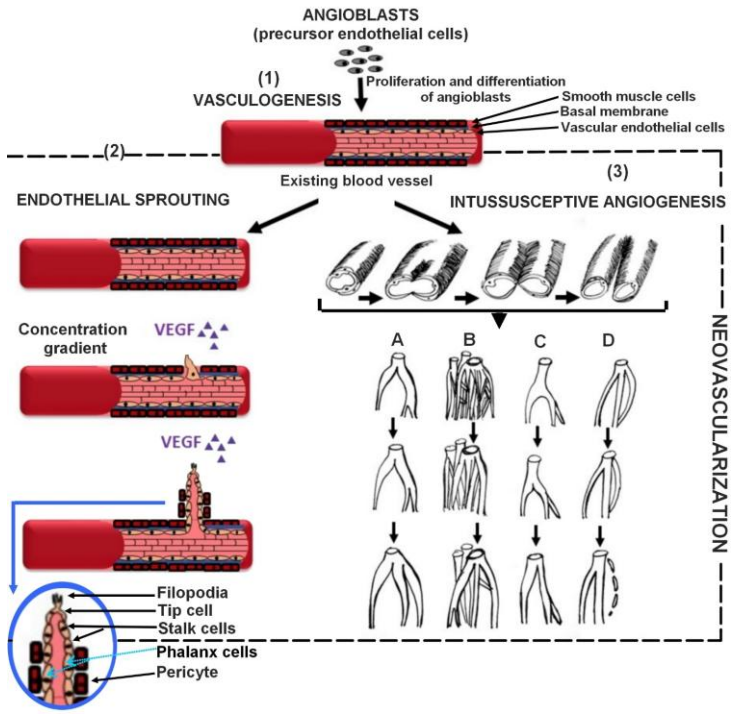

Figure 1. Mechanisms of formation and remodeling of blood vessels. Vasculogenesis (1), endothelial sprouting (2), intussusceptive angiogenesis (3): intussusceptive microvascular growth (A), intussusceptive arborization (B), intussusceptive branching remodeling (C), intussusceptive pruning (D)

The key molecules in these processes are VEGF, NO, angiogenin, prostaglandins, as well as numerous growth factors, including transforming (TGF), platelet (PDGF), insulin-like (IGF-1) and fibroblast (basic: bFGF and acid: aFGF) (Lopes-Coelho, 2021; Yue, 2007; Zawierucha, 2012). These molecules initiate the process and take part in the subsequent stages of vessel formation and reorganization.

However, angiopoietins (Ang-1 and Ang-2) play an important role in the final stage of neovascularization, mediating vessel stabilization and basement membrane formation, as well as endothelial cell regression (Yu, 2020).

It should not be overlooked that the extracellular matrix plays a significant role by exerting an indirect impact on cell adhesion, proliferation and migration cannot. Likewise, MMPs, by using their proteolytic activity, release important growth factors from the compartments of the ECM (Senger, 2011; Wang, 2021).

Apart from molecular stimulation, angiogenesis can be initiated chemically or mechanically. In the first case, the process begins in response to hypoxia (Abou Khouzam,

2020; de Mendonca, 2020; Zeng, 2020) or inflammation (Dimberg, 2010). In hypoxia, the HIF-1α factor interacts with the sequence in the VEGF promoter region thus leading to an increased expression of this gene (Abou Khouzam, 2020; Zawierucha, 2012).

Whereas, in the inflammatory process, the accumulation of macrophages, cells and thrombocytes contributes to the local release of angiogenic factors. In this mechanism, the key action is attributed to chemokines which, by binding to the extracellular matrix, enable leukocytes to firmly adhere to the vessel walls. Some chemotactic cytokines can also activate G protein signaling pathways or indirectly modulate VEGF-mediated signaling (Dimberg, 2010). Mechanical stimulation most often occurs on increased physical exertion (Stevenson, 2020). The process of angiogenesis is stimulated by increasing blood flow. Research suggests that this phenomenon results in vessel formation through deep angiogenesis (Mentzer, 2014). Obviously, these changes can also accompany pathological conditions.

Antiangiogenic factors are also extremely important in regulating vascular formation. Among them there are molecules capable of inhibiting the migration, proliferation and adhesion of endothelial cells. So far, the most important ones are angiostatin, angiopoietin-2, thrombospondins 1 and 2 (TSP-1, TSP-2), endostatin, interleukins (Il-10, Il-12), troponin-1, as well as integrin and protease inhibitors (Lopes-Coelho, 2021; Madu, 2020; Saman, 2020; Yu, 2020). Recently, special attention has been paid to TSP-1 in the context of the treatment of diseases related to excessive angiogenesis. It should be emphasized that the shift of the vascular balance towards angiogenic processes also occurs in pathological states. An example is the development of neoplasms in which the phenomenon of excessive neovascularization is observed (Nishida, 2006). It is the tumor vasculature that largely determines metastasis probability and ability (Rajabi, 2017).

The described regulation of angiogenesis is schematically presented in figure 2.

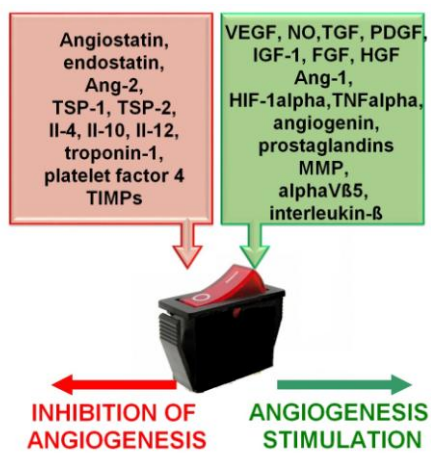

Figure 2. Proangiogenic and antiangiogenic factors regulating the angiogenesis process

DISORDERS OF ANGIOGENESIS IN DISEASES AND NEOPLASMS

Both uninhibited angiogenesis leading to pathological vascular hyperplasia as well as an excess of antiangiogenic factors resulting e.g., in regression of blood vessels, may be the causes of numerous dysfunctions that underlie various diseases (Dimberg, 2010).

Usually, the etiology of these diseases is associated with hypoxia, e.g. in the pathogenesis of stroke, ischemic heart and limb diseases, atherosclerosis, preeclampsia and hypertension. It may also be related to inflammation observed in asthma, psoriasis, ulcerative colitis, Crohn's disease or rheumatism (Campbell, 2010; Yue, 2007; Zduńska, 2019). Pathological neovascularization contributes to the development of retinopathy, vascular glaucoma, age-related macular degeneration (AMD), endometriosis, and even obesity (Abcouwer, 2013; Matsuo, 2015; Nijhawans, 2020). The significant role of angiogenesis in the pathophysiology of the above-mentioned diseases allows its modulation to be used for therapeutic purposes. For example, VEGF inhibitory therapies are used. This approach shows positive results in the treatment of cancer and AMD, protecting patients against blindness (Bressler, 2009). Currently, many substances with antiangiogenic properties (including TNP-470, endostatin and angiostatin) are subject to extensive laboratory and clinical testing (Zhang, 2020). In turn, substances such as bevacizumab or pegaptanib sodium have already been registered in the treatment of cancer under the brand names Avastin and Macugen, respectively (Garcia, 2020; Yue, 2007). To a large extent, these drugs are used as an adjunct to standard therapy. They have been shown to significantly reduce the side effects of the treatment used so far.

Neoplastic diseases are a frequent cause of death. Due to significant environmental pollution, high exposure to carcinogens, stress and unhealthy lifestyle, the number of patients increases every year. Prevention and rapid diagnosis, as well as metastasis prevention are milestones in the fight against cancer. It is well known that cancer foci are able to spread to tissues located distantly from the primary tumor through the network of blood or lymphatic vessels which enable altered cells to penetrate into the bloodstream or lymph and thus they are transported to new locations (Lugano, 2020; Nishida, 2006).

In the early stages of the process, tumor growth is limited to about 2 mm^3. At this stage, the tumor is able to take up oxygen and nutrients by diffusion. In the non-vascular phase, which may last for years, a balance is created between the proliferation of neoplastic cells and their apoptotic death (Jiang, 2020; Saman, 2020). There are even cases where a tumor becomes necrotic.

The vascular stage is initiated by tumor hypoxia(Jiang, 2020; Saman, 2020). The increase in gene expression followed by the synthesis of angiogenic factors initiates the process of neovascularization (Rajabi, 2017). The developing network of blood vessels provides oxygen and nutrients necessary for further development of cancer. In this phase, both sprouting and intussusceptive angiogenesis processes are of key importance (Lugano, 2020; Yue, 2007). An easier access to the bloodstream results in the metastatic possibility, and this in turn leads to increased expansion of neoplastic cells.

Currently, five classes of anti-cancer compounds based on the mechanism of angiogenesis are being tested in clinical trials. These include the following inhibitors:

- proteases;
- EC migration and proliferation;
- angiogenic growth factors (e.g. VEGF);
- proteins on the EC surface, e.g. integrins;
- other inhibitors with a unique molecular mechanism (Nishida, 2006).

Although treatment with anti-angiogenic substances prolonged the life of patients, a better effect was observed in combination with radio- or chemotherapy (Hurwitz, 2004; Zhang, 2020). Inhibiting tumor angiogenesis prevented tumor growth and reduced the rate of metastases. Interestingly, recent reports suggest that by normalizing the existing tumor vasculature, anti-angiogenic agents may also improve drug distribution to neoplasms, thus leading to a therapeutic success (Lopes-Coelho, 2021; Rajabi, 2017).

THROMBOSPONDIN

STRUCTURE AND CHARACTERISTICS OF THROMBOSPONDIN-1

Thrombospondin-1 belongs to the family of extracellular matrix proteins whose main task is to condition the phenotype of cells, regulate their proliferation, migration and apoptosis, as well as mediate cell-cell and cell-ECM interactions (Bradshaw, 2014; Murphy-Ullrich, 2019; Zhang, 2020). Previous studies have shown that apart from the ECM, in which TSP-1 was first identified, a significant reservoir of this protein are also α-granules of platelets, as well as osteocytes, chondrocytes and ECs (Krishna, 2013; Wierzbowska, 2004).

Depending on the structure, the TSP family of proteins can be divided into two groups. The first one includes two homotrimers. i.e. TSP-1 and TSP-2, while the second one includes pentameric molecules such as TSP-3, TSP-4 and TSP-5 / COMP (Isenberg, 2020; Krishna, 2013). Among the proteins mentioned, TSP-1 and TSP-2 are those best characterized ones.

Thrombospondin-1, with a molecular weight of 420kDa, consists of three identical subunits made up of six domains (shown in Figure 3). The presence of specific peptide sequences in individual domains determines the variable effect caused by the interaction of TSP-1 with numerous membrane proteins, namely CD47 (IAP, integrin associated protein), CD36 (platelet glycoprotein 4), integrins and proteoglycans. In the central region of the molecule there is a fragment consisting of three repetition sequences:

- type I (TSR1, thrombospondin type-1 repeats) – involved in the activation of TGFβ;
- type II (TSR2) – similar to epidermal growth factor (EGF);
- type III (TSR3) – containing calcium ion-binding fragments (Gutierrez, 2021; Mustonen, 2013).

Ca^{2+} association in this region leads to conformational changes in the molecule, thus allowing the attachment of TSP-1 to the cell surface. At the two opposite ends of the chain, there are globular domains, i.e. N-terminal, binding heparin or integrins, and C-terminal, conditioning interaction with the CD47 membrane receptor. What is also noteworthy is the region homologous with type I procollagen (called the von Willebrand C repeat) and the oligomerization domain connecting it to the N-terminus, containing thiol bonds that enable the formation of the trimer structure (Chistiakov, 2017).

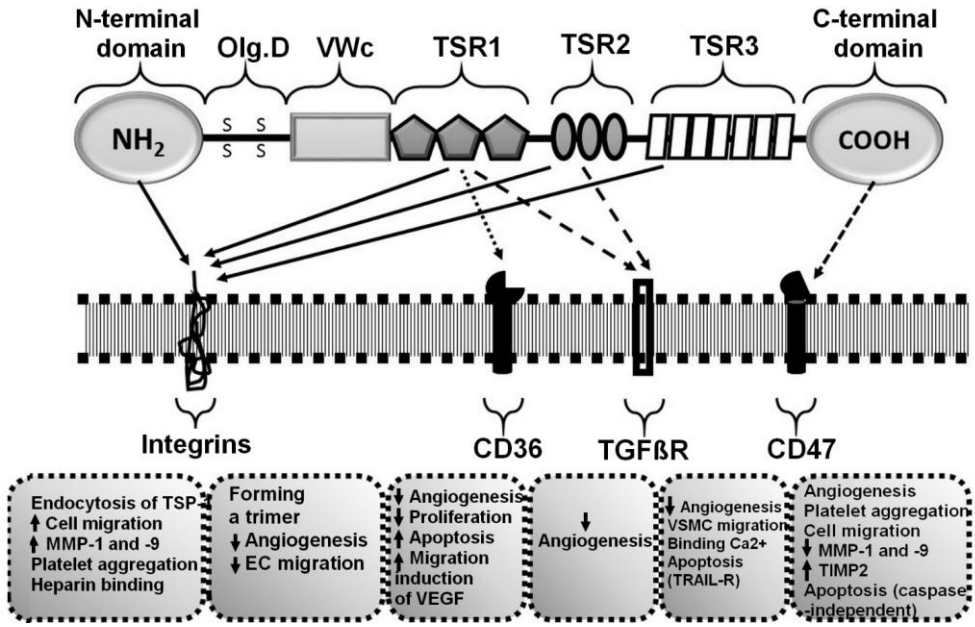

Figure 3. The structure of a single TSP-1 subunit and presentation of the basic effects conditioned by individual domains

REGULATION OF TSP-1 LEVELS

As in the case of most of nonstructural ECM proteins, TSP-1 concentration is low under homeostatic conditions, while its expression increases during pathological changes (Isenberg, 2020; Kim, 2016). Among the numerous factors regulating the concentration of this protein, a large part is played by TGFβ, p53 supersort protein, PDGF, interleukin-6 (IL-6), as well as cell hypoxic conditions and heat stress. Reports also suggest the role of adenosine and its derivatives in the regulation of TSP-1 synthesis via A2A and A2B receptors (Ernens, 2015). It should be emphasized that the expression levels for individual members of the TSP family are tissue specific (Mentzer, 2014).

The elimination of thrombospondin-1 is possible owing to the involvement of the N- terminal domain. TSP-1 molecules released into the vessel lumen become a substrate for thrombin. Another degradation mechanism may also be lysosomal endocytosis (Wierzbowska, 2004).

THE ROLE OF THROMBOSPONDIN-1 IN ANGIOGENESIS

The overwhelming number of studies prove the anti-angiogenic properties of thrombospondin-1. The exceptions are processes in which TSP-1 stimulates polynuclear cell chemotaxis and myofibroblast migration (Binsker, 2019; Osada-Oka, 2008).

The contribution TSP-1 makes to the regulation of angiogenesis is complex. Thrombospondin-1 may regulate the angiogenesis process directly by binding to CD36 and CD47 receptors and integrins or indirectly through interaction with proargiogenic factors

(Kale, 2021; Morandi, 2021). Interestingly, the research results indicate that the constitutive level of TSP-1 in blood vessels is sufficient to counteract proangiogenic signals, thus maintaining angiostasis (Krishna, 2013).

In the first mechanism, the key role is played by the TSR domain (in the structure of TSP-1) which modulates the processes of migration and apoptosis of endothelial cells via the CD36 receptor (Lawler, 2012; Morandi, 2021). Thrombospondin-1 induces apoptosis by modulating the activity of the tyrosine kinases, p38MAPK and caspase-3 (Huang, 2017; Morandi, 2021). It has also been proved that it regulates the expression of apoptotic factors reducing the number of inhibitors of programmed cell death, including Bcl-2, and increasing the level of pro-apoptotic proteins such as Bax (Li, 2003; Wang, 2019). TSP-1 may also be involved in promoting apoptosis by interacting with integrins αvβ3 (Wang, 2019). This has been shown to occur in cells lacking CD36 receptors. The interaction of TSP-1 with the CD36 receptor or with β1 integrins is also responsible for the inhibition of endothelial cell migration (Lawler, 2012; Wang, 2019).

Another mechanism by which TSP-1 directly modulates the angiogenesis process is based on interaction with the CD47 receptor via the C-terminal domain (Kale, 2021; Krishna, 2013). It consists in inhibiting the activity of endothelial nitric oxide synthase (eNOS) and guanylate cyclase (sGC), and consequently antagonizing NO/cGMP signaling (Chistiakov, 2017).

Moreover, the anti-angiogenic effect of thrombospondin-1 may be associated with inhibition of the cell cycle (Gao, 2016). It includes the p53 and p21 proteins (in the EC of large vessels) or the very low density lipoprotein receptor (VLDLR) and the Akt/MAPK pathway (in small blood vessels) (Gao, 2016; Lawler, 2012; Yamauchi, 2007).

However, the indirect action of TSP-1 in the regulation of angiogenesis consists in inhibiting the secretion of proangiogenic factors, as well as disturbing their binding to receptors. An example is the inactivation of the MMP-9 metalloproteinase which reduces the release from the extracellular matrix of the most important proangiogenic cytokine, vascular endothelial growth factor (VEGF) (Gao, 2016). In turn, the second indirect model involves a phenomenon where TSP-1, due to its affinity for heparan sulfate, competes with FGF-2 for a co-receptor site, thus blocking the binding of this proangiogenic molecule (Skora, 2006). The described mechanisms are shown in figure 4.

IMPORTANCE OF THROMBOSPONDIN-1 IN THE PATHOGENESIS OF CANCER AND CARDIOVASCULAR DISEASES

CANCERS

Because of its antiangiogenic properties, thrombospondin-1 is a potential therapeutic target in the treatment of neoplastic diseases. In some cases, low levels of the protein expression may also be a prognostic factor for disease relapse or low survival (Chistiakov, 2017; Krishna, 2013). Released from stromal cells, TSP-1 inhibits the migration and proliferation of endothelial cells and promotes their death by apoptosis, thus contributing to the inhibition of the expansion of the tumor vascular network.

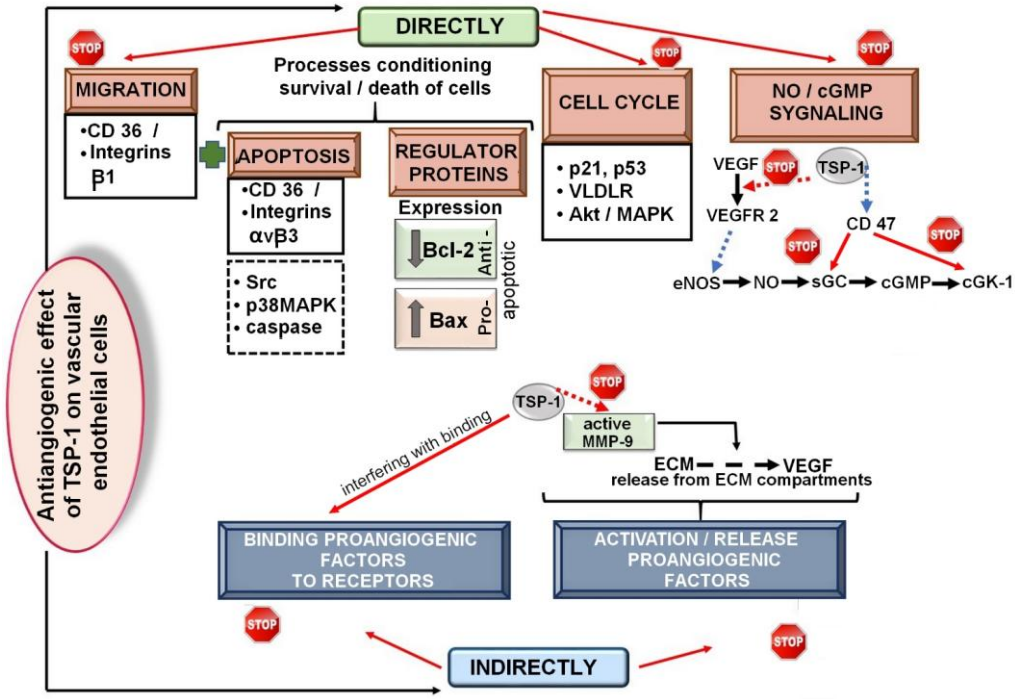

Figure 4. Antiangiogenic mechanisms of thrombospondin-1 in vascular endothelial cells

The antitumor activity of thrombospondin-1 may be based on direct induction of the apoptotic pathway by the CD36 receptor. There is also an indirect regulatory mechanism by which TSP-1 modulates transforming growth factor beta (TGF-β) and metalloproteinase-9 (Bradshaw, 2014). Thus, activation of the TGF-β cytokine enables its interaction with receptors on the cell surface, which triggers signaling pathways leading to inhibition of tumor growth (Bradshaw, 2014). Whereas inhibition of MMP-9 by TSP-1 contributes to a reduction in the level of pro-angiogenic VEGF released from the extracellular matrix. The described mechanisms are shown in figure 5.

CARDIOVASCULAR DISORDERS

In recent years, there has also been an increased interest in the TSP-1 molecule in the context of cardiovascular disorders. It has been shown that it is constitutively present in blood vessels and interacts with a number of proteins that are of key importance for maintenance of vascular homeostasis (Aburima, 2021; Krishna, 2013). The complex structure provides numerous ligand binding sites, which may explain the varied effects produced by TSP-1. On the one hand, its antiangiogenic properties are well known, and on the other hand, the participation in the accumulation of inflammatory cells and proliferation of myofibroblasts leads to increased secretion of proangiogenic factors (Ernens, 2015). Recent reports also suggest an involvement of TSP-1 in the processes of heart remodeling, since an increased expression of the protein is particularly observed as a result of myocardial damage. The results of *in vivo* studies also demonstrated the protective role of TSP-1 in the prevention of left ventricular

hypertrophy (LVH) during overload caused by arterial hypertension (Ernens, 2015; Vanhoutte, 2011). Apart from that, scientific studies also prove the participation of this protein in the reduction of inflammation in the border zone of the infarction (Ernens, 2015).

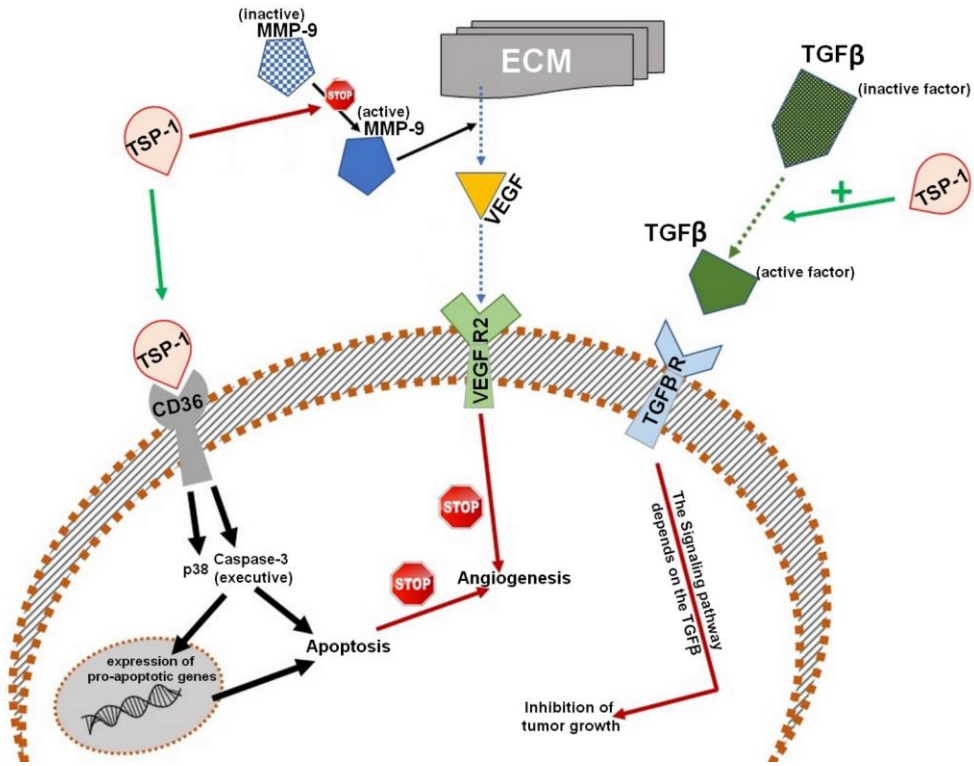

Figure 5. The role of thrombospondin-1 in inhibiting tumor growth: direct induction of apoptotic processes (A), indirect interference with pathways determining cell survival (B)

It is well known that vascular permeability and blood flow are very important elements for the proper functioning of the cardiovascular system. Nitric oxide (NO) plays a decisive role in these processes. Endogenous NO is produced in ECs, being a vasoactive molecule responsible for the transient vasodilation, inhibiting angiogenesis, preventing platelet aggregation, and even promoting cell death (Aburima, 2021; Gutierrez, 2021). Of the wide spectrum of effects, the changes induced in the vessel are determined by the concentration and duration of action of this molecule. It is known that at low concentrations, nitric oxide paracrinely dilates the vessels and increases their permeability. Moreover, it exhibits anti-inflammatory effects (Lopez-Dee, 2011). On the other hand, long-term exposure of ECs to increased levels of the oxide may result in cytostasis or cell death, significant vasodilation leading to a drop in blood pressure, as well as excessive permeability that stimulates formation of edema. A frequent effect is also a modification of the response to common vasoactive substances (Huang, 2017; Zhao, 2015).

Thrombospondin-1 by binding to the CD47 receptor inhibits the NO-cyclic guanosine-3', 5'-monophosphate (cGMP) pathway. TSP-1 ensures the maintenance of normal blood pressure and hemostasis by reducing the level of cGMP and antagonizing the effect of nitric oxide (Gutierrez, 2021). Moreover, thrombospondin-1 increases the local blood flow in ischemic tissues, thus regulating the cardiac response to vasoactive stress (Krishna, 2013).

Previous studies also emphasize the involvement of TSP-1 in the pathophysiology of age-related chronic diseases, including atherosclerosis, cardiovascular disorders or hypertension (Bradshaw, 2014; Gao, 2016). The results of these observations suggest a relationship between an increase in TSP-1 level, which is dependent on life expectancy, and NO deficiency, which is a key factor in the development of senile diseases (Bradshaw, 2014). The involvement of thrombospondin-1 has also been observed in other mechanisms underlying numerous cardiovascular diseases (Zhang, 2020) .

Thrombospondin-1, like many extracellular matrix proteins, modulates the activity of cytokines (Krishna, 2013). It determines the conformational transformation of the TGF-β molecule into its active form, thus allowing its binding to cell surface receptors (Murphy-Ullrich, 2019). This triggers the signaling pathway and produces a specific effect. The dual role of TGF-β in the cardiovascular system is well understood (Parichatikanond, 2020; Saadat, 2020; Yousefi, 2020). While there are many studies on anti-atherosclerotic, protective and profibrotic properties, in some circumstances it also exerts a pro-inflammatory effect, promotes fibrosis and heart failure (Bradshaw, 2014; Saadat, 2020; Wong, 2018). Thus by activating TGF-β, TSP-1 indirectly participates in the repair mechanisms of the heart muscle, prevents LVH, and contributes to the formation of fibrosis, inflammation and diabetic cardiomyopathy (Krishna, 2013).

A key role in the numerous pathological processes that accompany cardiovascular disorders is attributed to the malfunctioning of the extracellular matrix. ECM is not only a scaffold for the heart and blood vessels, but it also regulates the vital processes of cells through the functional proteins and growth factors present in the structure (Silva, 2020; Vanhoutte, 2011). Moreover, the matrix enables vessels to adapt to the changing blood flow and determines the protective reactions of cardiomyocytes in response to stress stimuli (Chistiakov, 2017). Cardiovascular diseases are very often accompanied by unfavorable changes in the structure of ECM and the expression of proteins such as TSP-1, tenascin, periostin, osteopontin, and osteonectin (Chistiakov, 2017). An example is a change in the release of the key pro-angiogenic factor, VEGF, from ECM, or myocardial hypertrophy characterized by the advantage of the synthesis of type I and III collagen molecules over their degradation (Zhang, 2020). It is known that thrombospondin proteins are components of the extracellular matrix that builds the cardiovascular system. Expression of TSP-1 from the constitutive level may increase in response to injuries, stress stimuli and changes in the tissue microenvironment (Chistiakov, 2017). Numerous studies indicate the involvement of different TSP-1 domains in promoting ECM reorganization.

A noteworthy phenomenon is the induction of MMP-2 which plays a key role in VSMC migration. An increase in the activity of MMP-1 and MMP-9 is also observed, as well as a decrease in the expression of TIMP-2 (an MMP inhibitor) as a result of the

action of the heparin binding domain. In turn, the participation of the C-terminal fragment of TSP-1 results in an increased level of TIMP-2 expression and inhibition of the functioning of MMP-2 and MMP-9. Similarly, the TSR region is responsible for lowering the activity of MMPs and promoting the secretion of TIMP-1 (Krishna, 2013; Yang, 2020; Zhang, 2020).

Getting to the crux of the matter, it may be concluded that different regions of the TSP-1 domains alter the phenotype of endothelial cells, influencing the MMP/TIMP balance as well as regulating the MAPK signaling cascade. Moreover, TSP-1 determines the adhesion, shape and mobility of cells, and modulates their interaction with the matrix (Bradshaw, 2014; Morandi, 2021).

It seems obvious that by interacting with key matrix factors at such multiple levels, thrombospondin-1 plays an important role in remodeling and repairing of ECMs that underlie many diseases.

THERAPEUTIC IMPORTANCE OF THROMBOSPONDIN-1

So far, a number of proteins analogous to TSP-1 have been generated. Some of them, due to positive results of laboratory tests, have been implemented in phase I and II clinical trials. Among them there are potentially anti-cancer peptides, ABT-510 and ABT-526, the structure of which uses sequences of functional domains TSR1, TSR2 or the N-terminal fragment (Krishna, 2013; Uronis, 2013). Although the results of phase II clinical trials on the effect of the ABT-510 mimetic administered alone did not bring the expected results since when used as a supplement to standard treatment, it significantly enhanced the anti-cancer effect (Recouvreux, 2012; Uronis, 2013). It has been shown that by removing and shortening abnormally branched blood vessels through ABT-510-stimulated apoptosis, the distribution of chemotherapeutic agents was significantly improved (Campbell, 2010; Morandi, 2021).

Other peptides homologous to the TSR fragment, such as: Wispostatin-1 (WISP-1), Vasculostatin (BAI-1), Lexatumimab are also subject to continuous laboratory analyzes (Cork, 2012; Gaustad, 2016; Moon, 2018). Moreover, new isomers of the thrombospondin-1 molecule, e.g., D1-TSP or D1-TSPa are still being designed, as well as analogs to Angiocidin, which is a TSP-1 binding protein (Gaustad, 2016; Lopez-Dee, 2011; Tuszynski, 2013). The action of most of these compounds is based on the anti-angiogenic, anti-proliferative and pro-apoptotic properties of individual domains.

Apart from therapeutic strategies based on recombinant TSP-1 molecule or newly synthesized analogs, there is also an approach to enhance or suppress the effects of endogenous protein. An example is the suppression of TSP-1 expression in ischemic tissues in order to regenerate them by promoting angiogenesis (Gutierrez, 2021; Lawler, 2012; Morandi, 2021). In experiments carried out on mice genetically deficient in TSP-1, improved skin flap healing in a model damage test was observed, as well as a lower percentage of rejected skin transplants as compared to wild-type mice (Isenberg, 2008). Therefore, the results of these studies are a promising harbinger of therapy based on the signaling axis of TSP-1 and CD47 which could promote wound healing after transplantation.

Another issue is metronomic dosing which increases the level of TSP-1 circulating in the vessels, which contributes to the effectiveness of anti-cancer therapy (Cazzaniga, 2021). This therapeutic solution is based on the continuous administration of low doses of chemotherapeutic agents. The results of the studies confirmed that the level of TSP-1 expression increases, while Fas receptors in endothelial cells become more susceptible to the pro-apoptotic effects of drugs (Cazzaniga, 2021; Lawler, 2012).

Although most of the research conducted so far has focused on the importance of thrombospondin-1 in the context of solid tumors, there are indications of a positive effect of this protein in the treatment of different types of leukemia. It was observed in cells isolated from patients with diagnosed acute leukemia, as well as in culture lines such as HL-60 (human leukemia cells) or Meg-01 (chronic myeloid leukemia cells) that TSP-1 inhibits cell proliferation and activates apoptotic pathways (Li, 2003). It has also been shown that cells with low CD36 expression have a weaker response to thrombospondin-1, which suggests a direct involvement of this receptor in the final result.

In the context of the cardiovascular system, apart from the treatment of hematological neoplasms, there are attempts to use thrombospondin-1 in the treatment of atherosclerosis and cardiac pathologies such as LVH, cardiac failure and myocardial infarction (Chistiakov, 2017). The suggested therapeutic solution for the last two diseases is the inhibition of CD47 receptor dependent signaling to enhance the angiogenesis process. Positive experiments of blocking this receptor by monoclonal antibodies have been carried out on *in vivo* models in preclinical studies (Ansell, 2021; Kaur, 2020; Takimoto, 2019; Zhang, 2017).

Recently, there are also attempts to use the profibrotic activity of thrombospondin-1 to counteract myocardial fibrosis. Based on the amino acid sequence of the TSP-1 fragment responsible for the activation of the TGF-β pathway, the LSKL peptide was developed (Chang, 2017; Chistiakov, 2017; Xu, 2020). The molecule interacts with the latency associated protein (LAP) and prevents it from binding to TSP-1. Thus, the release of TGF-β from the latent complex becomes impossible and the cytokine-dependent profibrotic pathway is inhibited (Restini, 2017). The cardioprotective effect of the LSKL molecule was confirmed by studies conducted in a rat model of diabetes type I in which the TGF-β pathway stimulated by glucose and angiotensin II was selectively blocked (Cohn, 2007; Restini, 2017; Wong, 2018). Novel TSP-1 antagonists that inhibit TGF-β signaling are also being sought in order to prevent the processes leading to cardiac fibrosis.

Another important issue is the growing proportion of the population affected by the problem of heart attacks. It is well known that a loss of cardiomyocytes during myocardial infarction is associated with the development of failure which may lead to death. A promising solution is the therapy with stem cells implanted in the sites of damaged tissue in order to regenerate it. However, such a process is associated with certain difficulties resulting from the low chance of acceptance and proliferation of the implanted cells and the problem of their proper differentiation into cardiomyocytes. In order to overcome this barrier, cells are properly prepared. Interestingly, this process uses a peptide derived from the thrombospondin-1 molecule. It has been confirmed that

this protein significantly increases the survival and adhesion of progenitor cells by stimulating the expression of TSP-1, P-selectin and integrins (Cointe, 2017).

CONCLUSION

Blood vessels are a fundamental element in the body which, by providing efficient gas exchange and supplying every cell with nutrients, hormones and growth factors, ensures proper development and regeneration. In physiological conditions, the process of new vessel formation known as vasculogenesis takes place mainly during the embryonic development. In turn, angiogenesis plays a key role in the reorganization of the vascular network, wound healing, and the female reproductive cycle. The regulation of such a complex process is possible due to the involvement of numerous pro- and antiangiogenic factors the proportion of which is shifted in favor of the inhibitors, thus ensuring angiostasis.

Thrombospondin-1 is a strong endogenous angiogenesis inhibitor. It is an important component of the extracellular matrix which, through its ability to interact with key surface receptors such as CD36, CD47 and integrins, modulates the functioning of fibroblasts, VSMC, ECs and inflammatory cells. The domain structure of the protein determines the occurrence of various effects depending on the activated pathway. On the other hand, the levels of TSP-1 expression specific for a given tissue, often result in discrepancies in the final result. The multitasking nature of thrombospondin-1 makes this protein a promising solution in treatment of numerous diseases. The direct effect of TSP-1 on the migration, proliferation and apoptosis of endothelial cells, as well as its indirect participation in the inhibition of VEGF activity, make it a potential therapeutic target in cardiovascular disorders. On the other hand, the ability to inhibit the abnormal vascular growth observed during tumor progression creates new possibilities in counteracting cancer.

Despite numerous studies on thrombospondin-1, this protein remains a subject of intense interest, sometimes arousing controversies. The discoveries made in the last decade have significantly contributed to the understanding of its role in the molecular regulation of angiogenesis. In turn, the designed peptides, which are TSP-1 analogues, resulted in positive effects in preclinical management and phase I and II clinical trials.

However, given the multiplicity of TSP-1 functions in the body and the opposing effects in different cell types, there is a concern about undesirable effects caused by the activation of different receptors. Therefore, it is important to continue research that will not only expand the current knowledge on the role of thrombospondin-1 in neoplastic processes, cardiovascular diseases and other disorders related to abnormal angiogenesis but will also precisely assign characteristic functions to specific regions in the structure of this molecule.

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Does physical activity improve the functioning of the thyroid gland among people with subclinical or overt hypothyroidism?

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ABSTRACT

Introduction: Health behaviors play the major role in creating human health, and among them the key determinant is systematic physical activity. Thyroid hormones play a fundamental role in the body's adaptation during exercise and therefore determine physical fitness. In hypothyroidism, there is a reduction in the oxidative capacity of energy substrates, decreased muscle performance, faster onset of fatigue and decreased exercise tolerance.

Aim: The paper reviews the literature if physical activity may affect the work of the thyroid gland among people with subclinical or overt hypothyroidism.

Material and methods: A literature review was made on the basis of keywords related to physical activity and thyroid function in major medical databases.

Results: There are no unequivocal results of studies indicating the improvement of the thyroid gland through exercise in people with subclinical or overt hypothyroidism.

Conclusions: There are currently no precise guidelines on the type, duration and intensity of physical activity for people with thyroid diseases. It is most appropriate to use the recommendations of the World Health Organization for the general population.

Keywords: hypothyroidism, physical activity, health

INTRODUCTION

Health behaviors are of paramount importance in creating health. These behaviors include diet, physical activity, dealing with stress, the no use of stimulants (smoking, alcohol and psychoactive substances abuse). They undoubtedly play the greatest role in creating human health, and systematic physical activity (PA) is one of the first among the factors that have a proven beneficial effect on health.

The benefits of rationally programmed, regular PA are reflected in the positive functioning of many systems in the human body, especially the circulatory and musculoskeletal systems, as well as the respiratory and immune systems. PA can prevent many civilization diseases, including obesity, hypertension, coronary heart disease and diabetes. People with a physically active lifestyle get sick less (Wojtasik, 2015).

We define subclinical hypothyroidism when the level of TSH exceeds the upper limit of the norm, and the levels of thyroxine and triiodothyronine are normal. Overt hypothyroidism refers to elevated TSH levels, and decreased levels of T3 and T4. In hypothyroidism, there is an insufficient production or inappropriate action of hormones, responsible for many important functions, including participation in the metabolism of proteins, carbohydrates, fats and cholesterol, cardiac stimulation, the nervous system and brain (Ponichtera, 2008). Thyroid hormones are responsible for regulating metabolic

changes in the body, so they also play a role in energy processes during exercise. They can condition physical fitness by participating in the adaptation of the body during physical exercise (Kanaka-Gantenbein, 2005). In diseases of the thyroid gland, especially hypothyroidism, a reduction in the oxidation capacity of energy substrates (ATP and phosphocreatine) is observed. Reduced muscle performance and faster appearance of fatigue due to intracellular pH drop and earlier glycogen depletion. In addition, hypothyroidism shows a reduction in VO_2 max (maximum VO_2), decreased cardiac output, increased amount of lactate, which also contributes to worse tolerance of physical effort [Sabini, 2015]. Substitution treatment in hypothyroidism may improve exercise tolerance, but more research is needed to confirm this relationship [Lankhaar, 2014]. Regular PA has many health benefits. WHO recommended average weekly volumes of 150–300 min of moderate intensity or 75-150 min of vigorous intensity PA, or an equivalent combination of both (WHO Global Recommendations). The main issue of this work is to answer the question how PA affects the level of thyroid hormones and which type of PA is especially recommended for hypothyroidism.

MATERIAL AND METHODS

LITERATURE SEARCH

Relevant studies were identified by searching PubMed (NCBI), ScienceDirect, SpringerLink and WHO website for the reports and recommendations. The search included studies published from last the ten years up to December 31, 2020. The computer-aided search strategy was adapted for each of the databases searched and included common text words, database-specific keywords, and Medical Subject Headings terms related to hypothyroidism and exercise. Keywords used in this search were "physical activity and thyroid disease", "physical activity and hypothyroidism", "exercise and hypothyroidism". The searches were limited to studies in English.

INCLUSION AND EXCLUSION CRITERIA

The inclusion and exclusion criteria for studies are described in Table 1. Studies with at least three patients with only over or subclinical hypothyroidism were included in the review. Only studies that reported patient characteristics with at least serum concentrations of TSH and/or one of the parameters T4, T3 (or T3/T4 ratio), free T4, free T3 were included. The intervention period for inclusion was required to be at least 6 weeks in duration. Studies with comorbidities other than hypothyroidism have not been allowed. Potentially studies eligible for further review were selected by screening their abstracts and title.

Table 1. Inclusion and Exclusion Criteria as Based on the PICOS Elements

PICOS	Inclusion Criteria	Exclusion Criteria
Participants	Patients with overt/ subclinical primary hypothyroidism	Patients with congenital or nonprimary hypothyroidism, undernutrition, tumor, diabetes, other autoimmune diseases
Interventions	Hypothyroidism treated for at least 6 weeks Physical exercise	

Comparators	Patients with primary hypothyroidism after restoration to euthyroidism Healthy subjects
Outcomes	Influence of physical activity on the level of thyroid hormones
Study design	All types of research

PICOS = participants, interventions, comparators, outcomes, study design

DATA EXTRACTION

Two researchers reviewed all titles and abstracts individually and extracted related results. All selected papers were reviewed and duplicate results were omitted. Table 2 summarizes the data extraction. Observational and intervention studies were selected from 10 qualified articles.

Table 2. Summary of the extraction criteria

Keywords / Medical base- number of results	physical activity and thyroid disease	physical activity and hypothyroidism	exercise and hypothyroidism	Elimination criteria
Pubmed	1,085	477	397	more than 10 years old science article books other thyroid diseases than subclinical and overt hypothyroidism comorbidities animal research influence of physical activity on the functioning of the thyroid gland (no influence on the level of TSH and/or fT4, fT3, T4, T3, T3/T4 ratio) intervention time less than 6 weeks
ScienceDirect	60,674	60,634	60,480	
Springer	208,552	208,416	208,101	
Qualified articles: 10				

RESULTS

The latest observational studies assessing the effect of thyroid hormone levels (based on a questionnaire) designed by Roa, pointed that there are no significant differences in the level of TSH and fT4 depending on the level of PA among healthy people (Roa, 2020). On the other hand, the older ones from 2015 show that among young people with hypothyroidism, there are significant differences in the regulation of thyroid function under the influence of PA (Bansal, 2015). Serum TSH was found to be significantly decreased and also a significant increase in serum T3 and T4 levels in patients of regular exercise group compared with nonexercise group (Bansal, 2015). At the same time, intervention studies show heterogeneous results. Most studies indicate that, there are no statistically significant differences in the levels of thyroid regulating hormones, TSH, and thyroid gland hormones, both compared to the control group and the post-training period (Ahn, 2019; Onsori, 2015, Rahmi, 2013; Beyleroglu, 2011). However, other studies (Masaki, 2019) indicate that the decrease in serum TSH concentration after exercise was greater in the subclinical hypothyroidism group than in the euthyroid group. Werneck et al. indicate that exercise intensity was negatively correlated with TSH and positively correlated with the thyroid secretory capacity and the thyroid sensitivity index T4 in patients with subclinical hypothyroidism (Werneck, 2018). In both cases, this suggests that PA positively influences the regulation of the thyroid gland. Regular PA, especially in the young population of people with hypothyroidism, may improve the functioning of the thyroid gland and reduce the amount of thyroxine ingested (Bansal, 2015).

It seems that both aerobic (Werneck, 2018) and resistance PA (Cinar, 2017) can support the work of the thyroid gland in people with hypothyroidism. More studies are required to confirm the impact as well as to provide recommendations for the duration of the training, type and frequency of PA in patients with hypothyroidism.

Table 3 summarizes selected observation and intervention research results concerning the influence of exercise on the level of thyroid hormones.

Table 3. Results of selected observation and intervention studies on the effect of exercise on the level of thyroid hormones

(Author, year)	Study group	Type of research	Type of physical activity	Changes in hormone levels	Comments / Conclusions
(Roa, 2020)	Men with hypothyroidism (n = 20)	A population-based cohort study	Information on PA was collected using a validated questionnaire	There was no association between TSH or fT4 and physical activity	Further studies need to be performed to evaluate whether thyroid hormone replacement therapy is associated with PA

(Ahn, 2019)	Middle-aged women in the subclinical hypothyroidism group (n = 20) vs obese (body mass indices [BMI], ≥ 25 kg/m^2) women without hypothyroidism in the obese group (n = 20)	Interventional study	12-week combination of exercise training program	The 12-week exercise training program did not have a similar significant impact on the hormones related to thyroid functions in both groups (there were no differences in the levels of T3, T4, fT4, T3/T4 ratio and TSH between the groups before and after the 12-week training program)	More research is necessary to explain which exercise training can effectively induce changes in the hormones associated with thyroid functions in patients with subclinical hypothyroidism
(Masaki, 2019)	Patients with untreated subclinical hypothyroidism (n = 53) compared with euthyroid subjects (n = 55)	Interventional study	Ramp cycle ergometer test	A decrease in serum TSH from baseline to after exercise were higher in the subclinical hypothyroidism group than in the euthyroid group	Acute aerobic exercise decreased serum TSH levels in patients with subclinical hypothyroidism and euthyroid subjects
(Werneck, 2018)	Women with subclinical hypothyro-idism (n = 20)	A cross-sectional study	60 minutes of aerobic activities (bike and treadmill), three times a week, for 16 weeks	Exercise intensity was negatively correlated with TSH and positively correlated with thyroid's secretory capacity and thyrotroph T4 sensitivity index among patients with subclinical hypothyro-idism	Moderate and intensity exercise is related to lower TSH, better thyroid secretion and lower thyrotropin resistance among patients with subclinical hypothyroidism

(Cinar, 2017)	Males: sedentaries (n = 20) and individuals who do physical exercises (n = 20)	Interventional study	6 weeks of resistance training (weight lifting) in young men + zinc supplementa-tion (3 mg/ kg/day)	Significant differences were observed in the levels of TSH, fT3, and fT4 hormones in all groups (3 non-exercising, supplemented, training and supplemented and training groups), except for the control group. There was a decrease in the level of hormones after the training period	Regular resistance training along with zinc supplementation leads to significant changes in thyroid hormones and can positively affect athletic performance
(Bansal, 2015)	Ambulatory treated hypothyroid patients (n = 22)	Observational study	3 months of moderate physical activity, 1 hour a day	A statistically significant reduction in TSH concentration was found in patients who exercised regularly, but at the same time such a significant difference was not observed in the non-exercising group. The levels of T3 and T4 were significantly increased in the exercise group, but there was no such significant difference in T3 and T4 in the inactivity group. Compared between the groups, there was a significant decrease in	Young people with hypothyroidism should regularly engage in PA to improve the functioning of the thyroid gland and reduce the dose of substitution thyroxine

				TSH and a significant increase in T3 and T4 in people who exercised regularly	
(Onsori, 2015)	Overweight inactive women (n = 30)	Interventional Study	12 weeks moderate aerobic training, 3 times a week for 60 minutes for women with excess body weight, not physically active	No statistical differences in TSH, T3 and T4 levels after 12 weeks of training. TSH and T3 levels increased slightly immediately after training, but the changes were not statistically significant	Moderate-intensity aerobic exercise does not significantly alter plasma TSH levels
(Rahimi, 2013)	Male students (n = 22)	Interventional study	8 weeks of resistance training with increasing intensity	There were no statistically significant differences in the levels of TSH, T3 and T4 (pre and post values)	More research is required to confirm that resistance exercise can positively affect thyroid function
(Hackney, 2012)	Highly trained males (n = 15).	Interventional study	Rest period, stationary endurance exercise session, and high-intensity interval exercise session until exhaustion for men with a minimum training period of 4 years	Compared to the training sessions and the rest period, there was a statistically significant increase in fT3, fT4, and rT3. After 12 h, there were no differences in thyroid hormone levels between the rest period and stationary endurance exercise. However, fT3 statistically decreased and rT3 increased comparing the	High-intensity interval exercise suppresses the peripheral T4 to T3 conversion, which means a longer recovery period after exercise to return hormone levels to normal levels

				12 h period after interval exercise versus 12 h after the rest period and stationary exercise	
Beyleroglu, (2011)	male field hockey players (n = 14)	Interventional study	Shuttle run test - leading to exhaustion, hockey players - 14 young men (average age 19, with 7 years of training experience)	There were no statistical differences in the levels of TSH, T3, T4 before and immediately after training. However, there was a statistically significant decrease in TSH and T3 an hour after exercise. T4 decreased slightly. There was a slight increase (not statistically significant) in TSH and T3 levels immediately after strenuous exercise, and a decrease in T4	Exercise did not lead to increased T3 to T4 conversion as there were no significant changes in serum fT3 levels immediately after exercise

DISCUSSION

Even older studies on the metabolism of thyroid hormones during exercise give heterogeneous results. Research by Ciloqlu et al. (Ciloqlu, 2005) suggest that with the increase in training intensity, the level of TSH increases. In contrast, hormonal changes in the thyroid gland are most pronounced with moderate exercise, and increasing the intensity leads to a further increase in TSH, fT4 and T4 and a decrease in fT3 and T3 levels. However, other authors do not confirm these results. In their studies, Onsori and Galedari (Onsori, 2015) concluded that moderate-intensity aerobic exercise did not significantly change the plasma TSH level. The results obtained by Hacney and Dobridge (Hacney, 2009) show that after exhaustive exercise there is an increase in TSH, fT3 and fT4 levels, but after 90 minutes of rest they return to the baseline values, and only after 24 hours the values are lower than the initial values.

A large literature review (Lankhaar, 2014) on the effects of both overt and subclinical hypothyroidism on the ability to adapt to exercise shows that the disease has adverse effects on physical well-being. Which applies to both untreated and treated hypothyroid

patients. At the same time, patients are more intolerant to physical exercise than healthy people. This intolerance contributes to the refusal of PA, deterioration of physical condition (muscle atrophy and weakness, fatigue, increased body fat) and an increased risk of cardiovascular events. As the authors emphasize, regular PA contributes to the improvement of the quality of life (increased physical and mental adaptation). According to this review, there are inconsistent data on the effects of training programs in patients with primary hypothyroidism, which also makes it difficult to make recommendations for secondary prevention. Therefore, research is needed with an emphasis on the implementation of an active lifestyle and disease prevention through effective exercise programs for this group of patients (Lankhaar, 2014). Werneck and others also suggested that exercise training improves quality of life (improved functional capacity, general health, emotional aspects, mental and physical component) in women with subclinical hypothyroidism (Werneck, 2018).

The main slogan of the WHO Discussion Paper is "More Active People for a Healthier World" (WHO Discussion Paper). WHO emphasizes that lack of PA is a risk factor for premature death from non-communicable diseases. At the same time, regular PA is associated with a reduced risk of heart disease, stroke, diabetes, and breast and colon cancer, as well as improved mental health and quality of life (WHO Discussion Paper). As emphasized by many authors (Lankhaar, 2014; Bansal, 2015; Werneck, 2018), PA should be regularly practiced by people with hypothyroidism, as it will positively affect the regulation of thyroid gland and improve well-being.

However, there are no precise recommendations as to the type of PA and its duration for people with thyroid diseases. The most appropriate is to use the recommendations of the World Health Organization for the general population.

CONCLUSIONS

In the past 10 years, a few research has focused on the influence of exercise on the level of thyroid hormones among people with overt/subclinical hypothyroidism. Some studies show a positive effect of PA, especially regular one, on the level of thyroid hormones. However, more research is required to confirm what type, duration and frequency of training should be to support thyroid function among people with hypothyroidism.

LIMITATIONS

Researchers focus only on English literature. Selecting only studies with overt and subclinical hypothyroidism, which limits the number of studies and conclusions.

LIST OF SHORTCUTS

ATP – adenosine triphosphate, the main energy carrier in cells;
fT3 – free triiodothyronine, a thyroid hormone not bound to proteins;
fT4 – free tyrosine, a thyroid hormone not bound to proteins;
PA – physical activity;
T3 – triiodothyronine, a thyroid hormone;
T4 – tyrosine, a thyroid hormone;
TSH – thyrotropic hormone, secreted by the pituitary gland, regulating the level of thyroid hormones;
rT3 – reverse triiodothyronine, an inactive form of the hormone;
VO_{2max} – maximal oxygen uptake;
WHO – World Health Organization.

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Psychobiotics: a new trend in neurobiology

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ABSTRACT

Intestinal microbiota produces the nutrients necessary for the regulation of homeostasis, and interacts with the host organism. Disruption of this delicate balance may be associated with numerous disturbances, including neuropsychiatric disorders. Pediatric microbiome imbalance can affect neurological and mental development in the long-term, through the interaction of the immune system with the gut-brain axis, which may involve serotonin and cytokines. Interestingly, changes in specific species in the host's intestinal microbiome may contribute to the development of psychiatric disorders like depression, and, on the other hand, depressive states may affect the specific species of intestinal microbiome. In this review, we focused on the correlation between intestinal microbiome and host's psyche. We have collected information about living organisms whose consumption in appropriate amounts can provide health benefits in patients suffering from mental illnesses. We reviewed information on the effects of specific bacteria on psychiatric disorders and health in general. The review was based on the search of literature performed in the PubMed database, comprising years 1980-2020.

Keywords: brain-gut-microbiome axis, neuropsychiatric disorders, psychobiotics

OLD COMPANIONS WITH NEW PROPERTIES

Eli Metchnikoff was a pioneer, Nobel Prize winner, who first noticed positive effects of bacteria on the human body. He was the first to emphasize that it is possible to turn harmful microbes into beneficial microbes (Metchnikoff, 1907). At the same time, Tissier, a French pediatrician noticed that there was a difference between the bacteria in the stools of healthy children and those with diarrhea. It has been suggested that transferring healthy bacteria from healthy, to those with diarrhea, could restore the body's balance (Tissier, 1906). A series of observations confirmed that the addition of a feed supplement may have a positive effect on the health of the host, while maintaining the intestinal balance (Fuller, 1989; Guarner and Schaafsma, 1998).

In 2001, the Food and Agriculture Organization of the United Nations and the WHO formulated a definition of probiotics that applies to a wide range of products while setting the basic requirements, describing probiotics as living organisms that benefit the body. It was emphasized that the probiotic construct is not the product of metabolism of dead microorganisms or other non-viable products (WHO, 2001). In 2013, the International Scientific Association for Probiotics and Prebiotics, confirmed that a probiotic must show proven health benefits, specific for a group or strain, can have different methods of administration and, most importantly, must be safe for the patient, and that microbiological products, microbial ingredients and dead microorganisms do not belong to the probiotic classification (Hill et al., 2014).

In recent years, bacteria have played an important role in the treatment of various diseases, by shaping the intestinal microbiome and potentially influencing physiology, cognition and immunomodulation (Suez et al., 2019). As evidenced recently, probiotics – living microorganisms that can benefit the host's organism – taken in specific amounts, can bring beneficial effects to patients suffering from mental disorders, by affecting the gut-brain axis. This finding laid the foundation for the term "psychobiotics" (Dinan et al., 2013).

The aim of the study is to determine the potential role of probiotic microorganisms among patients suffering from mental diseases on the basis of informations presented in medical literature.

In our review, we present the current state of knowledge on routes of communication between the gut microbiota and the brain, as well as effects of specific bacteria on the host organism. The review covers a wide range of scientific reports, from molecular research to behavioral observations.

MICROBIOME – ETERNAL COMPANIONS

Human intestinal flora contains approximately 10^{13}-10^{14} microorganisms, whose genome contains at least 100 times more genes compared to the human genome (Gill et al., 2006). Intestinal microbiota, called also "colonization resistance factor", because it has the ability to inhibit colonization and growth of certain pathogenic microbes, initiates host defense and reduces inflammation (Lawley and Walker, 2013). Microbiota produces nutrients regulating the homeostasis and interacts with the host organism. Disturbance of this equilibrium may lead to the development not only infectious diseases, but also conditions such as obesity or diabetes. Moreover, the latest reports suggest a link with neuropsychiatric disorders (Rieder et al., 2017). Recent papers show that the composition and development of intestinal microbiome in early childhood determines health in adult life (Milani et al., 2017).

The contact with the microbiome begins at an early stage of development. It has been shown that vaginal microbiome affects the chance of ovum implantation. Vaginal microbiome dominated by Lactobacillus (> 90% *Lactobacillus* spp.) affects the positive reproductive outcome, while lowering the level of bacteria correlates with a reduction in the chance of embryo implantation (Moreno et al., 2016). Human microbiome is acquired at an early stage of life, probably through contact with placental microbiome, which – as many suggest – comes from the maternal microbiome (Perez-Muñoz et al., 2017). What's more, the microbiome of children delivered by vaginal delivery resembles the mother's vaginal microbiome, where *Lactobacillus* spp, *Prevotella* spp, or *Sneathia* spp. predominate, while the microbiome of children delivered by caesarean section resembles the microbiome of mother's skin, where Staphylococcus, Corynebacterium and *Cutiibacterium* spp. predominate (Dominguez-Bello et al., 2010). In addition, babies born via caesarean section have a smaller number of Bifidobacteria and *Bacteroides* species, including *B. longum* and *B. catenulatum*, compared to vaginally-delivered newborns (Biasucci et al., 2010). Changes in the vaginal microbiome appear to be associated with the occurrence of recurrent miscarriage. It has been observed that vaginal microbiome in the group of women with recurrent miscarriages is dominated by *Atopobium*, *Prevotella* and *Streptococcus*, while healthy women were characterized

by the occurrence of *Lactobacillus* and *Gardnerella* (Zhang et al., 2019). Isolates obtained from breast milk of lactating women showed the presence *Bifidobacterium* spp. – *B. breve, B. longum* and *B. bifidum*, which served as antipathogens (Eshaghi et al., 2017). Also, the use of antibiotics is of great importance for the diversity of infants' bacterial microbiome, as it reduces the *Bifidobacterium* population and increases population of *Enterococcus* spp. (Tanaka et al., 2009).

It is postulated that colonization of fetal intestines begins with the transport of effective microbiome to the placenta, amniotic fluid and the mammary gland. Similar microorganisms are found in the amniotic fluid, placenta and meconium (Collado et al., 2016). Bacteria like *B. adolescentis*, *B. bifidum*, *B. catenulatum*, *B. longum* subsp. *longum*, and *B. pseudocatenulatum* have been detected in both mother and infant faeces (Makino et al., 2013). Hence, it has been hypothesized that the maternal intestine is an important source of infant microbiota.

Interestingly, mothers who gave birth prematurely, had lower microbiome diversity than mothers who gave birth on time. Premature birth correlated with the occurrence of a lower abundance of Bifidobacterium and Streptococcus, and to the Clostridiales orders in the maternal intestines, which could lead to the development of inflammation and preterm labor (Dahl et al., 2017). Interestingly, premature birth can lead to depression and anxiety in the offspring later in life (Van Lieshout et al., 2018).

The gut microbiome has been suggested to be involved in the development of autism spectrum disorders. It has been hypothesized that a high-fat diet of the mother may contribute to a change in the microbiota profile of the offspring, reduce the number of *Lactobacillus reuteri* and lead to neurodevelopmental disorders and behavioral changes in autism models (Buffington et al., 2016).

There are studies in which, after examining 537 placentas, the authors emphasize that the human placenta does not have a microbiome, but is a potential place via which pathogens and pathogenic bacteria such as *Streptococcus agalactiae*, can be acquired (de Goffau et al., 2019). Both Lauder et al. and Theis et al. concur that resident microbiota cannot be identified in placental tissues using qPCR analysis (Lauder et al., 2016; Theis et al., 2019). On the other hand, studies highlighting non-sterile intrauterine environment emphasize that during pregnancy, microbiota may be transferred from the mother to the developing fetus. Consequently, there is a hypothesis that the nutrition of a pregnant mother affects the developmental processes in the uterus, which translates into the risk of future diseases of the offspring (Chu et al., 2016). In studies on placenta samples collected from 320 patients, a small but metabolically rich microbiome was found, consisting of nonpathogenic commensal microbiota from the *Firmicutes*, *Tenericutes*, *Proteobacteria*, *Bacteroidetes* and *Fusobacteria phyla*. The placental microbiome profile was observed to be most similar to that of the oral cavity, but this study included oral microbiome profile of non-pregnant women (Aagaard et al., 2014).

However, the presence of bacteria in the placenta is debatable as most research is based on molecular techniques that can detect live microbiota as well as microbial components. Hence, there is insufficient evidence of bacterial viability (Perez-Muñoz et al., 2017).

Nutrition is an important factor shaping the pediatric microbiome. It has been reported that the cessation of breast-feeding in 12-month-old infants leads to the development of microbiota similar to adults, where *Clostridia*, *Roseburia*, *Clostrium* and *Anaerostipes* predominated, while in infants still breast-fed the level of *Bifidobacterium* and *Lactobacillus* continued to increase (Bäckhed et al., 2015). Also, antibiotic consumption is a significant problem, transforming the normal microbiome for several years and spreading the resistance strains, which may result in colonization by microorganisms harboring drug resistance genes (Jandhyala et al., 2015; Cox et al., 2014) .

It has been documented that infant's temperament depends on gut microbiota. A study performed on a group of 301 fecal samples collected from children aged 2.5 months, assessed the temperament of children at the age of 6 months. It has been found that negative reactivity and fear correlated with reduced diversity of intestinal microbiome. Positive emotionality was attributed to high concentrations of *Streptococcus* spp. and *Bifidobacterium* spp. (Aatsinki et al., 2019). Pediatric microbiome imbalance can affect neurological and mental development in the long-term, through the interaction of the immune system with the gut-brain axis, which may involve serotonin and cytokines (Herba et al., 2016). Microbial colonization modulates brain development, including the developing serotonergic system, possibly by affecting the tryptophan metabolism. It is possible that the intestinal microbiome, via the kynurenine pathway, influences the metabolism of tryptophan, reducing the fraction intended for the synthesis of serotonin and influencing the production of neuroactive metabolites. Local changes in serotonin levels affect the nervous processes in the digestive tract, which affects CNS development and neurotransmission by activating the gut-brain axis (O'Mahony et al., 2015). Moreover, the ability of the microbiota to degrade tryptophan into tryptophol, which has a large influence on the production of IFNγ (interferon gamma), which, in turn, may influence TNFα (tumor necrosis factor alpha) expression (Schirmer et al., 2016). Increased TNF-α levels were observed in children with autism spectrum disorder and correlated with severity of the symptoms (Xie et al., 2017).

EFFECT OF INTESTINAL MICROBIOTA ON THE BRAIN AND BEHAVIOR

One of the dominant groups of microorganisms present in the intestines of mammals and social insects that may interact with the host organism are Bifidobacteria (Milani et al., 2014). It has been reported that *Bifidobacterium* spp. is able to modulate host's mood, thanks to the effect on the secretion of neurotransmitters and neuromodulators such as GABA (gamma-aminobutyric acid) (Dinan et al., 2015). Moreover, the therapeutic effect of antidepressants affects GABA re-uptake, resulting in an increased expression of BDNF (brain-derived neurotrophic factor) in the hippocampus (Calabrese et al., 2013). The time required to observe the effects of probiotics may vary depending on neurotransmitter tested - it has been demonstrated that glutamate increases after 2 weeks and GABA after 4 weeks (Janik et al., 2016).

Due to the significantly lower load of *Bifidobacterium* and *Lactobacillus* in the stool samples of people with MDD (major depressive disorder) compared to control, it was hypothesized that these bacteria can affect the severity of the disease. Bifidobacterium counts correlate negatively with serum cortisol concentration, which is related to the HPA axis (hypothalamic-pituitary-adrenal axis), playing an important role in the stress

response. Interestingly, a negative correlation between the *Lactobacillus* counts and sleep on the Hamilton Depression Rating Scale (HAM-D) was noticed (Aizawa et al., 2016). Another study showed that *Lactobacillus casei* administration significantly suppressed stress-related high plasma corticosterone levels, and reduced the number of CRF-expressing (corticotropin releasing factor) cells in the PVN (paraventricular nucleus). It was presumed that this effect was caused by an effect on the gastric vagal afferent activity (Takada et al., 2016).

Bifidobacterium longum is a promising strain of *Bifidobacterium* spp., which may have therapeutic potential in patients with psychiatric diseases (Messaoudi et al., 2011). It has been shown that *B. longum* can affect the brain, changing its electrical activity, as *B. longum* fermentation products significantly decrease action potential of intestinal neurons (Dinan and Cryan, 2017). It has been hypothesized that *B. longum* has the ability to communicate with the CNS through the intestinal neural system (Bercik et al., 2011). In response to *B. longum*, changes in brainwave frequency were demonstrated in frontal cortex and cingulate cortex (increased theta band) and in hippocampus, fusiform gyrus, temporal cortex and the cerebellum (reduced beta-2 band), which correlated with the vitality of subjects (Wang et al., 2019). In addition, it has been reported that the presence of *B. longum* contributes to the modulation of inflammation, by increasing the concentration of IL-4, interferon-γ, and IL-17A (Nicola et al., 2016).

Based on the review of current literature, it seems that the influence of *B. longum* on anxiety-like behavior requires stimulation of vagal nerve pathways at the level of intestinal nervous system (Bercik et al., 2011). It has been demonstrated that the presence of potential pathogens can activate vagal sensory neurons (c-Fos proto-oncogen expression) through peripheral sensory neurons (Goehler et al., 2005). In the study conducted on 22 volunteers, it has been demonstrated that *B. longum* supplementation may alleviate the psychological and physiological reactions to a severe stressor, and induce improvement of hippocampal spatial memory (Allen et al., 2016). In addition, in laboratory animal studies, it has been noted that *B. longum* may reduce hyperthermia induced by an external factor such as exposure to stress (Savignac et al., 2014).

The mother's milk microbiome is rich in Bifidobacterium, which colonizes the intestines of mollusks and influences the assimilation of human milk oligosaccharides (Sakanaka et al., 2019). Additionally, Bifidibacterium supports the maturation of the immune system early in life (Pettersen and Arrieta, 2020). Bifidobacterium supplementation has been documented to promote weight gain in premature infants, possibly by fermenting undigested carbohydrates into short-chain fatty acids which are used to synthesize glucose and lipids. It also contributes to the production of bile acids, which aid digestion and fat absorption (Oshiro et al., 2019). *Bifidobacterium* has a positive effect on development from the very beginning of infant's life.

The latest scientific reports indicate that the microbiome has the ability to produce and/or consume GABA. The bacterium involved in the metabolism of GABA is gram-positive bacterium of the *Ruminococcaceae* family, KLE1738. KLE1738 is a significant part of the human gut microbiome, it is found in 19.08% of human gut metagenomes contained in the Integrated Microbial Next Generation Sequencing database. On the other hand, KLE1738 is a small percentage of the entire microbiome, with a relative

abundance >1% in only 0,19% of human gut metagenomes. Growth of KLE1738 is possible only in the presence of GABA, and by using the unique dependency of KLE1738 from GABA, KLE1738 was used to identify additional GABA producers in the co-culture test. It turned out that in order to grow, KLE1738 required the presence of a common gut bacteria, *Bacteroides fragilis* which induces GABA production. Notably, in 23 patients with MDD, the low abundance of *B. fragilis*, which promotes the development of KLE1738, was associated with high activity of the part of the brain, whose activation is characteristic for depression – cerebellum, hippocampus, and frontal regions of the brain (Strandwitz et al., 2019).

Potential mechanisms of communication between intestinal microbiota and the brain may probably include five pathways: intestinal neural network, neuroendocrine-pituitary-adrenal axis, intestinal immune system, neurotransmitters and nerve regulators synthesized by intestinal bacteria and barrier pathways, including intestinal and mucous barrier and blood-brain barrier (fig.1) (Wang et al., 2016).

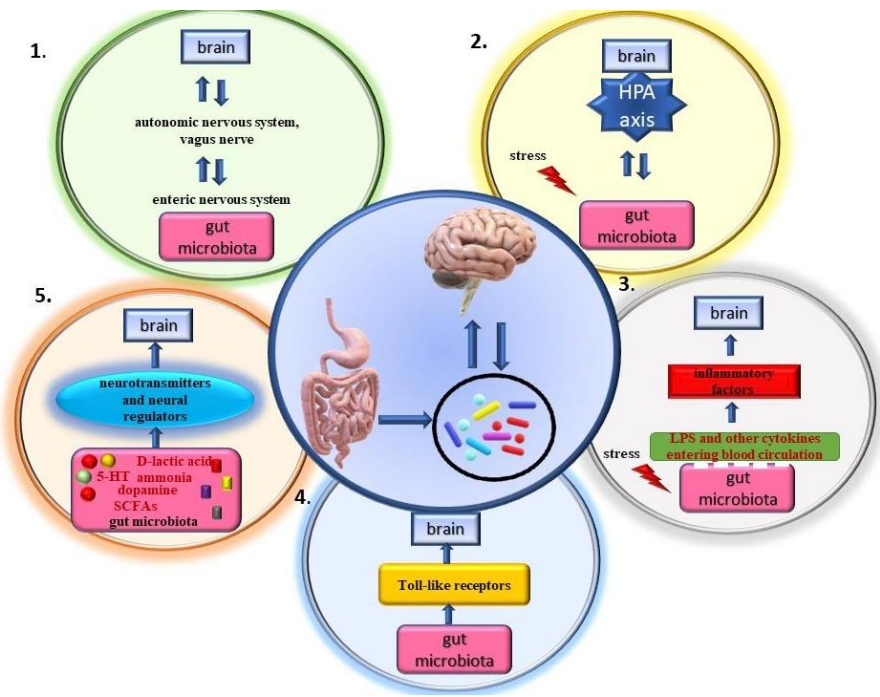

Figure 1. Five putative communication pathways between the gut microbiome and the brain. 1. The neuroanatomical pathway: two-way information exchange between the intestinal nervous system and the autonomic nervous system and the vagus nerve in the spinal cord. 2. Neuroendocrine axis – HPA axis – neuroendocrine maturation: microbiota stress modulates HPA axis and, conversely, HPA axis affects composition of the microbiome. 3. Barrier system: stress can change the permeability of the intestinal mucosa, and LPS penetrating into the cryovascular system and other cytokines stimulate the production of inflammatory cytokines that directly affect the brain. 4. The gut immune system: bacteria communicate with the host via a Toll-like receptor that is involved in the cytokine response. 5. Neurotransmitters and neural regulators synthesized by gut microbiome: bacteria can produce: 5-HT, D-lactic acid, ammonia, dopamine, SCFAs which affect the host's brain (Wang et al., 2016)

Moreover, one should take into account mediators that act as relays in the intestinal, peripheral and central nervous systems and share transduction mechanisms with other biologically active peptides, such as intestinal hormones and neuropeptides (Holzer and fanzi, 2014). Several neuropeptides relevant for the brain-intestinal axis have been identified: YY peptide (PYY), pancreatic peptide (PP) and Y peptide (NPY), which play an important role in the pathophysiology of depression and energy homeostasis regulation, anxiety, and resistance to stress (Holzer et al., 2012). The microbiome can contribute to the secretion of PYY through two pathways: either indirectly through metabolites and biomolecules produced by bacteria, or through the conversion of nutrients by bacteria. The microbiota communicates with enteroendocrine cells, L-cells, via the toll-like receptors (TLRs). As a consequence, the nuclear transcription factor NF-kB is activated, which plays an important role in the immune response (Covasa et al., 2019). NPY has been documented to have 4 (Y1, Y2, Y4, Y5) receptors, and the Y4 receptor knockout in the brain correlated with antidepressant activity and modulated locomotion associated with anxiety (Tasan et al., 2009). In addition, the direct connection between hormonal and bacterial changes in the intestinal microbiota and the brain takes place via the vagus nerve (Perez-Burgos et al., 2013). The vagus nerve may be a potential conduit involved in the control of cytokine production, inhibiting inflammation through the cholinergic anti-inflammatory pathway. The reduction of the immune response is carried out by the secretion of vagus nerve neurotransmitter, acetylcholine. Acetylcholine attaches to acetylcholine receptors on macrophages which may prevent tissue damage and death from cytokine release (Tracey, 2007). In an experiment on laboratory animals it has been shown that subdiaphragmatic vagotomy leads to impaired immune response during *Salmonella* Typhimurium (STM) infection. STM was introduced into the stomach, in order to mimic natural bacterial infection and then subdiagphragmatic vagotomy was performed. This caused attenuated immune response, because of decreased number of CD4+ and CD8+ T cells in the circulation. As a result of the experiment, it has been found that STM can induce appearance of Fos-immunoreactive cells in hypothalamic paraventricular nucleus (PVN), supraoptic nucleus (SON) and subpopulation of T cells. Subdiagphragmatic vagotomy attenuated the reaction induced by STM, which proves that an unimpaired vagus nerve is necessary to maintain the immune balance of the host (Wang et al., 2002).

It is postulated that inflammation plays a key role in the pathophysiology of depression, and the severity of disease symptoms may be related to a change in the concentration of pro-inflammatory and anti-inflammatory cytokines (Kim et al., 2008). C-reactive protein (CRP), interleukin-6 (IL-6), IL-1β, TNF-α, and IL-1RA (interleukin-1 receptor antagonist) are used most commonly as inflammatory biomarkers in depression studies (Howren et al., 2009). Cytokine-induced oxidative stress can damage glial cells, deregulate glutamate levels leading to excitotoxicity and, consequently, reduce the level of neurotrophic factors, for example BDNF (Eyre and Baune, 2012). BDNF supports the proper functioning of neurons, is responsible for neuroplasticity and the process of neurogenesis in the brain. Interestingly, the regulation of BDNF expression is associated with intestinal microbiome: concentration and expression of BDNF in germ-free animals, free of all detectable microorganisms and parasites, is reduced, while probiotic supplementation reverses this state (Shan et al., 2018; Nobuyuki et al., 2004). Further-

more, depression can increase intestinal permeability and thus increase the displacement of endotoxins induced by inflammation, therefore a higher antibody titer against intestinal bacteria has been reported among patients with depression compared to controls (Maes et al., 2008).

BDNF, when bound to the TrkB receptor (tyrosine receptor kinase B), induces receptor dimerization and autophosphorylates tyrosine residues which initiates secondary cascades to regulate neurogenesis, synaptic plasticity, or apoptosis (Huang and Reichard, 2003). It is postulated that the function of BDNF is based on the relationship with the PI3K/Akt kinase pathway, MAPK, ERK, and the loss of this function contributes to the development of neurodegenerative diseases (Deng et al., 2016). Interestingly, physical exercise can affect the reduction of stress-induced changes in the hippocampus in rats, by activating the PI3K/Akt pathway, thereby inducing a therapeutic effect (Fang et al., 2013). Moreover, therapeutic effect was observed after activation of the Akt/GSK-3β pathway in the hippocampus in mice with memory and learning impairment associated with apoptosis of neurons in the hippocampus (Huang et al., 2015).

The microbiota can synthesize and recognize many neurotransmitters that activate neural afferents or enterochromaffin cells that transmit information to the brain. Probiotics can take part in the transmission of the microbiota-gut-brain axis through the fermentation product of starch and dietary fiber, i.e. short chain fatty acids (SCFA), mainly sodium butyrate. Sodium butyrate increases the level of ten-eleven translocation methylcytosine dioxygenase 1 (TET1). TET1 is responsible for DNA demethylation and increases the concentration of BDNF (Maqsood and Store, 2016). Moreover, it is hypothesized that the gut microbiota could potentially be involved in the regulation of serotonin transporter (5-HTT) and brain neurotrophins (NT) expression promoting neuronal survival through the release of BDNF from the hippocampus. The theory of BDNF balance regulation by gut microbiota suggests the influence of probiotics on brain function and development (fig. 2) (Ranuh et al., 2019).

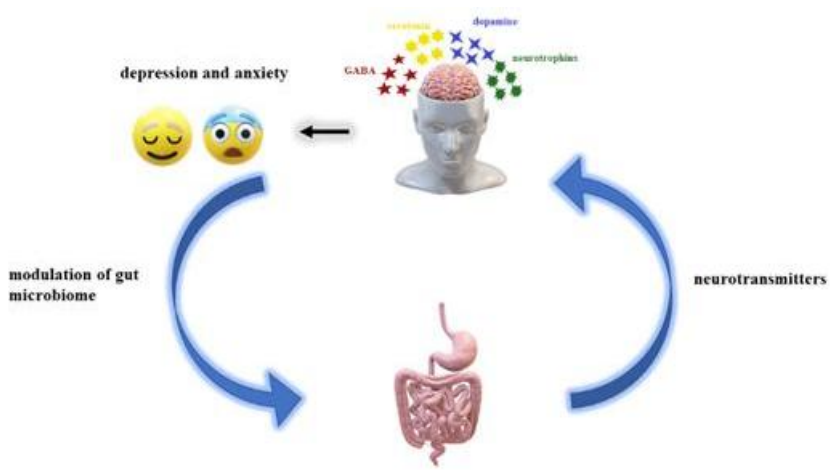

Figure 2. Putative effect of microbiome-secreted neurotransmitters (GABA, serotonin, dopamine, neurotrophins) on the development of psychiatric disorders

The transfer of the maternal microbiome to the offspring may be disrupted by prenatal stress (Jašarević et al., 2018). Maternal stress reduces the abundance of *Lactobaciulli* in the mother's vagina and in the intestines of the offspring. This change of the microbiome may be involved in abnormal amino acid profiles in the developing brain (Jašarević et al., 2015). Moreover, prenatal stress increases blood pressure, enhances the response of the HPA axis, and impairs cognitive function in adulthood (Golubeva et al., 2015). Animal studies suggest that maternal stress exposure may increase the risk of autism spectrum disorders and aggravate symptoms of the disease (Varcin et al., 2017). In addition, stress experience may affect dopamine receptors in the ventral striatum of the offspring, which may translate into susceptibility to mental disorders such as depression (Lovic et al., 2013; Seo et al., 2019). Hence, it is very important to prevent prenatal stress, which affects the developing brain and may cause dysfunction later in life.

THE FUTURE OF PSYCHOBIOTICS

Interestingly, changes in specific species in the host's intestinal microbiome may contribute to the development of depression, and, on the other hand, depressive states may affect the specific species of intestinal microbiome (Winter et al., 2018).

Another evidence confirming the important role of microbiome in an episode of depression is the transplantation of mycobacteria from individuals affected by depression to healthy people, resulting in a change in behavior characteristic for anxiety and depression, measured by behavioral tests (Kelly et al., 2016). In monkeys, it has been observed that the reduction of *Bifidobacterium* and *Lactobacillus* levels may be caused by stress resulting from separation of offspring from the mother (Bailey and Coe, 1999). The withdrawal from mothers for 3 hours a day between 2 and 12 days after birth changes the proper functioning of the microbiota-brain axis, increases sensitivity to stress and may result in increased susceptibility to diseases such as depression in adulthood (O'Mahony et al., 2009). In clinical trials, it has been shown that the microorganisms of the Bacteroides family correlate with the appearance of depression (Naseribafrouei et al., 2014). Interestingly, intestinal microorganisms (e.g. *Enterococcus faecalis*) can affect the bioavailability and efficacy of the drug, because it has been demonstrated that microorganisms are able to alter the activity of tyrosine and L-dopa decarboxylase in Parkinson disease patients (O'Neill, 2019).

Due to numerous observations of the influence of microbiota on the host's psyche, a new term "psychobiotic" was introduced, defined as a living microorganism, whose consumption in appropriate quantities provides health benefits in patients suffering from mental illness (Dinan et al., 2013; Liu et al., 2018; O'Connor, 2017).

However, there is data of insufficient efficacy of psychobiotics in relieving anxiety. Liu et al. in 2018 performed a study on a group of 1551 people taking placebo and probiotics and found no differences in the context of relieving the symptoms of anxiety (Liu et al., 2018). Although the relationship between stress-related mental disorders such as depression and the gut-brain axis has not been fully explained, psychobiotics may be potential pharmaceuticals for patients suffering from mental illness (O'Connor, 2017; Liu et al., 2018). Due to their effect on the microbiota-gut-brain axis, probiotics

have antidepressant potential. Compared to pharmaceuticals, they are more effective in reducing symptoms associated with major depressive disorders and have fewer side effects (Yong et al., 2020). Probiotics can also have negative effects on the host organism, especially among immunocompromised patients, where probiotic translocation may take place. *Lactobacilli* are able to cause opportunistic infections, *Lactobacillus* and *Bifidobacterium* infections contribute to endocarditis and bacteremia (Liong, 2008; Kubiszewska et al., 2014). Addition of probiotics may modulate immune system and increase the response to allergens or vaccines, it may also produce billiard salt hydrolase, the excess of which is converted into secondary bile acids, which in turn increases the risk of colorectal cancer (Zawistowska-Rojek and Tyski, 2018).

Thus, nutritional interventions have enormous potential for treating diseases related to mental disorders such as depression at an early stage of exposure. But general understanding of bacterial transfer and of intervention plans for the uptake of beneficial bacteria to the offspring for normal organism development remains limited. We should also learn more about the side effects of probiotics and their effectiveness on a large scale. Hence, more research is needed to check the exact interaction of probiotics, which have a significant impact on overall health. Numerous studies conclude that the correlation between intestinal microbiota and human health requires future research and will be the focal point of interest for neuroscience in the next decade (Smith, 2015).

There is a relationship between stress-related mental disorders such as depression and the brain-gut-microbiota axis.

There are many publications describing the relationship between the gut microbiota and the brain.

The links between intestinal microbiota compounds with neuropsychiatric disorders.

Psychobiotics may be potential pharmaceuticals for patients suffering from mental illness.

LIST OF ABBREVIATIONS

5-HT – serotonin;
5-HTT – serotonin transporter;
BDNF – brain-derived neurotrophic factor;
CNS – central nervous system;
CRF – corticotropin releasing factor;
CRP – C-reactive protein;
GABA – gamma-aminobutyric acid;
GF – germ-free;
HAM-D – Hamilton Depression Rating Scale;
HPA axis – hypothalamic-pituitary-adrenal axis;
IFNγ – interferon gamma;
IL-1RA – interleukin-1 receptor antagonist;
MDD – major depressive disorder;
NPY – Y peptide;
NT – neurotrophins;
PP – pancreatic peptide;
PVN – paraventricular nucleus;
PYY – YY peptide;

SON – supraoptic nucleus;
TET1 – Ten-Eleven Translocation methylcytosine dioxygenase 1;
TLRs – toll like receptors;
TNFα – tumor necrosis factor alpha;
TrkB receptor – tyrosine receptor kinase B;

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Supply of fat-soluble vitamins in the daily food rations of five-year-old children from the urban environment

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ABSTRACT

A rationally balanced diet provides the child with the right amount of energy and all the nutrients necessary for the proper development and functioning of the body. Scientific reports emphasize the essence of the influence of vitamins supplied with the diet and dietary supplements on the health condition in childhood, which at the same time translates into optimal health condition later in life. The aim of the study is to assess the content of selected fat-soluble vitamins in daily food rations of children attending public and non-public kindergartens in Biała Podlaska. The group of 454 children (251 boys and 203 girls) aged 5 was included in the observations. The vitamin supply in the daily food ration was assessed on the basis of the average daily intake of vitamins with meals during the child's stay in kindergarten (the method of current recording of consumed products, dishes and drinks) and outside the preschool institution (3-day dietary record with parents of the studied children). Estimation of the supply of selected vitamins was carried out using of the computer program "Dieta.5.0" (IŻŻ). The assessment of the degree of implementation of the nutritional recommendations was based on the Polish nutrition standards for children aged 4-6, updated in 2012 and standards of Institute of Medicine (US). The conducted studies indicate insufficient supply of vitamin D, E and too much high intake of vitamin A.

Keywords: children, vitamins, food rations

INTRODUCTION

A rationally balanced diet provides the child with the right amount of energy and all nutrients necessary for the correct development and functioning of the body. The daily food ration of a child should contain products from all groups, because an appropriate model of nutrition satisfies the demand of the developing organism for dietary macro- and micronutrients, including vitamins (Charzewska, 2011; Gidding, 2015; Voortman, 2015; Huk-Wieliczuk, 2017). Scientific reports emphasize an important effect of vitamins supplied with food and dietary supplements on the state of health during childhood which, at the same time, is translated into an optimum health condition in later life.

Vitamins are divided into two groups according to their solubility: water-soluble (including vitamin C and B-group vitamins), and fat-soluble (vitamins A, D, E, K). Fat-soluble micronutrients participate in many physiological processes taking place in the human body. Many scientific studies indicate that the deficiency of these vitamins may contribute to an increased mortality risk due to cancerous diseases, type 2 diabetes, may disturb the function of the immune system, and favour the development of metabolic syndrome (Szostak-Węgierek, 2008; Peixoto Paes-Silva, 2019; Huk-Wieliczuk,

2020). The deficiency of fat-soluble vitamins is frequently observed in persons with fat absorption disorders, or when the supply of these vitamins with food is insufficient (Peckenpaugh, 2011). Interactions between these nutrients, especially between vitamins A and D, also exert an influence on the effect of individual vitamins (Albahrani, 2016, Zhang, 2020).

The aim of the study is assessment of the content of selected fat-soluble vitamins in daily food rations of children attending public and non-public nursery schools in Biała Podlaska.

MATERIALS AND METHODS

The study was conducted in 2016 in 12 nursery facilities (public and non-public) in Biała Podlaska, in which the consent for observation of children was obtained from parents. The study was approved by the Ethics Committee for Scientific Research at Jozef Pilsudski University of Physical Education in Warsaw (Research project DS. 246)

The observations covered a group of 454 children (251 boys and 203 girls) aged 5 years. Based on the threshold values for BMI established by Cole (2000, 2007) nearly 70% of the examined children had a normal body weight, while every eighth was underweight or overweight, including obesity – 4%.

The supply of vitamins in daily food rations was estimated based on their daily consumption with meals during the stay of the child in the nursery school (the method of keeping regular records of products, meals and beverages consumed), and outside the nursery facility (3-day dietary record with parents of the children in the study). While carrying out the dietary questionnaire the "Album of photographs of food products and dishes" (Szponar, 2000) was used developed by the Institute of Food and Nutrition in Warsaw, which enabled the assessment of the size of portions of products, dishes and beverages consumed by the examined children The content of the selected vitamins in menus was evaluated using the computer software "Diet.5.0" (Institute of Food and Nutrition). The results obtained were referred to the up-dated in 2012 Polish dietary standards for children aged 4-6 developed by the Institute of Food and Nutrition (Jarosz, 2012); in the case of supply of vitamin A – Estimated Average Requirement (EAR), vitamin E and D – Adequate Intake (AI).

RESULTS

Table 1 presents the mean content of fat-soluble vitamins in a daily food ration of children at pre-school age, according to gender, whereas Figure 1 presents the degree of implementation of the recommended dietary standard. The mean values of vitamin A in daily meals of the children ranged from 801 μg (in girls) to 837.89 μg (in boys). Also, the diets of boys, compared to those of girls, had a higher content of retinol (270.52 μg and 256.94 μg, respectively) and β-carotene (3,462.02 μg and 3,356.98 μg). With respect to dietary recommendations an excessive supply of vitamin A was noted in the whole group (by 274%); in the diets of girls the recommended value was exceeded by more than 267%, while in boys – by 279%. An opposite tendency was observed in the case of two other vitamins analyzed. The daily food rations of the examined children showed a very low level of implementation of the recommended

standard for vitamin D (17%). Boys consumed a greater amount of this vitamin in meals than girls – 1.82 vs. 1.45 μg. The supply of vitamin E, for which the estimated mean content in a daily food ration for the whole group was 5.14 mg, ranged from 4.95-5.37 mg, according to gender.

The implementation of dietary recommendations for this nutrient remained on the level of 86% for the whole group of pre-school children in Biała Podlaska, and was slightly higher in the diets of girls than boys – 89% vs. 82%.

Table 1. Content of fat-soluble vitamins in daily food rations is test group of girls (n = 203) and boys (n = 251)

Nutrient	girls		boys	
	x ±SD	Min-max	x ±SD	Min-max
Vitamin A (μg)	801 ±355.51	127.18-212.89	837.89 ±440.51	102.07-2481.02
incl. retinol (μg)	256.94 ±149.83	24.18-902.76	270.52 ±212.21	15.29-1305.68
β-carotene (μg)	3356.98 ±2742,15	95.51-3,091.74	3462.02 ±2535,87	92.83-18584.28
Vitamin D (μg)	1.45 ±1.60	0.08-12.33	1.82 ±3.75	0.11-54.53
Vitamin E (mg)	5.37 ±5.97	0.62-75.98	4.95 ±2.79	1.18-29.71

Source: own study

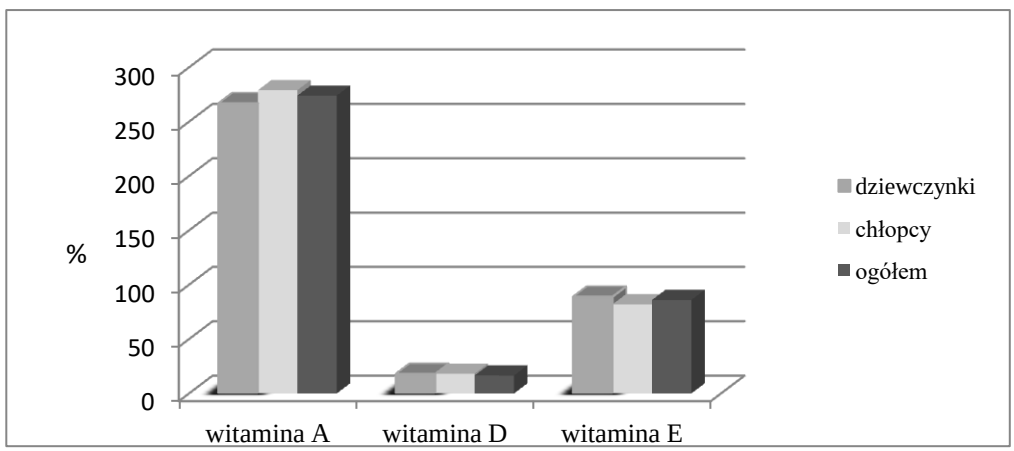

Figure 1. Implementation of standards [%] for vitamins A, D, E in daily diet of test group of is test group of girls (n = 203) and boys (n = 251). Source: own study

While analyzing the percentage of children from Biała Podlaska with an excess or deficiency of fat-soluble vitamins in their diet it was found that the content of vitamin A in a daily food ration of 3.74% of children (1.94% of girls and 5.22% of boys) was below the recommended standard. Meals of more than 94% of pre-school children (95.15 % of girls and 93.15% of boys) were characterized by an excessive (beyond dietary recommendations) amount of a given nutrient. In the context of the conducted study it was noted that the daily food rations of 72% of children (73.08% of girls and 71.31% of boys) did not supply the proper amount of vitamin E. In turn, in the diet of 62% of the examined children (a higher percentage of boys than girls) an excess of this

vitamin was observed. In the model of nutrition of nearly all the pre-school children in the study the content of vitamin D was below the recommended values.

DISCUSSION

Unbalanced content of fat-soluble vitamins in the diets of children is the cause of many current diseases and an unfavourable prognosis for their health in the future.

Vitamin A exerts an effect on the proper development, growth, proliferation and differentiation of cells, and plays an important role in the processes regulating vision. With the deficiency of this vitamin there occurs an impaired functioning of the immune system, and an increase in the percentage of occurrence of contagious diseases in the population. However, also an excess of this vitamin may lead to an excessive excitability and irritability of children, dermal changes, gum bleeding, loss of calcium from the bones and, in consequence, the development of osteoporosis (Penniston, 2006). The presented study showed that the supply of vitamin A in meals exceeded the daily demand for this nutrient nearly 3 times, irrespectively of gender of the examined pre-school children. The results of own study are close to those obtained by Górnicka (2011), who analyzed the content of the selected vitamins in planned food rations in nursery facilities. According to the researchers the amount of vitamin A in menus of pre-school children from Warsaw remained on the level of 914.5 μg, which is a value 3 times higher than the standard. Also, Orkusz (2016) observed that the supply of vitamin A in the analyzed pre-school rations was more than twice as high as the standard; the demand for vitamin A was satisfied in 227.9% in spring, in 254.5% in summer, in 279.4% in the autumn, and in 251.1% in winter. Similar findings were reported by Rogalska-Niedźwiedź (2008), who focused on the population of children (mean age 4.6 years) living in urban centres of different sizes; the mean content of vitamin A in the diet of the examined children ranged from 133-1,400 μg. Also, Hamułka (2003), while assessing the energy and nutritional value of ten-day menus of pre-school children confirmed the proper content of vitamins E, C, PP and B1, whereas the consumption of vitamin A was too high, compared to the recommended dietary standard. The supply of this vitamin in the amount of 1,686 μg in a daily food ration 2.5 times exceeded the recommended values. Similar results were obtained by Sadowska (2010), who monitored the mode of nutrition of children attending the selected nursery schools in Szczecin – every fifth child was overweight or obese, and the recommendations concerning consumption, including vitamin A were exceeded more than twice. The observed excessive supply of a given nutrient was interpreted by many researchers. Orkusz (2018) stated that a high content of vitamin A in the meals of children results from serving excessive amounts of butter and margarine, which are present in a mainly invisible form, in cakes served in the afternoon, and in egg pastes prepared on the basis of egg yolks. In turn, Kozłowska-Wojciechowska (2005), as well as Szczepaniak, (2002) reported that children most willingly choose vegetables with slightly sweet taste (carrots, potatoes, tomatoes, cucumbers), which may result in an unbalanced content of vitamin A in their daily food ration. The excess of this vitamin may also result from the fact that parents of the examined children add dietary supplements to their diet.

According to scientific reports an elevated level of β-carotene decreases obesity (preventing the oxidation of LDL shows an anti-atherosclerotic effect), and correlates with a lower frequency of occurrence of the metabolic syndrome (Beydoun, 2018;

Coronel, 2019). In own observations β-carotene contributed more to the supply of vitamin A, compared to retinol, which is a favourable phenomenon. In the examined group of pre-school children from the area of Biała Podlaska, a too low consumption of vitamins D and E was observed, both among girls and boys.

The mean content of vitamin D in the diets of children aged 5 years remained on the level of 11.23% of implementation of the recommended standard, and this is a result which is very alarming. In the given group of pre-school children as many as 40% of them did not consume fish at least once a weak, boys more often than girls (42% and 36%, respectively). The deficiency of vitamin D seriously impairs the physiological balance of the body, and may contribute to the development of many diseases, such as rickets, dental caries, growth disorders, or neurological disorders. An insufficient amount of this endogenous vitamin in the body results in an increased morbidity due to infections. The children are apathetic, may feel chronic fatigue, bone pain and spontaneous joint pain, vision disorders, or sleep problems. There may be a relationship between vitamin D deficiency and headache (Donmez, 2018).

The role of this vitamin in the development and treatment of autoimmune diseases is emphasized (Lisowska, 2017). The results of own study correspond with those obtained by Dymkowska-Malesa (2013), who observed that the mean supply of vitamin D in the diets of children from Koszalin was only 11% of the implementation of the recommended standard, and none of the ten-day menus supplied pre-school children with the sufficient amount of this vitamin. A study by Merkiel (2015) confirmed an insufficient consumption of vitamin D in the group of children aged 4-6; the mean content of this vitamin in children's diet ranged within 1.25-1.83 μg.

The results of own study also demonstrated an insufficient supply of vitamin E in the diet of children from the area of Biała Podlaska, on the level of 14%. The results obtained are in accordance with the data concerning the nutritional model for children aged 1-3 years living in Germany, Russia, and the USA; deficiencies of vitamin E reached over 20% (Hilger, 2015). The cause of too low consumption of this nutrient may be the unwillingness of children to eat vegetables, because its main source in meals are vegetable oils added to salads (Sadowska, 2010; Johnson, 2016).

Inappropriate content of fat-soluble vitamins in the diets of the examined children indicates the need for undertaking actions in order to increase knowledge concerning the role of these nutrients in food, in the context of prevention of civilisation diseases, including those cancerous.

SHORT CONCLUSION

The daily ration of pre-school children from Biała Podlaska with respect to the supply of fat-soluble vitamins did not satisfy dietary recommendations. A high supply of vitamin A was accompanied by too low consumption of vitamins D and E. The observed anomalies in the mode of nutrition evidence the necessity for monitoring nutrition in pre-school facilities in the area of Biała Podlaska, and for undertaking actions related with the nutritional education of parents and nursery school staff.

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Dietary supplements, OTC drugs and other alternative treatment options in fighting the COVID-19 pandemic

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ABSTRACT

The COVID-19 pandemic poses an enormous challenge to health care professionals, as well as researchers and scientists, seeking effective and safe ways to prevent SARS-CoV-2 infection and treat COVID-19 in a variety of clinical conditions. In addition to antivirals, inflammatory inhibitors, or anticoagulants currently used in therapy, dietary supplements and OTC – over-the-counter – medications are also being considered in these deliberations. This paper presents a review of studies and available literature on the usefulness of selected medicinal preparations in the prevention of new coronavirus infection and in the treatment of patients suffering from COVID-19. Biological mechanisms of their effect on body cells are presented, which justifies their postulated usefulness in the fight against COVID-19 pandemic according to the authors of the cited works. A critical analysis of available source materials allows us to conclude that there is a great need for multicenter clinical trials of high quality, in accordance with the principles of Evidence Based Medicine. They will allow to obtain the best reliable up-to-date data, thanks to which the available scientific evidence can be accurately and precisely used in everyday clinical practice. In the preparation of this paper, source materials were used from PubMed, a publicly available database of articles in medicine and the life sciences, and the U.S. National Institutes of Health website, which contains information on registered clinical trials conducted around the world.

Keywords: COVID-19 pandemic, dietary supplements, OTC drugs

INTRODUCTION

COVID-19 is an infectious disease caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) which leads to severe respiratory illness with accompanying vascular damage, microangiopathy and thrombosis. The initial symptoms of infection, taking form of typical upper respiratory tract infection may relatively quickly develop and cause dyspnea, pneumonia and worsening of general condition. In the following stage of disease, the ongoing cytokine storm and hyperinflammation may lead to multiorgan failure, exhaustion of regeneration capacity of the system and death. The analysis of epidemiological data of the Polish Society of Epidemiology Infectious Diseases (Feb 26, 2021) shows that the death rate due to COVID-19 is closely related to the age of patients. In Poland the death rate amounts to 7,3% in total and it reaches 22,6% in the age group over 80 (SARSTer database by Polish Society of Epidemiology and Infectious Diseases). At the same time, the mortality rate among people requiring mechanical ventilation is set at 66.7%, and the total number of deaths due to COVID-19 has exceeded 50,000 per year since the first case of infection with the new coronavirus appeared in our country (data from COVID-19 Dashboard by the Center for Systems Science and Engineering at Johns Hopkins University).

Increasing daily number of infections and emergence of new virus variants: B1.351 ("South Africa" Variant), B.1.1.28 ("Brazil" Variant) and B.1.1.7 ("U.K." Variant) with potentially greater infectivity than that causing the primary outbreak in China in 2019 and then in Poland in March 2020, presents medical services with a huge challenge, and the return to normality before the outbreak of the pandemic seems to be very distant. Apart from the emphasis on respecting sanitary rules and attempts to limit the horizontal transmission infections, as well as active detection of infected people, the most important activities currently include the development of effective and safe preventive vaccinations and therapeutic regimens that will increase the survival rate of patients with COVID-19 in various clinical conditions. This goal was achieved partially. It was possible thanks to the development and introduction of several vaccine formulations and the initiation of mass vaccination of the population. Gibraltar and Israel are among the fastest vaccinating countries by population (data from The Our World in Data – Coronavirus (COVID-19) Vaccinations by University of Oxford).

Potential therapeutic options include the use of antiviral drugs (e.g. remdesivir), inflammation inhibitors (tocilizumab, anakinra, baricitinib, glucocorticoids, quinoline derivatives), anticoagulants and the use of convalescent plasma (Stasi. In the face of a pandemic, drugs are being sought to alleviate and shorten the course of the disease, as well as substances that will potentially avoid infection or reduce the risk of severe COVID-19. They also include dietary supplements and the so-called OTC drugs – dispensed without a prescription. This paper presents some of the most popular preparations from these groups, as well as selected drugs registered in other clinical states, the effectiveness of which is currently being considered by researchers, scientists and clinicians.

SEARCH STRATEGY AND SELECTION CRITERIA

In the preparation of this study, source materials from the public database collecting articles in the field of medicine and life sciences – PubMed (https://pubmed.ncbi.nlm.nih.gov) and the website of the U.S. National Institute of Health (clinicaltrials.gov) containing information on registered clinical trials, were used.

REVIEW

DIETARY SUPPLEMENTS AND OTC PREPARATIONS IN PREVENTION AND THERAPY COVID- 19

In works concerning dietary supplements and OTC drugs, presenting their influence on the course of SARS-CoV-2 disease, and including both prospective and retrospective studies, fulfilling the conditions of Evidence Based Medicine, the influence of vitamin D is most often analyzed. This interest stems from the properties described at the beginning of the 21st century by which it can regulate the immune response (Griffin, 2000). The most common form of vitamin D in humans is cholecalciferol (D3). In the process of hydroxylation occurring in the liver and kidneys, it is converted to the active form – calcitriol (1,25-(OH)2D). In this form, due to its structure and numerous functions it performs, vitamin D is called a hormone (Chen, 2007). Circulating in the blood, it binds with specific receptors (VDR), present, among others, in bone, muscle, adipose

tissue, skin, intestine, kidneys, pancreatic islets, as well as in T and B lymphocytes, monocytes and antigen presenting cells (APC) (Myszka, 2014).

Apart from well-known influence on calcium-phosphate balance and role in maintaining correct structure and function of bones, vitamin D has also a number of actions modulating immunological processes – it influences activation and proliferation of lymphocytes, differentiation of Th lymphocytes, production of specific antibodies and regulation of immunological response. It inhibits the production of proinflammatory cytokines and stimulates the production of anti-inflammatory substances, as well as antimicrobial peptides (PAD): cathelicidin and defensin, establishing a balance of the immune response to anti-inflammatory actions with the predominance of innate mechanisms (Witkowska, 2008; Yin, 2014).

The participation of vitamin D as a regulator of immune response and the mechanisms of this action have been presented in works on infectious diseases (including viral: HRV – Human Rhinovirus, RSV – Respiratory Syncytial Virus, influenza virus, HCV – Hepatitis C Virus) and chronic diseases: cancer, cardiovascular, autoimmune, diabetes and others (Siddiqui, 2020; Wang, 2017). The immunomodulatory role of vitamin D is now extensively described in papers on the course of COVID-19. One available analysis presents a 6-week continuous prospective observational study involving 154 patients aged 30-60 years. The purpose of this study was to determine whether vitamin D levels in patients hospitalized with COVID-19 (infection confirmed by RT-PCR) affect disease severity. Asymptomatic patients were designated as Group A (91 patients) and those requiring medical care in the intensive care unit were designated as Group B (63 patients). The parameters measured in both groups were 25(OH)D levels and inflammatory markers: IL-6, TNF-α and serum ferritin. The authors used standard statistical analysis and estimated that the mean vitamin D level in group A (27.89 ±6.21 ng/mL) was higher than in group B (14.35 ±5.79 ng/mL), and the difference was statistically significant. The prevalence of vitamin D deficiency was 32.96% in the asymptomatic group and 96.82% in the severe COVID-19 group. Of all 154 patients, 90 had vitamin D deficiency (group A: 29; group B: 61). They also found that serum levels of inflammatory markers were higher in patients with vitamin D deficiency, and the mortality rate for vitamin D deficiency was estimated to be high (21% vs. 3.1%) (Jain, 2020).

Inflammatory markers in COVID-19 and the correlation between their levels and vitamin D levels were also analyzed in another study of 216 patients. The authors showed that COVID-19 patients had statistically significant lower vitamin D levels than population controls (13.8 ±7.2 ng/mL vs. 20.9 ±7.4 ng/mL). Vitamin D deficiency was found overall in 82.2% of COVID-19 cases and 47.2% in the control population. Vitamin D concentration was significantly negatively correlated with serum ferritin and D-dimer levels. A similar trend was also observed with C-reactive protein levels, but it was not statistically significant ($p = 0.083$). However, there was no statistical correlation between serum vitamin D and IL-6 levels in COVID-19 patients. In assessing the clinical course, the authors observed a statistically significant better oxygenation index (PaO_2/FiO_2), less frequent radiographic progression of disease, shorter intensive care unit stay, and shorter total hospitalization in patients with vitamin

D levels equal to or greater than 20 ng/mL compared with vitamin D deficient patients (Hernández, 2021).

The results of these and other previous studies suggest that vitamin D, as an immunomodulator and suppressor of pro-inflammatory factors, prevents the development of the so-called "cytokine storm", which is an excessive, abnormal immune response to a pathogen. During this process IL-2, IL-7, IL-10, TNF-α, G-CSF, CCL2, CCL3, CXCL10, CRP, ferritin, D-dimers and others are released in significant amounts. This unrestrained stimulation of the immune response, in the case of COVID-19, leads to a severe course of the disease with frequent clinical features of ARDS, thromboembolic complications and multi-organ failure (Hojyo, 2020).

Studies on geriatric populations are also available. One of these (GERIA-COVID) compared the course of COVID-19 in three groups of patients. The first was comprised of patients who had been supplementing vitamin D at a dose of 50,000 IU/month or 80,000-100,000 IU/2-3 months for at least one year prior to the disease. The second group consisted of patients who had not supplemented vitamin D prior to onset of illness, but started vitamin D several hours after diagnosis of COVID-19. The third group consisted of patients who had not supplemented vitamin D before or after onset of COVID-19 (no patient consent). Patients were evaluated according to the 8-point WHO Ordinal Scale for Clinical Improvement (in which 0 indicates no symptoms of disease and 8 indicates death). A score equal to or greater than 5 indicates a severe course of COVID-19. Patients were evaluated during admission and then repeated when clinical status changed. The study authors assessed the 14-day risk of death in the three groups of patients, finding a statistically significant negative correlation between regular vitamin D supplementation (group 1) and 14-day mortality. In contrast, starting supplementation after COVID-19 diagnosis (group 2) was not associated with a lower risk of mortality within 2 weeks of hospital admission (Annweiler, 2020).

Another important mechanism by which vitamin D may be beneficial in COVID-19 is by modifying the effect that SARS-CoV-2 virus has during cell invasion on ACE2. Angiotensin-converting enzyme (ACE) is part of the Renin-Angiotensin-Aldosterone system and causes the conversion of angiotensin I to angiotensin II, increasing blood pressure by increasing the volume of circulating blood retained. ACE2 under normal conditions results in less angiotensin I as a substrate for ACE (it cleaves angiotensin I and angiotensin II, and the end result of this process is an increase in vasodilator angiotensin 1-7) (Vickers, 2002). ACE2 is also a receptor used by SARS-CoV and SARS-CoV-2 viruses to enter the host cell. The down-regulation of available ACE2 that occurs in this manner results in an increase in free angiotensin II, which has deleterious effects on epithelial, lung, kidney, and gastrointestinal cells that have been shown to express ACE2 (Harmer, 2002). In practice, high levels of angiotensin II can cause ARDS or cardiovascular injury in COVID-19. Renin, a proteolytic enzyme and positive regulator of angiotensin II, is strongly inhibited by vitamin D. Thus, vitamin D supplementation prevents the accumulation of angiotensin II and reduces its pro-inflammatory activity by inhibiting renin release in COVID-infected patients, thereby reducing the risk of ARDS, myocarditis or cardiac damage (Mercola, 2020).

A dominant number of studies on the impact of vitamin D on the course of COVID-19, similarly to the cited works, are observational or retrospective in nature. Although most meta-analyses agree on the significance of 25(OH)D levels in the course of COVID-19, there are also publications in which the authors did not confirm the relationship between vitamin D deficiency and the course of coronavirus infection. In their conclusions, they indicate that this correlation is still largely hypothetical and requires further analysis (Hastie, 2020; Rubin, 2021). On the basis of available data, it is difficult to formulate new recommendations on the doses of vitamin D supplementation and, therefore, in Poland it is still assumed that the guidelines used so far, presented in the recommendations edited by Agnieszka Rusińska – "Principles of vitamin D supplementation and treatment – amendment 2018" are valid and sufficient (http://mavipuro.pl/jourarch/PN2018001.pdf). However, it should be emphasized that the situation regarding vitamin D research is very dynamic. Currently, 62 clinical trials on the effect of vitamin D on COVID-19 are registered (clinicaltrials.gov). It allows to suspect that subsequent publications (especially randomized trials) will provide more reliable conclusions and practical solutions in this regard.

Another, very popular supplement also considered in the context of modifying the course of SARS-CoV-2 infection is zinc. In the cell, zinc is an essential element for many enzymatic activities. It acts as a cofactor in the synthesis and metabolism of proteins, carbohydrates, fats and nucleic acids (McCall, 2000). Zinc deficiency may cause hair loss, diarrhea, growth retardation, delayed puberty, and hypogonadism in males. It is also associated with impaired immune function. Zinc is critical for the normal development and function of cells mediating innate immunity, neutrophils and NK cells. Its deficiency also affects macrophages. It impairs phagocytosis and cytokine production, and has adverse effects on the maturation and function of T and B lymphocytes (Prasad, 2008). Zinc has been proven to inhibit the synthesis, replication, and transcription complex of coronaviruses. It can also interfere with virus replication and synthesis of its proteins, providing a therapeutic effect in viral infections (Skalny, 2020).

Zinc by itself, however, does not have as much antiviral significance as originally suspected. It's action is possible by combining with ionophores – hydrophobic molecules capable of transporting ions through the lipid layer into the cell (e.g. polyphenols). Chloroquine and hydroxychloroquine, organic chemicals with antiprotozoal, antiviral, and anti-inflammatory activities, can also play the role of ionophore. In in vitro studies, the introduction of zinc into the cell due to the presence of ionophore allowed the demonstration of antiviral properties of zinc in the form of inhibition of RNA polymerase activity of SARS-CoV virus (te Velthuis, 2010). Zinc thus plays a supportive role in viral infection in patients already treated with e.g. hydroxychloroquine. This conclusion was also drawn from an observational study comparing the effects of treatment in patients diagnosed with COVID-19. A 24% reduction in the risk of in-hospital mortality was observed in these patients, while neither zinc alone nor ionophore alone reduced it (Frontera, 2020).

Unfortunately, the vast majority of available studies on zinc concentration or supplementation in the context of SARS-CoV-2 infection are reviews, often contain conflicting findings, or present only a very loose association between the two. An example is the

work linking the presence of zinc deficiency in subjects to the presence of risk factors for severe COVID-19, rather than to actual disease incidence or course (Mossink, 2020). There are currently 20 registered clinical trials investigating the role of zinc in COVID-19 (clinicaltrials.gov). Three of them have the status of completed, and only one of them is a randomized clinical trial with more than 200 patients. Its objective was to compare COVID-19 course and symptom reduction in four groups of patients diagnosed with SARS-CoV-2 infection treated on an outpatient basis. The following interventions were conducted: group 1, zinc supplementation; group 2, ascorbic acid supplementation; group 3, zinc and ascorbic acid supplementation; group 4, no supplementation. In the results obtained, the authors did not show a significant reduction in the duration of symptoms in patients in the study groups compared with the group receiving standard care (Thomas, 2021). Considering the mechanism of antiviral action of zinc, the results of intervention studies in which hydroxychloroquine was used in addition to zinc supplementation seem to be relevant. There are currently 6 such clinical trials and another 3 involve zinc supplementation and the use of ivermectin (clinicaltrials.gov).

Selenium is another element whose importance in the context of pandemics is under scientific consideration. Together with vitamin E, it acts through antioxidant pathways, increasing NK cell activity and stimulating T cell activation, proliferation and differentiation. A selenium derivative has been proven to have the ability to react with the sulfhydryl groups in the viral isomerase (PDI) active site, converting them to an inactive disulfide. In this way, the virus loses its ability to enter the cytoplasm of a healthy cell (Kieliszek, 2020; Shakoor, 2020). An association between selenium concentrations among residents of specific regions of China and COVID-19 cure rates in those locations has also been described. In a retrospective population-based analysis, it was shown that in Enshi city, Hubei province, which has the highest selenium intake in China, the cure rate was almost three times higher than the average for all other cities in Hubei province. In contrast, in Heilongjiang province, where selenium intake is among the lowest in the world, mortality from COVID-19 was nearly five times higher than the average for all other provinces except Hubei. However, this study has significant limitations. The analysis did not take into account the age of the patients or their comorbidities. There is also no information on their treatment – site and drugs used, and data on selenium concentrations by region are from 2011 or older (Zhang, 2020).

In a European observational study of 33 COVID-19 patients, selenium levels, levels of the trace element transporter (SELENOP), and the enzymatic activity of glutathione peroxidase 3 (GPx3), which is an enzyme composed of four subunits, each containing a selenium atom, were evaluated. The authors reported that patients suffering from COVID-19 are deficient in all three assayed parameters. They suggested that individuals living in areas with a poor baseline selenium supply or on diets that exclude selenium sources, and COVID-19 patients with pre-existing comorbidities or a long course of disease, are particularly vulnerable to severe selenium deficiency and may benefit from adequate selenium supplementation. They emphasized that the observed association of mortality risk with selenium deficiency and the likely feedback mechanism support the initiation of intervention studies according to the highest quality standards (Moghaddam, 2020). They also registered a randomized clinical trial to

evaluate the early and late response to influenza and COVID-19 vaccination in subjects taking a combination formulation of beta-glucans and a probiotic rich in selenium and zinc (ABBC1) (clinicaltrails.gov).

OTHER SELECTED MEDICINAL PRODUCTS-THYMOSIN, BCG VACCINE AND AMANTADINE IN THE PREVENTION AND THERAPY OF COVID-19

Thymosin is a thymic peptide hormone found in the α1 and β4 form of thymosin. T α1 acts as an immunomodulatory drug, accelerating maturation and inhibiting apoptosis of T cells and regulating inflammatory mediator activity. Its benefits in sepsis patients have been described in terms of reduced 28-day mortality and modulation of the anti-inflammatory response. In addition, thymosin may also prevent the phenomenon of cytokine storm (Liu, 2016). Its pleiotropic effect, stimulating antiviral response while reducing inflammation caused by bacterial infections, has been pointed out in the past (Giacomini, 2015). As an immune modulator, it exerts a major biological effect on the regulation of immune system function in many diseases, including sepsis, chemotherapy-induced immunosuppression, and acquired immunodeficiency syndrome. Currently, there are no reliable data on the clinical efficacy of thymosin α1 in patients with COVID-19.

The available retrospective study evaluated the potential therapeutic efficacy of thymosin α1 in critically ill COVID-19 patients. It collected clinical data from 8 centers in China. A significant difference in 28-day patient mortality was observed between the thymosin and no-thymosin treatment groups. At the same time, no significant difference in 60-day mortality and overall survival time was described. The reduction in short-term mortality was in critically ill patients, especially those older than 64 years, with leukocyte count > 6.8 × 109/l, neutrophil count > 5.3 × 109/l, lymphocyte count < 0.73 × 109/l, PaO_2/FiO_2 parameter < 196, SOFA organ failure score > 3, and APACHE II clinical status score > 7. The authors of this study concluded that the results suggest that thymosin α1 treatment may reduce early mortality and alleviate organ dysfunction in critically ill COVID-19 patients. The results provided clinical information regarding the selection of the population that may benefit most from thymosin α1 treatment for SARS-CoV-2 infection (Wu, 2020).

There are also reports of gender differences in response to thymosin treatment of critically ill COVID-19 patients. Statistically significant differences have been shown in the levels of the inflammatory markers CRP and IL-6, which is also associated with the development of more symptoms. After thymosin treatment, in women the mentioned parameters showed a decreasing trend. The decrease in CRP in men was not significant, and the level of IL-6 was significantly higher in them than in women. In particular, in patients in the age group above 65 years, the levels of IL-6, CRP and the ratio of IL-6 to IL-10 in men were significantly higher than in women. According to the authors' suggestion, after observing the mentioned relationships, the next step should be to identify gender-specific risk factors that could explain these differences (Li, 2021).

Among the registered clinical trials, there is currently one interventional randomized trial evaluating the effect of thymosin on the recovery time and course of COVID-19 in patients diagnosed with SARS-CoV-2 infection, and one exploratory trial evaluating

the efficacy of thymosin in preventing COVID-19 in patients with end-stage renal disease undergoing dialysis (clinicaltrials.gov).

The BCG (short for Bacillus Calmette-Guérin) vaccine was developed in France in the first half of the 20th century and first used in 1921. The isolated strain of bacteria causing tuberculosis in cattle served as a basis for the development of an attenuated live strain used for prevention of tuberculosis in humans. Most countries (including Poland since 1995) have a national vaccination policy using BCG. The exceptions are Germany, France, Spain, Great Britain and the United States, where the vaccine is not widely used (http://www.bcgatlas.org/). Apart from the prevention of tuberculosis-related mortality, an unexpected effect of BCG vaccination is a strong immunostimulation, which may also have a protective effect against infections with pathogens other than Mycobacterium tuberculosis – e.g. *Salmonella*, *Shigella* and especially the respiratory virus RSV and associated acute inflammation of the lower respiratory tract (Stensballe, 2005).

Important evidence showing that BCG protects against viral pathogens (DNA and RNA, and including herpes and influenza viruses) comes from studies in a mouse model. An experimental study also demonstrated the effect of BCG on viral infection in humans. Shortly after vaccination began in the 1920s, epidemiological studies showed that BCG vaccination strongly reduced infant mortality, and this was not just related to a reduction in the incidence of tuberculosis in infants. The reduction in deaths was primarily due to less frequent severe respiratory infections and neonatal sepsis. In later years, similar studies in other locations, including randomized trials with a control group, showed a 50% and even 70% reduction in mortality in the youngest age group with BCG vaccination (O'Neill, 2020).

These effects are thought to be mediated by induction of immunological memory and lymphocyte activation, resulting in increased cytokine production, macrophage activity, induction of T-cell responses and the presence of higher antibody titers (Moorlag, 2019). An important concept in the described mechanism is the so-called "trained immunity". It denotes a long-term functional, epigenetic reprogramming of transcriptional pathways, which is induced by exogenous or endogenous factors, and which leads to an altered response of innate immune cells to a second stimulation after returning to an inactive state. The secondary response to a subsequent nonspecific stimulus can be tailored to be stronger or weaker than the primary response, as needed (Netea, 2020).

The immunomodulatory effect of BCG vaccine is also used in oncology. The greatest success has undoubtedly been achieved in the treatment of superficial, epithelial, non-invasive bladder cancer, and intravesical BCG immunotherapy is described in the guidelines of the Polish Urological Association.

These nonspecific effects of BCG vaccination gave much hope in the first quarter of 2020, when consideration of the role the BCG vaccine could play in the fight against the SARS-CoV-2 virus pandemic began to appear en masse. According to the authors of an observational study, COVID-19 incidence and mortality were significantly lower in countries with continued population-based BCG vaccination compared to countries where vaccination is not universal – for every 10 percent increase in BCG vaccination

rates, there was a 10.4 percent decrease in COVID-19 mortality. However, these initial reports were quickly verified (Hensel, 2020) (Kleen, 2020; Kumar, 2020). Many of the epidemiologic observations conducted did not take into account potential confounding variables, including socioeconomic factors, comorbidities, and the rate of testing for SARS-CoV-2 infection. The last variable proved to be the most important element, fundamentally altering the results obtained and conclusions drawn. After taking these observations into account, the authors of a cross-sectional study evaluating the epidemiological situation of 74 countries concluded that there was no correlation between BCG vaccination policy and the spread of COVID-19. Their conclusions indicated that countries with continued universal BCG vaccination had significantly lower rates of SARS-CoV-2 testing per population than countries that had abandoned mandatory BCG vaccination.

The topic of the potential immunomodulatory effect of BCG vaccine in the context of preventing SARS-CoV-2 infection or severe COVID-19 remains of interest. The 24 registered interventional clinical trials on the effect of BCG vaccine, mostly aim to evaluate its efficacy in the prevention of COVID-19 in health care workers. One of them is an ongoing multicenter, randomized, placebo-controlled interventional study in Poland and is currently in phase 3 – "Clinical Trial Evaluating the Effect of BCG Vaccination on the Incidence and Severity of SARS-CoV-2 Infections Among Healthcare Professionals During the COVID-19 Pandemic in Poland" (clinicaltrials.gov).

Another agent described in the context of potential prevention of severe COVID-19 is amantadine. Amantadine is one of the adamantane derivatives that exhibit several biological activities, including antiviral, antibacterial, anti-inflammatory, antidiabetic, and central nervous system transmitter modification activity. Amantadine and rimantadine - amine derivatives – disrupt the replication cycle of influenza A virus (activity has been observed only against some subtypes). Their antiviral activity consists in damaging or blocking the function of the transmembrane domain of the viral M2 protein, which interferes with the process of viral clearance (Lis-Cieplak, 2012).

However, amantadine and rimantadine are not widely used in medical therapy due to the aforementioned limited activity, increasing viral resistance to adamantane derivatives, and due to side effects and interactions with other medicinal products (PubChem database https://pubchem.ncbi.nlm.nih.gov/compound/Amantadine). However, the main use of amantadine is in the treatment of dyskinesias associated with Parkinson's disease (PD). It exerts a blocking effect on NMDA receptors, thereby increasing dopamine levels in the central nervous system. Memantine is also the adamantane derivative most commonly used to slow cognitive decline in patients with dementia, including those with PD (Tipton, 2020).

One of the first papers to consider the use of amantadine in the prevention of severe SARS-CoV-2 infection was published in April 2020. Its authors presented the results of a study based on an interview questionnaire conducted with 22 patients with neurological disease (multiple sclerosis, PD or cognitive impairment) and SARS-CoV-2 infection, confirmed by rtPCR. Patients had been taking amantadine or memantine in fixed doses for at least 3 months prior to exposure. The authors of this study report that none of the patients developed clinical signs of infectious disease. They also reported

no significant changes in neurological status during the course of the primary nervous system disease (Rejdak, 2020). A similar paper was published in August 2020. This study included 15 patients who presented with symptoms of SARS-CoV-2 infection. The authors note that due to the lack of availability of serologic testing, the diagnosis was based on patient-reported symptoms in a questionnaire (loss of smell, gastro-intestinal distress, fever, dry cough, dyspnea, headache, nausea, rhinitis). Patients were treated with a combination of amantadine, azithromycin, celecoxib, and aspirin. In addition, some of them also received ipratropium/salbutamol by nebulization. Later, patients were confirmed to have IgG SARS-CoV-2 antibodies (Aranda-Abreu, 2020).

The observational studies presented here are the only ones currently available that link amantadine and SARS-CoV-2 infection. They are notable for the small groups of patients followed, the lack of control groups, the low quality of the study instruments, and the very bold final conclusions. In the introduction explaining the motivation of the study, the authors refer to the results of in vitro studies. However, we find there information about the lack of direct translation of the results obtained in them to the utility of adamantane derivatives against SARS-CoV. However, they encouraged to conduct further studies on this topic (Torres, 2007).

The use of antivirals (including amantadine), antibiotics, and their combination in treatment of patients with COVID-19 was assessed in a retrospective observational study conducted in 2020 in New Mexico (Mancilla-Galindo, 2021). The authors included 136,855 patients in various age groups (mostly middle-aged adults) with RT-PCR-confirmed SARS-CoV-2 infection. The study evaluated the survival of ambulatory and hospitalized, critical and non-critical patients according to the therapy used. Of all patients, 10.0% received antibiotics only; 3.0% – antivirals only; 3.6% – antivirals plus antibiotics; and 83.4% – no medications. The authors reported that the antiviral drugs analyzed (oseltamivir, zanamivir, amantadine, rimantadine, acyclovir and lopinavir/ ritonavir) did not improve survival among patients with COVID-19, whereas oseltamivir was associated with an increased risk of death. In the study of interest, oseltamivir was the most widely antiviral used (8414 patients) and the authors emphasize that it was a highly-prescribed medication in Mexico City during pandemic. According to the authors, the unsatisfactory response to oseltamivir might be due to its mechanism, impairing the immune response and limiting the capacity to eliminate the infection. Improvement in survival was noted in critically ill patients who received antibiotics, whereas in the general population, they did not result in increased survival. The authors noticed some limitations of their study, like cointerventions matter, low diagnostic testing rate and limited accessibility to the Intensive Care Unit in New Mexico. They conclude that the hypotheses raised in the research should be tested in clinical trials.

There are currently no registered interventional clinical trials or high quality prospective studies in the clinicaltrials.gov system evaluating the efficacy of amantadine in the prevention of SARS-CoV-2 infection or the prevention of severe COVID-19. However, as reported by the Agency for Medical Research, on behalf of the Ministry of Health, ABM funded a study on the use of amantadine in the prevention of progression and treatment of symptoms of COVID-19 (Minister of Health's response to parliamentary interpellation – PLD.050.24.2021.IM).

SHORT CONCLUSION

Despite the availability in the PubMed database of many publications and articles on the topics addressed in this study, surprisingly few of them constitute high quality studies and medical experiments in accordance with the principles of Evidence Based Medicine (randomized placebo-controlled trials or meta-analyses). Most are review papers, in vitro studies, or case reports. This makes it difficult to draw firm conclusions about the usefulness, and sometimes safety of investigational substances and it impedes to include them in guidelines for the treatment of patients with COVID-19. The authors of the cited papers unanimously emphasize the need for involvement of government representatives and the research community in high-quality multicenter clinical trials. These must be activities based on the principles of EBM, and thus allow to obtain the best reliable current data, so that the available scientific evidence can be accurately and precisely used in everyday clinical practice.

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An ideal definition of tattoo does it exsist? Historical and sociological, medical and psychological, philosophical and ethical aspects

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ABSTRACT

Tattooing is a phenomenon with a multitude of meanings and descriptions.

Any attempt at giving a definition of the tattoo should follow the standard practice and go back to the beginnings. Next, it seems necessary to focus on the not-always-obvious function of a permanent body drawing as well as motives for its performance. The perception of tattoos is known to vary. Their esthetical value tends to arise controversies. Thus, what requires analysis is the assessment of the psychological condition of as well as the perception of sexual attractiveness by people deciding to have a permanent body drawing. Finally, a question should be posed about the limits of a change to the image of the tattoo bearer and possible modification of earlier tattoos – if only in view of how common the laser method of tattoo removal has become. The latter invites reflection, first and foremost on the side effects and potential risk of complications. It also encourages to consider the psychological reasons for the performance of a permanent body drawing, in the first place, and for the desire to have it later removed.

The study endeavours to sum up a possibly broad array of issues related to the phenomenon of tattooing in order to develop its universal and thus seemingly ideal definition. Yet, what cannot be forgotten is that the development of such a definition might, even inadvertently, constitute a predictor of an unequivocal assessment of the permanent body drawing discussed.

Keywords: tattoo, definition, body decoration, body deformation

INTRODUCTION

There are ample grounds to consider tattooing *one of the most radical and expressive instruments of creating an individual identity* (Dziuban, 2013). On the one hand, it affects both the body and the psyche and, on the other, it requires exceptional commitment as well as awareness of its durability. A tattoo also issues an extremely important communique which can be read in different ways, frequently in a stereotype way as associated with the criminal world or with a specific subculture. As if contrary to what has been said above, we seem to be witnessing a revival of sorts in the performance of this modification to the human body, with tattooing becoming an ever more perfect instrument of self-expression. Neither can it be ignored that tattooing can be interpreted in a variety of ways depending, for instance, on the historical period, ruling culture or religion or even field of science. Is it consequently possible to attempt to give an unequivocal definition, a *perfect* definition of tattooing which would include all its aspects?

TATTOO – ORIGINS AND NEW SCOPE

In one of the studies, we can read that tattoo is a pattern on the skin deliberately, voluntarily (with the exception of some cases where [...] it has the form of compulsion) made with the help of different techniques, performing different functions and having different meaning for its owner. Its performance consists in the insertion into a densely punctured or cut skin of a dye, as a rule according to a pattern previously drawn or impressed on the body (Snopek, 2009). The variety in the description of the phenomenon is most likely to result from the multitude of functions the tattoo performs such as, for instance, ink, pattern, emblem, engraving or dot in penitentiary environments. What should be drawn attention to is the nomenclature of the pattern-related tattoos dependent on a particular environment – zinc prison tattoos, anchor criminal tattoos or simply decorative tattoos (Przybyliński, 2007; Snopek, 2009).

According to a 1688 report, the word tattoo referred, initially, to a drum or trumpet signal calling soldiers or sailors back to their billets at night. The term was borrowed from the Dutch word taptoe meaning turning off the water as police had then the habit to visit taverns for exactly this purpose in the late hours of the night. The author of the first description of the tattoo as we understand it today was James Cook. In 1769, he wrote in his logbook that the Tahitians used the terms tatau and tatu to denote body marking with pigments. The definition was first officially published in 1774, and the word tattooing in 1777. In Europe, till the 18th century, the term tattoo was seen as standing for the techniques of body decorating and marking and was referred to with the Latin word signum, borrowed from the Luther Bible of 1534, stigma, graphism, hieroglyph, mark – from Les Miserables by Victor Hugo or, in Western Europe, an engraved drawing or the French word piquage. Tattooed people were called made-up or bulleted (Skrzypek, 2017; Snopek, 2009; Goldstein, 2007; Przybyliński, 2007).

In 1856, a Belgian, P. Nysten, was the first to place the word tattoo in the Dictionary of Medicine he authored. In his 1971 textbook, Thomas B. Fitzpatrick mentions briefly a traumatic entry of a foreign substance (for instance, a lead pencil), scarring due to a fall from a bicycle or a motorcycle [...] as well as chemicals such as gun powder (Goldstein, 2007). According to The Great Medical Dictionary of 1996, tattoo is a unicolour or multicolour picture fixed on the skin by injecting in the skin insoluble dyes (ink, cinnabar, ultramarine, red lead), being a specific sign.

The American Academy of Dermatology (2004) specifies 5 types of tattoos. They include, among others, traumatic, undesired formation of dirty or debris deposits under the skin, leaving a pigmentation area after healing – most commonly after cycling or motorcycling accidents, traumatic pencil stabbing (the so-called graphite or pencil tattoo). Amateur tattoos are usually made by people themselves or by their friends. Their artistry is poor. They are not rich in details. They tend to be performed through a subcutaneous insertion of ink, coal or ash with the help of a pin. Professional tattoos can be divided into cultural and modern. For instance, on the South Pacific Islands, tattoos are characterised by the use of traditional methods as well as the heritage of a given community. Modern tattoos, of a varying degree of artistry, including even ideas of fantasy origin, are made with a special pistol. In turn, medical tattoos consist in marking fixed orientation points by a doctor and cosmetic tattoos include, among others,

permanent make-up, vitiligo-masking or coverage of an undesired, previously made, permanent drawing on the body. The latter was introduced in 1984 as a new technique in ophthalmology – a pigment was used to give a uniform colour to the eyelid. The first publication on the subject, entitled Micropigmentation, appeared two years later. In 1992, the American Institute of Permanent Colour Technology was established and began to conduct seminars in the latest techniques and equipment for micropigmentation. They are now continued under the auspices of the American Academy of Micropigmentation (Goldstein, 2007).

TATTOO – A MULTITUDE OF MEANINGS AND FUNCTIONS

"Homo depictus" – "a man decorating himself" is next to Ernst Cassirer's "animal simbolicus", Jahan Huizinga's "homo ludens" or Adam Smith' "homo economicus", one of the most adequate descriptions of the species-specific nature of man. In all and any epochs and in all and any societies we can find evidence confirming man's ability to beautify or modify their body with the help of a variety of techniques: make-up, garments, jewellery, drawing or even radical deformation (Dziuban, 2013).

Tattoos have always performed and still perform a variety of different functions. Practically all of them still apply. And thus, for instance, the function of group membership – once linked to being a member of a specific tribe – can today be applied to both isolated environments, among others, the penitentiary environment, and to liberation movements, for instance, youth, musical or sports subcultures. One had to be painted to be a man; who remained in the natural state, did not differ from the animal. [...] Facial paintings give the person concerned, first and foremost, the dignity of the human being – they confirm the transition from nature to culture, from the "reasonless" animal to the civilised man. The tattoo was then a civilisation mark, thanks to it the body was changing from a purely biological entity to an element of the social category order, classification and practices, was transformed into a social phenomenon, culturally interpreted and understandable to all members of the community (Dziuban, 2013). It served both to emphasise the social position and to mark slaves, prisoners of war, criminals or prisoners of Nazi extermination camps. Some time around the 18th century, in Japan, it reached the rank of art. Its functions included also: military – the first military distinction, repressive-warning – physical stigma, patriotic – manifestation of protest against or support for a system or prophylactic – for instance, blood group marking. In some cases, it played yet other functions magic-religious – a protective amulet, social – among others, an indicator of the social position, aesthetic – a beautifying element, and even sexual – an alleged contraceptive. What should not be forgotten is its psychological function – an expression of emotion, value, interest or commemoration of essential or precious events and memories (Skrzypek, 2017; Snopek, 2009).

The present perception of the tattoo and attitude to it, whether negative, positive or neutral, depends mainly on the domain defining it, be it religious studies, law, pedagogy, medicine or psychology. In accordance with the words of The First Letter to St. Peter: *Your beauty should not come from outward adornment, such as elaborate hairstyles and the wearing of gold jewellery or fine clothes. Rather, it should be that of your inner self, the unfading beauty of a gentle and quiet spirit, which is of great worth in God's sight.* The Bible warns the faithful against tattooing. Yet, it is extremely difficult to find

a direct and unambiguous attitude of the Church to the issue, neither in the past not today, as it frequently involves ethical dilemmas, if only those concerning a change to the image of man created in the image of God. There are countries in which law forbids the convicted to make tattoos and to allow for their performance on their body as well as to encourage to make them or have them made, or to assist in making them and having them made, and provides strict sanitary requirements for their performance. Tattoos or body piercings also disqualify people from being blood donors for a period of six months or four months in the case when molecular biology tests for the presence of viral hepatitis Type B or C infection is negative. Pedagogy attempts to treat a drawing on the body surface as a form communication between the pupil and the tutor, a means of conveying information which is neither obvious nor easy. However, it should not be forgotten that in no circumstances is it reason for assessing the tattooed teenager in terms of the latter being good or bad. The tattoo is here the question of aesthetics not ethics (Snopek, 2009), especially so, since in tattoo studios the principle of not making body decorations to people below 18 years of age is not observed everywhere, and even where it is, amateur tattoo cannot be excluded. What seems to be important is to raise the awareness of the advantage of delaying the decision to undergo the procedure in view of a possible change of both the penchants and the appearance of the body. On their part, doctors focus on a possible risk to health. Tattooing increases the risk of infective agents, whether bacterial (syphilis, tuberculosis, purulent dermatoses), or viral (hepatitis Type B and C, HIV, HPV), being introduced. Also, over time, dyes can cause contact dermatitis. Neither should it be forgotten that complications may develop. From the point of view of psychology people with tattoos can be divided into three groups: those having one or a few decorative tattoos, those having a large number of decorations covering the whole body and those in whom the presence of a tattoo is evidence that its bearer belongs to a particular social group (Snopek, 2009).

TATTOO – A MIRROR OF LIFE

There is a broad array of motives underpinning the decision to have a tattoo. They can be divided into three main ones, namely: *the so-called subconscious call for help,* the need to be unique and distinct as well as the already mentioned need to manifest one's belonging to a group and aesthetic circle (Sagoe, 2017). *Tattooing involves giving the body personal features, making it a true home and temple of the soul inhabiting it. The tattoo is a symbol of not only who we are at a given moment in our life, but also an expression of everything what has led us to it. [...] it can become a symbol of the road we have covered in our life as well as our deep reflections and considerations. [...] For some people, it can be the 'unknown island', to be known only by who practises tattooing, others will call it magic and yet others will compare a tattoo to a drug being a source of satisfaction and joy. A tattoo can be treated as a companion-friend to the end of life, as a 'search for one's own self' or as a life of philosophy of its own kind* (Snopek, 2009).

In 1999, Bryan Turner presented one of the most controversial interpretations of the phenomenon of the late modern tattoo. The conclusion he reached was that while in traditional societies the decoration of the body had a socially meaningful form, in modern times it constitutes merely a decorative attribute of the individual, a narcistic

expression of the *Ego*. It is thus, according to him, one of the forms of transforming or even improving one's body, along with diet, physical exercise, cosmetic procedures and even cosmetic surgery. This opinion is supported by Susan Benson (2000) who adds that a person permanently modifying their body aims at gaining control of it and defining the limits of their identity in an unambiguous way. What is also of no lesser importance is the celebration of the so-called magic moments. *These events are referred to as the rites of transition or the rites of initiation. Some people would instinctively feel a need for them and if the society does not provide them with such a rite of transition, they will come up with it on their own! [...] It has to be physical, painful, bloody and leave a permanent trace. This is characteristic of the rites of transition* (Dziuban, 2013). The late modern tattoo can be defined in three ways – as an empty sign, a consumer good, allowing for a *game* with one's own *Ego* or with the help of concepts emphasizing the identity-creating and individual character of a permanent body drawing or, on the contrary, in terms of a renaissance of its community-ritual character. Yet, the majority of narrations seems to indicate that it is its individualizing character that prevails these days. It is so because the choice of the body modification results from individual aesthetic preferences, being simultaneously a sign of self-actualization, the *'packaging' of the identity, a way of its, if only momentary, materialization or enclosure in an aesthetic form – a picture, a symbol or a word – written on the skin* (Dziuban, 2013).

Treating tattoos as a record of the features of one's own *Ego,* we can distinguish its three basic dimensions – the already mentioned identity dimension, the biographical dimension and the emotional dimension. In the first case, it is worthwhile to seek answer to the question of whether the performance on the body of a permanent drawing constituted the moment of revealing or/and expressing one's identity with the help of the procedure or whether it created it through the modification of the body. Neither should it be forgotten that the very act of the choice and then of impressing a given picture on the body can be psychologically defined as carrying out an *Ego* expressing-oriented identity work. This is in turn linked to the so-called aesthetic moment of reflectiveness, anchored in culture, for instance, in a phrase read, a lyric heard or a memorable image, as well as to the attribution to it of personal meanings. The latter allows to exert pressure on oneself as well as on the surrounding world, among others, in the context of the currently so crucial issues related to *the cult of the body* – an individual self-presentation as well as improvement of the external appearance – which increases the feeling of pleasure and even admiration (Skrzypek, 2017; Dziuban, 2013).

The entanglement of tattoos with the individual biography defines, on the other hand, these body decorations as *commemorative places,* deeply rooted in individual experiences, important to be perpetuated, such as, for instance, the passing of school finals, the commencement of studies or/and first work, a change of the place of living, birth or new relationship. Not infrequently, a tattoo becomes a carrier of the reorganization of one's internal life or style. Body drawings can thus constitute materialized memory traces, orientation points often marking the crucial biographical moments of particular individuals. In this context, they are often given special names by their owners who refer to them as – *an archeologic site, a photo album, a chronicle, a memory carrier*. The reading of the history of one's life often involves nostalgic return to certain

contexts which allows to re-experience the emotions accompanying them. Yet, it is often a non-obvious biography, extremely difficult and even impossible to be deciphered by others, and, first and foremost, unique. This last-mentioned feature seems to be confirmed also by the possibility of using a permanent body drawing to identify corpses or to create a psychological picture of a particular person. And thus, in 2014, two cases were described, of a man and a woman, who died by suicide, both having commemorative tattoos. The nineteen years old man was found hung on an electric cable in a barn in which his brother, whose name and the letters RIP he tattooed on his arm, died in the same way nine years and two days earlier. The forty seven years old woman was found in similar circumstances – hung on a cord attached to a metal beam in a barn close to her house. She died a year and five days after her daughter's death, in a similar way, in the same place, and the tattoo gave her name, date of birth and date of her suicidal death. The perceptation of the tattoo as a biographical map can also be linked to its identity dimension presented above as a search for and repeated interpretation of memory traces inscribed on one's body invites reflection on the continuity of one's identity and with time allows for the reconstruction of one's own personality having thus a potentially autotherapeutic function (Skrzypek, 2017; Byard, 2014; Dziuban, 2013).

No less interesting seems to be the question of the tattoo-mediated management of emotions – conscious use of a tattoo to influence one's own emotions and emotional states. It can constitute a form of gratification as well as a way of externalizing one's cumulating emotions, in particular, when it is to difficult to express them in a straightforward way. Tattooing also frequently proves, especially in men, to be a way of masking negative emotions (Skrzypek, 2017; Dziuban, 2013).

The results of a 2016 survey, conducted in Chicago on a group of 363 people with at least one tattoo, indicate that what seems to dominate is the desire to commemorate crucial events, religious motives as well as, surprisingly, the desire to simply have fun, to give oneself a treat. What seems significant is also the fact that as many as 70% of the respondents completed studies, 71% had no health problems and only 6%, with medium or lower education, had the permanent body drawing made under the influence of psychoactive substances. On the other hand, Norwegian studies (2017) point mainly to motives linked to spiritual aspects, fashion or boost of one's own value (Dimitropoulos, 2016; Sagoe, 2017).

TATTOO – A SOURCE OF EXCLUSION

Tattoo can be seen as one of the most controversial procedures of body decoration. In the majority of people, it generates negative associations and emotions. It is treated in a different way than painting, drawing, graphics or sculpture. [...] The most frequently encountered as well as the most embedded stereotype connected with the phenomenon of tattooing [...] is its association with being a feature of the criminal world. This conviction [...] can contribute to the emergence of many problems such as, for instance, conflicts in partnership relations, emotional problems, etc. (Snopek, 2009).

It was Cesare Lombroso, an Italian scientist, psychiatrist and anthropologist, a professor of forensic medicine and founder of the anthropological school of criminal law, who is

largely responsible for the treatment of tattoos as a manifestation of deviation and psychopathology. In his studies on a group of criminals, prostitutes and soldiers-criminals, he proved that tattooing is an inherent feature of every born criminal. In his 1891 work Man-criminal in relation to anthropology, jurisprudence and penitentiary discipline, he concludes that a permanent drawing on the body is a psychological rather than biological features: One of the specific features of the properties of the primitive human in a wild state is the ease with which the latter surrenders to a procedure, surgical rather than aesthetic, which bears the name of tattooing taken from the oceanic languages (Lombroso, 2014). This concerns solely the lower classes and, among them, most frequently criminals whom it gives a special anatomical-legal property. What Lombroso failed to understand in the first place was the fact that a mentally healthy man can volunteer to submit himself to the pain of the procedure solely to embellish his body. However, today's observations show that in a world of social isolation resistance to physical pain ranks high and allows to build up fellow prisoners' trust.

In addition, Lombroso (2014) stressed that criminals acquire their tattoos largely yet before the age of sixteen. And although he also noticed, already then, the possibility of the occurrence of health complications, mentioning, for instance, body ulceration or gangrene, he drew attention to the fact that it was criminals rather than representatives of primitive cultures that tattoo their intimate parts more frequently as well as that who has more tattoos is more firmly embedded in the lawless life and is considered a person of higher stature among other criminals. His theories were debunked already at the beginning of the 20th century while still exerting an enormous influence on the development of psychiatry and on public opinion.

Another cause of the negative treatment of a permanent body drawing is its unequivocal identification with the already mentioned amateur tattoo, most frequently resembling prison rather artistic tattoo in terms of both the method of performance and aesthetics. What cannot be forgotten is also the shortage of knowledge about this method of body modification and the already mentioned need for legally regulated observance of the hygiene of work. The spread of stereotypes is also caused by the limited number of publications on the subject.

What remains controversial is also the aesthetics of tattoos. For some, a tattoo can constituts a decoration and even a piece art while for others a disfiguration, irrespective of the way it is executed and the pattern. At present, probably due to the existence of ever more interesting designs and ever better techniques of performance, the tattoo is ever more frequently assessed in aesthetic terms (Snopek, 2009).

Contemporary studies show that it is most frequently men with lower education, dissatisfied with their physical appearance, having a positive attitude to tattoos, younger and less educated fathers and a larger number of tattooed family members and friends who decide to have a permanent drawing on their body. Interestingly, there seems to be no statistically significant correlation between religious beliefs and behaviours and attitude to tattoos and their acceptance (Cegolon, 2010; Roberts, 2006; Koch, 2004).

The majority of reports tends to concentrate on risky behaviours among people deciding to have a permanent drawing on their body. They seem to indicate that adult tattooed

people are characterized by a significantly higher level of reactive rebelliousness, anger or verbal aggression. Yet, the size of this effect is relatively small and of no significant difference in terms of proactive rebellion, physical aggression and hostility. It can therefore be concluded that though these stereotypes may contain a grain of truth, they are not that likely to represent the actual psychological picture of people with tattoos. However, numerous studies confirm the actually more frequent occurrence of high-risk behaviours: cigarette smoking, uncontrolled alcohol drinking and use of psychoactive substances as well as casual sexual activity. The performance of a permanent drawing on the body allows to raise the level of self-assessment and positive perception of one's body as well as to reduce the number of impulsive behaviours and symptoms of depression and anxiety, though David Goldberg's Questionnaire for the General State of Health shows absence of statistically significant differences in the occurrence of depressive and somatic symptoms between the group of 308 people with body modification and the control group. This confirms that conclusions of the 2011 British study that the improvement of self-perception is present solely immediately after the execution of a tattoo. Sleep and function disturbances are also rarer (Pajor, 2015; Swami, 2015; Iannaccone, 2015; Owen, 2015; Swami, 2011).

What cannot be ignored are the numerous studies of the personality features of people with tattoos. Different reports assessing the features of the so-called Great Five – agreeableness, conscientiousness, extraversion, openness to experience and neuroticism – testify to the occurrence of significantly higher results in the last three scales and lower results in conscientiousness. Symptoms of depression and eating disorders, sexual abuse experiences and suicides among tattooed people are also more common. Findings concerning personality disorders are not unequivocal – some studies point to more common diagnoses of borderline, sadistic or anti-social personality disorders than in the general population. The latter can be a predictor of criminogenic behaviours. The research carried out in 2014 on the basis of an analysis of data concerning tattooing and criminal behaviours of 973 minors proceeding from administrative documentation seems to indicate that people below the age of 18, with a permanent body drawing are 13% more likely to commit assault, 9% to commit murder, 3% to commit fraud. This is likely to be related also to the above mentioned more frequent occurrence of risky behaviours. Interestingly, in this group, tattoos are more frequently made in visible places, among others, on the neck or arms, and tend to be more numerous. An important question thus arises why serious crimes rather than minor offences, such as theft, tend to be more common in the study group. This may have its source in the desire to have better achievements and thus increase the chance of gaining a higher rank function in criminal groups and the latter, as it has already been mentioned, are marked with attention-drawing and permanent drawings on the body (Liao, 2014; Owen, 2015; Swami, 2012; Swami, 2011).

TATTOO – A SOURCE OF SEXUAL ATTRACTIVENESS

What seems to be interesting are differences between tattooed men and women studied in Australia on a sample of 4290 men and 4366 women. It turns out that women with tattoos more frequently have a regular partner with whom, however they do not dwell together. Contrary to stereotypes, in neither sex is tattooing connected with sexual

identity and the tattoo is the least likely to be made under the age of twenty and above the age of forty. According to earlier postulates, tattooing is linked to lower education and specific risky behaviours as well symptoms of worsening mental health – in females, mainly with a higher likelihood of tobacco and cannabis smoking over the past year as well as a higher number of sexual partners in her life while in males also with symptoms of depression (Heywood, 2012).

As Ovid writes in *The Art of Love: Hearts have as many moods as faces expressions. Gaining one thousand hearts requires one thousand ways.* Unfortunately, the sole awareness of what is expected of a partner is no guarantee of the fulfilment of the desires. Women have always given attention to the high social position of the partner while men to women's beauty and health. Common evolution, full of competition of one's own sex with the opposite sex has led to the development of psychological mechanisms. However, it should not be forgotten that the effectiveness and ways of attracting a partner are strongly dependent on whether the partner is sought for keeps or just for a transient relationship. In the case of the latter, the possibilities of presenting the desired features in too positive a light are incomparably larger. *Briefly speaking, transient contacts are a treacherous ground full of traps concerning primarily pretending having the features which the opposite sex desires most. In the case of men, it will involve pretending having a higher social position, larger resources and greater emotional engagement. In the case of women – pretending to be beautiful and sexually faithful. [...] The strategies applied by women to gain men consist not so much in showing their true appearance as in attempts to expose and present these of their features that comply with the beauty criteria developed in men* (Buss, 2014). And thus, for instance, in response to male expectations, women use make-up, strengthen their hair with egg yolk or hair sprays, apply cosmetic procedures and even undergo plastic surgeries. *They wear artificial nails so that their hands would seem longer, high heels so that their legs would look longer and slimmer, dark or vertically striped clothes to look slimmer, padded clothes to make their body look fuller* (Buss, 2014).

What follows from the evolutionary point of view discussed above is that men are under the pressure of spreading their genes and consequently of increasing the number of partners. Any signals informing of a woman's potential readiness for sexual intercourse are thus important, and tattoos seem to belong to these signals. Numerous studies confirm that tattooed women are often perceived as more sexually lax, and thus readier for having a faster and easier relation. Therefore, although minors often speak of women with a visible tattoo in negative terms, adult men perceive them in a more favourable way, are more likely to press for a fast date or sexual relation. Yet, the assessment of physical attractiveness is not unequivocally bound to a specific permanent drawing on the body. It should not be forgotten that a woman will have a bigger chance of choosing a relatively better partner in the case when it is attractive for a larger number of men. Thus, a tattoo seems to increase the likelihood of the possibility of choosing the best of them and numerous reports confirm that representatives of the fair sex are aware of the influence of their appearance on male behaviours. Also, many reports point out that tattooed women tend to begin sexual life earlier. What still remains to be answered is whether this results from the fact that they were more frequently interested in sexual contacts or from the fact that they begin to attract men earlier. One of the ways to check it seems to be the observance of the changeability of behaviour in response to the

presence or absence of body modification in the fair sex. Some studies point to the perception of women with tattoos as less intelligent and competent which is stereotypically linked to a lower socioeconomic status and the latter, in turn, with their easier sexual accessibility (Gueguen, 2013; Buss, 2014).

The above postulates are further supported by the 2010 research findings. The latter show that not only have a significant majority of the 42 respondents – 31 women and 11 men, aged 18-65 – never felt discriminated against or had to cover tattoos, but, on the contrary, they believed that permanent body drawings have made them definitely more attractive. They treated the tattoo as as an expressive and aesthetic instrument, declaring that they did not perform it under the influence of psychoactive agents and would not have surrendered to the procedure if there were a risk of having any problems at work as well as that they were aware of the difficulty of their possible removal. In addition, they also said that they did not perceive tattooing as a possibility of distracting attention from signs of aging and even less so as a result of obsession about their ow body, its appearance or beauty, or a way to reinforce the frequently unattainable media-promoted beauty canons. They confirmed having had at least one tattoo prior to employment – performed between 14 and 48 years of age – and declared readiness to undergo subsequent body decoration procedures. Over 70% of the respondents claimed that tattooing is not a form of cultural rebellion and assessed the relayed pain as minimal to bearable. Almost 65% of the respondents could not point to any particular, meaningful event that might have induced them to decide to have the procedure, declared family acceptance, simultaneously confirming that tattoos increase sexual attractiveness and expressiveness. In addition, approximately 40% of the respondents assessed a tattooed partner in an extremely positive way – as exceptional and wonderful (Lise, 2010).

According to what many respondents report, and as pointed out earlier, irrespective of the sexual identity, a tattoo can prove a perfect instrument not only for the *expression of one' s own self, reliving one's own biography or emotions or making oneself symbolically distinct from others, but also for communicating one's feelings to the close environment. [...] The subject of a tattoo inscribed on the skin of an individual is thus not so much passions or interests shared with others, [...] but, first and foremost, the feelings towards another person or emotional states experienced thanks to their involvement in an intimate relation* (Dziuban, 2013). The intimacy of the message was confirmed also by the choice of the place – most frequently the abdomen, the back, the neckline, the chest or the waist. If a more exposed area was chosen, the message was frequently coded to make it incomprehensible to the surrounding world. The name of the nearest person inscribed on the skin guaranteed *having the person always close as well as a feeling of merger, identity, ring-fencing,* the feeling of making the person concerned an inseparable part of one's own *Ego,* constituting simultaneously *a token of love* (Dziuban, 2013).

TATTOO – NOT NECESSARILY PERMANENT

For ages, the body has been a mystery. It has been treated as the first and natural instrument of man (Snopek, 2009). This is confirmed by Klaus Prahlhans in his 2005 book The most shocking ways of embellishing the human body, which discusses available

ways of beautifying the body. He mentions, among others, the lotus feet of Chinese women, scull deformation, neck lengthening, lip and ear stretching, elongated labia, subcutaneous implants and wasp waist performance. [...] The author mentions tattoo as the most popular and simultaneously mild way of body decoration (Snopek, 2009).

The body is known to have always been an object of creative interest. Essential changes in this respect became visible in the 60s of the 20th century when it came to be viewed not as a carrier of important contents and when a distinction was made between sensuality and intellect as well as the already mentioned attractiveness and disgust. The perception of carnality underwent a significant change thanks to the Viennese Actionism, the representatives of which used the body as a form of artistic expression. Unlike them, the approach of body art artists was more personal. Their first representative, a French lady called Orlan, was the first to film her own operation at the age of thirty one to create a sculpture of a woman's body on her own example and to simultaneously show the inaccessibility of objective beauty and the gruesomeness of the very process of attempting it. Since the 70s, the movement of the so-called contemporary primitivists has been developing, the movement of artists concentrating on surpassing their own limitations, for whom the cultural aspect of carnality has been as important as the biological one (Snopek, 2009).

At present, practically everybody can almost completely (de)form their body according to their needs (Snopek, 2009). As it has already been mentioned, the reasons can frequently be sought in an expression of individuality, identity, specific understanding of beauty and aesthetics, an attempt to reach one's own ideal of the human body. Tattoos may also be performed in the name of pain, protest as well as be a way of achieving or being coerced to achieve an intended goal by proving their courage or respecting subcultural norms as it is in the penitentiary environment (Snopek, 2009). Unfortunately, they also entail the risk of frequently even very severe complications. The latter may be of health, psychological or social character. The first mentioned can arise both during the procedure and after the procedure. The second involve negative psychological consequences resulting, for instance, from the necessity of a life-long acceptance of one's own new image and decision. The last mentioned refer primarily to the struggle with the lack of the openness of certain environments to body decorations of this kind. Yet, one may wonder whether there are any limits to the change of man's image. [...] It might be right to say that the most important, if not the only limit, to be encountered by a man intending to transform their body is nothing else but their own organism (Snopek, 2009). And the body does have its limits.

Tattooing does happen to prove a dangerous causing numerous side effects such as, for instance oedema or pruritus. In 2005, a study was conducted on a sample of 40 patients, among others, with the help of Dermatology Life Quality Index (DLQI) which includes the following scales: Symptoms and Emotions, Daily Activities, Entertainment, Work and School and Personal Relations. Its results point out that patients experience reduced quality of life, similar to that in severe dermatological diseases the symptom of which is pruritus. Another report informs that 20% from among 501 respondents had to face pruritus for a month following the execution of a tattoo (Carlsen, 2017; Carlsen, 2015).

The past few years have witnessed the growing commonness of the application of the laser method of tattoos removal. This is related to the ever better effects of removing tattoos as well as to the much lower prices of procedures of this kind (Snopek 2009). Unfortunately, not everybody can have a tattoo removed with the help of a laser. Every procedure should be preceded by an interview and by the so-called test intended to check whether pigments react to laser light with a change of colour as well as to assess the risk of allergic reactions. Difficulties can also arise due to a darker colour of the skin and the size of the body drawing, for instance, when it covers the whole back. A study carried out in 2017 points to a change of expectations among patients who are becoming more and more demanding. What seems to be crucial is the relation between the laser-operating doctor and the patient as the former often faces the task of persuading the patient to accept a realistic though not necessarily ideal solution (Carlsen, 2017; Liszewski, 2015; Snopek, 2009).

In spite of numerous publications devoted to medical aspects of tattoo removal, little is still known about the features and behaviours of individual people as regards the performance of a permanent body drawing and even less about the features and behaviours of people who want to have tattoos removed. A 2011 report distinguishes three main reasons for having a tattoo removed: dissatisfaction, embarrassment or shame and professional reasons. In earlier studies, they reasons were also divided into: social, family-related and desire-to-improve one's self-evaluation related. Interestingly, the latter turned out to be the most common. A question thus arises whether the performance of a tattoo is only seemingly a self-therapeutic act? Most probably, it constitutes a kind of immediate gratification. However, later, especially when the decision is impulsive, dictated by the previously mentioned personality features, the tattoo turns out to be an unwanted decoration, lowering the feeling of one's own value (Skrzypek, 2017; Latreille, 2011).

SUMMARY

It might seem that the multitude of approaches to the phenomenon of tattooing, the multitude of its interpretation and the considerations and discussions it has generated, as described in this study, would exhaust all the information related to the tattoo and, first and foremost, allow to face the title question.

Unfortunately, contrary to the expectations, only the 2017 Norwegian study assesses the occurrence of a permanent body drawing among sportsmen taking into account differences between features of people with overtly visible and hidden tattoos. For the time being, there are no reports concerning the type and detailed characteristic of risky behaviours in adults nor any including the history of respondents' life. From the medical point of view, further studies are also necessary as regards threats to health both in the context of somatic diseases, in particular contagious, and mental disorders and complications. As it has already been mentioned, there are still very few publications concerning the psychological aspects of the removal of tattoos. It is no doubt a source of serious limitations of the literature quoted above that in the majority of cases respondents are students, a group which has its own specific features. Moreover, not enough interest seems to have been given, for instance, to comparing people with few tattoos and people with extensive tattoos or differences related to the methods in which

the tattoo is performed whether professional or amateur. For the time being, it is thus difficult to decide whether tattooing is a positive or a negative phenomenon (Sagoe, 2017, Liszewski, 2015; Pajor, 2015; Guéguen, 2013; Heywood, 2012; Lise, 2010).

It is also worthwhile to draw attention to the fact that the now frequent perception of the tattoo as an instrument of self-expression, increasing the individual's attractiveness, might necessitate organization of awareness-raising educational campaigns concerning threats to health, especially since it is known that it is people more prone to risky behaviours who are more likely to have tattoos made.

Finally, it is worth adding that both the performance and the removal of tattoos can entail often difficult to resolve ethical dilemmas which is confirmed by the scenario of the 2017 case in which doctors were supposed to face the decision whether to remove a permanent body drawing as a punishment meted by dissatisfied parents or leave it in a fourteen year old who had the amateur tattoo made by a friend against their will (Chheda, 2017).

It thus seems obvious that, unfortunately, the title question must yet be left without an unequivocal answer and to repeat after Goldstein (2007) that answering the question: '*Should I have a tattoo made?'* it is best to answer: *'This is your canvas. But I also advise to choose a licenced tattoo artist' and add with a pinch of warning*: *'If you ever want to remove it, even with the use of many available techniques, scars may appear'.*

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The effect of plant extracts from *Lamiaceae* family on human fibroblasts and *Borrelia burgdorferi* spirocheates – *in vitro* study

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ABSTRACT

Lyme borreliosis is a multisystem disease affecting many human tissues and organs which is caused by infection with *Borrelia burgdorferi* spirochetes. The standard antibiotic therapy is often complemented by patients with medicinal plants possessing antibacterial and strengthening the body properties. In order to determine the effects of medicinal plant extracts on *Borrelia burgdorferi* spirochetes, several of them were used: *Thymus serpyllum* L., *Prunella vulgaris* L., *Glechoma hederacea* L., *Thymus vulgaris* L., *Rosmarinus officinalis* L., *Salvia officinalis* L., *Lamium album* L. and *Origanum majorana* L. The plants were extracted with 80% methanol, water, ethyl acetate and dichloromethane. Cytotoxicity tests were performed on human fibroblasts exposed to the concentration of 8 to 0.016 mg/ml of the extract. There was also determined MIC for *Borrelia burgdorferi*. The highest ability to inhibit the growth of *Borrelia burgdorferi* was demonstrated by *Salvia officinalis* L. (0.25 mg/ml), followed by *Thymus serpyllum* L. (0.5 mg/ml) and *Lamium album* L. (1 mg/ml). The weakest antibacterial properties were demonstrated by *Prunella vulgaris* L. (4 mg/ml). Among the analyzed plant extracts, *Salvia officinalis* L. has the greatest ability to inhibit the development of *Borrelia burgdorferi* spirochetes. However, further studies are recommended to select as many plants as possible that can complement modern antibiotic therapies in the treatment of Lyme disease.

Keywords: *Borrelia burgdorferi*, *Lamiaceae*, Minimal Inhibitory Concentration, human fibroblasts

INTRODUCTION

Lyme borreliosis is a multisystem disease affecting many human tissues and organs which is caused by infection with *Borrelia burgdorferi* spirochetes. It is tick-borne disorder and it creates many diagnostic and therapeutic problems. Serological tests for Lyme disease are often difficult to interpret. The life strategy of spirochetes, complex immune response of host and deficient diagnostic methods comprise main diagnostic difficulties. Among difficulties in the treatment of Lyme disease are: high efficiency of spirochetes in the initial colonization of tissues, their rapid spread, quick penetration of CNS. The spirochetes have the ability to penetrate the host cells and thus they are invisible for immune system (Biesiada, 2012; Chmielewski, 2010; Mączka, 2010). Moreover, life strategy of *Borrelia spirochetes*, complicated immune response and imperfect diagnostic methods constitute an essential problems with the correct diagnosis of Lyme disease. *Borrelia burgdorferi* characterizes also a high frequency of antigenic variation in relation to other pathogens. This contributes to the generation of many types of antibodies which make difficult diagnosis with the use of tests designed to capture a specific antibody (Akira, 2006; Fikrig, 2006).

The long-term oral or intravenous antibiotic therapy is administered to Lyme patients. The efficacy of this treatment will be greater when quickly is implemented antibiotic therapy. The safety of antibiotics is dependent on individuals. They usually are well

tolerated, however they can cause side effects such as: anaphylactic reactions, allergic reactions, diarrhea, sensitivity to light and many others. The interactions of antibiotics with food and other drugs also constitute the problems because they can influence on treatment process (Wasiluk, 2011).

Pharmacognostic methods can prove effective in the treatment of Lyme disease. The standard antibiotic therapy is often complemented by patients with medicinal plants possessing antibacterial and strengthening the body properties. Currently, many medicinal plants or active substances derived from theirs are used in treatment of diseases, including *Lyme borreliosis*. They contain rich chemical composition which can influence on bacteria in available and unavailable places for other chemical drugs. Therefore, the return to natural methods may contribute to the rapid eradication of Lyme disease. Moreover, understanding the mechanism of influence of herbs and *Borrelia burgdorferi* on human cells may be helpful in the development of novel diagnostic and treatment strategies of Lyme disease (Goc, 2016).

There are many of scientific reports confirming the use of some species of plants from the *Lamiaceae* family in the treatment of infections caused by bacteria. Many plants from this family show antibacterial, antifungal and antiviral properties (Nieto, 2017; Assis, 2018). In order to achieve the best therapeutic effects, it is necessary to determine what solvent should be used to make plant extracts. Several representatives of the *Lamiaceae* family were used for this purpose: breckland thyme (*Thymus serpyllum* L.), common self-heal (*Prunella vulgaris* L.), ground ivy (*Glechoma hederacea* L.), common thyme (*Thymus vulgaris* L.), rosemary (*Rosmarinus officinalis* L.), garden sage (*Salvia officinalis* L.), white nettle (*Lamium album* L.) and marjoram (*Origanum majorana* L.) by extracting dry matter with solvents of increasing polarity (dichloromethane, ethyl acetate, methanol, water). It was also decided to assess the toxic effect of these extracts on human cells and to assess the MIC (Minimal Inhibitory Concentration) on *Borrelia burgorferi* spirochetes.

MATERIALS AND METHODS

PLANT MATERIAL

The plant material used to prepare the extracts was purchased from producers of herbal products. The samples of dried plants were extracted using four solvents with different polarity such as water, methanol/water (80:20), ethyl acetate and dichloromethane (Kukula-Koch, 2013). Powdered plant material (2 g) was transferred into the round bottom flasks and treated with 100 cm^3 of solvents. The extractions were carried out for 30 minutes under reflux at the boiling point of the solvent. After extraction, the extracts were separated from the solid plant material by filtering process. Plant extracts were combined and evaporated in rotary evaporator under reduced pressure, and then were freeze-dried to completely remove the solvents. The lyophilized powder was weighed and next, serial dilution of extracts were prepared. Tween 80 was added to dissolve lipophilic compounds (Liebold, 2011).

CELL CULTURES

Normal human dermal fibroblasts (NHDF cell line) were obtained from the Clonetics (CC-2511; San Diego, CA, USA). The reference strain of *Borrelia afzelii* (VS 461, ATCC 51567) was obtained from the National Institute of Public Health – National Institute of Hygiene in Warsaw (Poland).

The bacterial culture was carried out in BSK-H medium (Barbour'a, Stoenner'a, Kelly'ego; Sigma-Aldrich, St. Louis, MO, USA) at 35˚C in microaerophilic conditions. The microscopic analysis of culture was performed upon 7 days of growth. Cell number was monitored by cell counting in the Bürker chamber.

Normal human dermal fibroblasts were routinely maintained in the FBM medium (Fibroblast Basal Medium; Lonza, Basel, Switzerland), supplemented with a human fibroblast growth factor-basic (hFGF-B), insulin and gentamicin (FGMTM SingleQuotsTM; Lonza, Basel, Switzerland) at 37˚C in a 5% CO_2 incubator (Direct Heat CO_2; Thermo Scientific, Waltham, MA, USA). Both, cell number and viability were monitored by cell counting in the Bürker chamber, after staining them with 0.2% trypan blue (Biological Industries, Beit HaEmek, Israel). The experiment was performed on cells in the logarithmic phase of growth under condition of ≥ 98% viability assessed by trypan blue exclusion. For the experiments, NHDF cells will be used at 4-6 passages.

ANALYSIS OF BIOACTIVE COMPOUNDS IN PLANT EXTRACTS

The content of total polar phenolic compounds in extracts was determined colorimetrically using Folin-Ciocalteu reagent. The reaction mixture contained of a extract, Folin-Ciocalteu reagent and a sodium carbonate solution. The final mixture was diluted with deionized water. The mixture was kept in the dark at ambient conditions for 60 min in order to complete the reaction. Then, the absorbance at 750 nm was measured using an Infinite 200 PRO NanoQuant (Tecan, Männedorf, Switzerland). The phenol content (mg/ml) was read from the calibration curve and was expressed in terms of gallic acid.

The content of flavonoids in extracts was determined colorimetrically using aluminum chloride solution. A certain volume sample solutions were mixed with 5% $NaNO_2$ solution for 6 min. Then 0.06 ml of 10% $AlCl_3$ solution was added and reacted with the solution. Six minutes later, 0.8 ml of 4% NaOH solution was joined into the solution and mixed thoroughly. The final mixture was diluted with deionized water. The mixture was incubated in the room temperature for 15 min and then the absorbance of the mixture was measured at 415 nm by using an Infinite 200 PRO NanoQuant (Tecan, Männedorf, Switzerland). The flavonoid content (mg/ml) was read from the calibration curve and was ex-pressed in terms of quercetin.

The total content of phenolic acids was determined spectrophotometrically using Arnova reagent and was read from the calibration curve and expressed in terms of caffeic acid. The absorbance at 490 nm was measured using an Infinite 200 PRO NanoQuant (Tecan, Männedorf, Switzerland). Then, the mixture was incubated in the room tempera-ture for 15 min and then the absorbance of the mixture was measured at 510 nm.

MINIMAL INHIBITORY CONCENTRATION (MIC)

MIC for *Borrelia burgdorferi* was determined by serial micro-dilution in BSK-H liquid medium (with the 25 μg/ml of phenol red) using 96-well titration plates (Hunfeld, 2000). A series of dilutions of the plants extract were made to concentrations ranging from 0.015 to 8 mg/ml. Final concentrations of the lyophilized plants extracts were reconstituted by adding of 200 μl of the final inoculum suspension in BSK containing phenol red as growth indicator. Microtitre plates with *Borrelia* samples and growth controls were sealed with sterile adhesive plastic and cultured at 35°C with 5% CO_2. The presence or absence of growth was examined after 0, 24, 48, 72, 96, 120, 144 and 168 h by kinetic measurement of indicator colour shift at 450:630 nm applying a commercially available ELISA-reader (Tecan Infinite 200 PRO; Tecan Austria, Grödig, Austria). Amoxycyline at a concentration of 0.5 μg/ml was used as a negative control (Sigma-Aldrich, St Louis, MO, USA) (Sicklinger, 2003).

CYTOTOXICITY

In Vitro Toxicology Assay Kit Sulforhodamine B based (Sigma-Aldrich, St Louis, MO, USA) was used to determine whether plant extracts at concentrations between 8 mg/ml and 0.016 mg/ml was toxic to the fibroblast cell cultures. Plant extract was prepared as a stock solution in PBS and then diluted in culture medium. Viability of cells was evaluated after 24 h of exposure to extracts. The effects of this extracts on cell viability was evaluated in two independent experiments.

Normal human dermal fibroblasts were seeded into 96-well culture plates (Nunc, Wiesbaden, Germany) at a density of 5000 cells/well and were treated with plant extracts for 24 hours. Absorbance at the wavelength of 565 nm was read on a microplate reader Wallac 1420 VICTOR (PerkinElmer, Waltham, MA, USA).

STATISTICAL ANALYSES

Statistical analyses were performed using Statistica 10.0 software (StatSoft, Tulsa, OK, USA), and the level of significance was set at $p < 0.05$. Values were expressed as means and standard deviation (SD) of two independent experiments. A one-way ANOVA test, which was followed by Dunnett's test, were used to assess any significant differences among the groups in cytotoxicity tests.

RESULTS

In this study, the amount of active substances (phenols, phenolic acids and flavonoids) that can be obtained in the extracts using solvents of different polarity was determined. Dichloromethane, ethyl acetate, methanol and water were used for this purpose.

In the case of *Lamium album* L., *Rosmarinus officinalis* L. and *Salvia officinalis* L. the highest concentration of phenols (approx. 80%) was obtained by using methanol as a solvent. The greatest amount of phenolic acids was obtained through the use of methanol and dichloromethane, where the yield in various plants was approx. 60%. Flavonoids, on the other hand, were best extracted with water (max 46.3%), methanol (max 51.4%) and ethyl acetate (max 34%). The results are summarized in Table 1.

Table 1. Concentration of active compounds (phenols, phenolic acids and flavonoids) in mg/ml and the percentage extracted using the solvents: D – dichloromethane, E – ethyl acetate, M – methanol, W – water

		Phenols:	Phenolic acids:	Flavonoids:
Glechoma hederacea L.	D:	0.021 (2.9%)	0.552 (22.8%)	0.218 (6.4%)
	E:	0.027 (3.8%)	0.319 (13.2%)	0.836 (34%)
	M:	0.601 (85%)	1.165 (48.2%)	1.326 (39%)
	W:	0.059 (8.3%)	0.383 (15.8%)	1.021 (30%)
Lamium album L.	D:	0.028 (1.9%)	0.492 (14.7%)	0.291 (11.6%)
	E:	0.104 (6.8%)	0.332 (9.9%)	0.412 (16.5%)
	M:	1.302 (86.1%)	2.139 (64.1%)	0.984 (39.4%)
	W:	0.078 (5.2%)	0.374 (11.2%)	0.812 (32.5%)
Origanum majorana L.	D:	0.043 (10.3%)	0.864 (37.2%)	0.319 (14.2%)
	E:	0.024 (5.8%)	0.368 (15.8%)	0.411 (18.3%)
	M:	0.244 (59.2%)	0.614 (26.5)	1.158 (51.4%)
	W:	0.102 (24.7%)	0.476 (20.5)	0.364 (16.2)
Prunella vulgaris L.	D:	0.023 (5.5%)	3.521 (69.6%)	0.236 (6.3%)
	E:	0.046 (11.1%)	0.336 (6.7%)	0.693 (18.4%)
	M:	0.310 (74.1%)	0.854 (16.9%)	1.391 (37%)
	W:	0.039 (9.3%)	0.345 (6.8%)	1.439 (38.3%)
Rosmarinus officinalis L.	D:	0.011 (1.3%)	1.911 (27.2%)	0.091 (3.4%)
	E:	0.105 (12.5%)	0.806 (11.5%)	0.447 (16.6%)
	M:	0.674 (80.2%)	2.837 (40.4%)	0.91 (33.8%)
	W:	0.05 (5.9%)	1.461 (20.8)	1.247 (46.3%)
Salvia officinalis L.	D:	0.021 (2.9%)	0.552 (22.8%)	0.226 (12%)
	E:	0.027 (3.8%)	0.319 (13.2%)	0.404 (21.4%)
	M:	0.601 (85%)	1.165 (48.2%)	0.839 (44.5%)
	W:	0.059 (8.3%)	0.383 (15.8%)	0.416 (22.1%)
Thymus serpyllum L.	D:	0.056 (13%)	1.115 (55.4%)	0.072 (5.4%)
	E:	0.013 (2.9%)	0.108 (5.4%)	0.155 (11.6%)
	M:	0.244 (56.2%)	0.313 (15.5%)	0.657 (49.4%)
	W:	0.121 (27.9%)	0.476 (23.7%)	0.447 (33.6%)
Thymus vulgaris L.	D:	0.034 (10.1%)	2.180 (60.2%)	0.441 (11.1%)
	E:	0.026 (7.7%)	0.378 (10.4%)	0.591 (14.8%)
	M:	0.232 (69.8%)	0.661 (18.3%)	1.563 (39.2)
	W:	0.041 (12.4%)	0.402 (11.1%)	1.389 (34.9%)

EFFECT OF PLANT EXTRACTS ON NHDF VIABILITY

According to the results of a cell viability test, plant extracts was slightly cytotoxic to the normal human dermal fibroblasts at concentrations of < 2 mg/ml. Significant growth inhibition was observed in cultures incubated with higher concentrations of plant extracts (fig. 1).

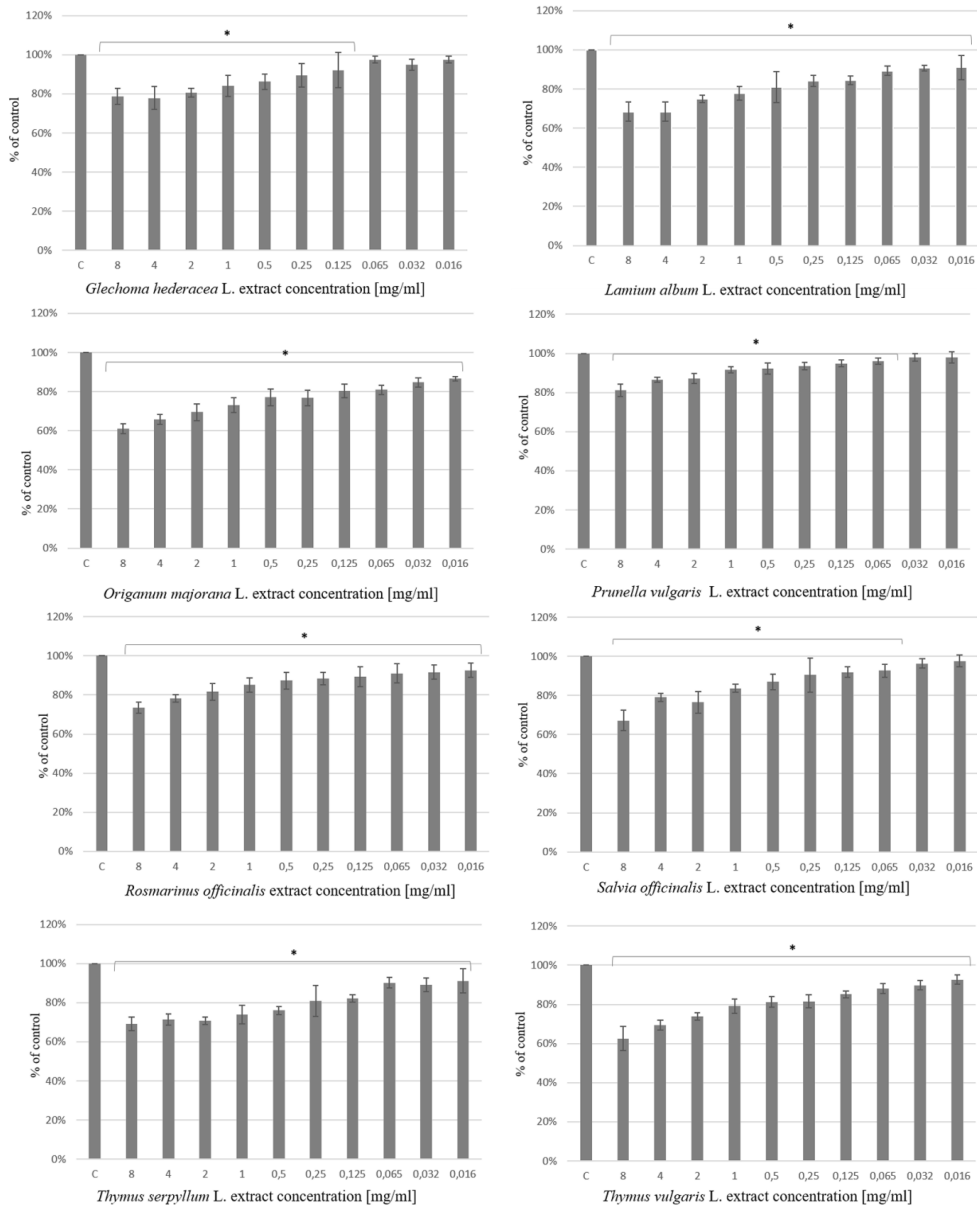

Figure 1. Cell viability in normal human dermal fibroblast cultures in the presence of plant extracts for 24 h. Each bar represents the mean ±SD of two independent experiments.
Statistical significance: *p < 0.05 vs. control (C)

MINIMUM INHIBITORY CONCENTRATION (MIC)

Compared to the control – amoxycilin (0.5 μg/ml), *Salvia officinalis* L. (0.25 mg/ml) showed the greatest ability to inhibit the development of *Borrelia burgdorferi* spirochetes. The MIC of *Thymus serpyllum* L. and *Lamium album* L. were 0.5 mg/ml and 1 mg/ml, respectively. The remaining plant extracts had a similar MIC level – 2 mg/ml. In turn, *Prunella vulgaris* L. was characterized by the lowest MIC index. The results are summarized in table 2.

Table 2. The minimum concentration of plant extracts inhibiting the growth of *Borrelia burgdorferi* spirochetes after 7 days

	MIC
Glechoma hederacea L.	2 mg/ml
Lamium album L.	1 mg/ml
Origanum majorana L.	2 mg/ml
Prunella vulgaris L.	4 mg/ml
Rosmarinus officinalis L.	2 mg/ml
Salvia officinalis L.	0.25 mg/ml
Thymus serpyllum L.	0.5 mg/ml
Thymus vulgaris L.	2 mg/ml
Amoxycilin (control)	0.5 μg/ml

MIC – Minimal Inhibitory Concentration

DISCUSSION

The use of herbal drugs in the treatment of bacterial infections is often used in medicine. Sometimes they synergistically support the action of antibiotics or chemotherapeutic agents in the fight against microorganisms. Their influence often also has a beneficial effect on the patient's well-being due to many compounds, e.g. with antioxidant activity, which to some extent support convalescence. A large number of plant preparations – including those for Lyme disease – registered as dietary supplements, would indicate that the subject of their antibacterial activity, including against spirochetes has been thoroughly tested. However, in the literature the number of works does not constitute such a large percentage. There are studies available describing e.g. the effect of essential oils on *Borrelia burgdorferi* spirochetes (Feng, 2018), single plant extracts from *Dipsacus sylvestris* (Liebold, 2011) or pure substances of natural origin (Goc, 2015). One of the numerous families is *Lamiaceae*, which has been tested for activity against some bacteria or fungi (Assis, 2018).

In this work, the content of active compounds obtained by using various solvents was analyzed in order to determine which of them allowed to obtain the greatest amount of active ingredients in the extract. Phenolic compounds are most often extracted with methanol, phenolic acids – methanol and dichloromethane, and flavonoids – water and methanol. Ethyl acetate had the lowest share. Due to the fact that the solvents differed in polarity, it was reasonable to use several in order to isolate as many active substances as possible.

In the case of plants of the *Lamiaceae* family that can be used in the treatment of Lyme disease, *Lamium album* L., *Salvia officinalis* L. and *Thymus serpyllum* L. deserve special attention due to their bactericidal properties and low toxicity. The lowest, approx. 90%, was found in garden sage, which is known for its antibacterial and antifungal properties (Gniewosz, 2012; Sookto, 2013). In research Paduch et al. methanol and ethyl acetate extracts from white nettle showed no toxic effects against normal human skin fibroblasts (Paduch, 2008). *Prunella vulgaris* L. shows the lowest ability to inhibit the growth of spirochetes, but its low toxicity to human cells could be used by producing preparations containing a higher concentration of its extract. It is also possible that the *Prunella vulgaris* L. extract would act synergistically with any of the antibiotics used. Such a synergistic effect was observed in studies where the plant extract was tested together with the drug cefixime in patients with urinary tract infection (Komal, 2018). The inhibitory effect on the spirochetes of *Borrelia burgorferi* of the others plants was at the level that Liebold et al. showed for *Dipsacus sylvestris* (Liebold, 2011). Among those described for the MIC – 2 mg/ml, the highest cytotoxicity (approx. 70%) to human cells was shown by *Origanum majorana* L. Extracts of this plant have proven inhibitory and bactericidal activity in the case of, e.g., *Staphylococcus aureus* (Abdel-Massih, 2014). The next *Thymus vulgaris* L. was characterized by cytotoxicity at the level of 75%. Thyme essential oil has proven antimicrobial properties (Carvalho, 2015; Al-Shuneigat, 2014). Also rosemary and ivy, the toxicity of which oscillated around 80%, were tested for antibacterial activity (Klancnik, 2009; Kumarasamya, 2002).

The above data should be one of many studies on the possibility of using plant extracts in the treatment of Lyme disease. Further studies are recommended to prove the antibacterial activity of specific plants, especially *in vivo* studies.

SHORT CONCLUSION

Among the analyzed plant extracts, *Salvia officinalis* L. has the greatest ability to inhibit the development of *Borrelia burgdorferi* spirochetes. However, further studies are recommended to select as many plants as possible that can complement modern antibiotic therapies in the treatment of Lyme disease.

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Conflicts of Interest: The authors declare no conflict of interest.

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Short brief about urogenital mycoplasma infections

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ABSTRACT

Urogenital mycoplasmas are often present on the mucous membranes of the men and women urogenital tract without causing any symptoms. On the other hand, they are taken into account as a possible cause of urethritis and cervicitis, and less often other diseases. The consequence of the mycoplasmas presence in the reproductive tract is colonization of the newborn, which occurs during delivery, and in most cases it subsides spontaneously. Systemic infections caused by urogenital mycoplasmas in immunocompetent patients are rare, they mainly affect immunocompromised people, including HIV-positive patients. The etiological agent of such infections may also be others mycoplasmas, which are very rarely isolated from biological materials.

In this review, we present an update from the current literature to discuss the potential of *Mycoplasma* spp. and *Ureaplasma* spp. to be a risk agent of male and female urogenital and extragenital infections, also in immunocompromised patients. Current treatment data of mycoplasma infections has been also included.

Keywords: *Ureaplasma*, *Mycoplasma*, urogenital infections

INTRODUCTION

Mycoplasma spp. and *Ureaplasma* spp. belong to the *Mycoplasmataceae* family, class of *Mollicutes*. Among *Mycoplasma* species important in the development of infections in humans are: *M. pneumoniae, M. genitalium*, *M. hominis* and others, such as: *M. fermentans, M. penetrans* and *M. pirum,* which are considered a possible etiological agent of opportunistic infections (Garrity, 2004; Waites, 2005). All mycoplasmas are the smallest bacteria (approx. 0.3 μm in diameter) capable of reproducing on their own outside of a living cell. All of them have a simple structure and a small genome of 0.75-0.76 Mpb (the size of about 1/10 of the *E. coli* genome) (Robertson, 2002). The characteristic feature of these bacteria is the lack of a cell wall, which determines their resistance to basic antibiotics, including β-lactam and glycopeptide antibiotics. These microorganisms have a simplified metabolism, they cannot synthesize fatty acids (which is a consequence of genome minimization). They have a high affinity for mucosal epithelial cells, mainly the urogenital and respiratory systems, as well as red blood cells and joint surfaces. They can also generate free radicals, ammonia and H_2O_2 causing damage to the host cells (Waites, 2005).

Ureaplasma spp. found in the human urogenital tract are currently divided into species: *U. parvum* and *U. urealyticum*, which can only be identified by molecular methods and before 1999, were considered as the same, one species *U. urealyticum.* It is now recognized that, *U. parvum* includes serovars 1, 3, 6 and 14 while *U. urealyticum* includes serovars 2, 4, 5, 7–13 (Kong, 2000; Schelonka, 2007).

Urogenital mycoplasmas are microorganisms whose primary colonization site is the epithelium of the lower urogenital tract. Colonization may occur through sexual contact and also through the perinatal route. Their presence depends on age, hormone levels, sexual activity and pregnancy. They may be part of the microbiota without any symptoms, but the incidence of urogenital mycoplasmas increases in people with clinical

symptoms of infection. The importance of this atypical bacteria in the development of inflammation both women and men urogenital tract, as well as the consequences of infections for the health of the newborn, are the subject of ongoing research. The species of urogenital mycoplasmas best known and most frequently included in the research are *U. urealyticum, U. parvum, M. genitalium* and *M. hominis*. In recent years, there have been studies showing the relationship with human diseases mycoplasmas other species, such as: *M. fermentans, M. penetrans* and *M. pirum* (Wu, 2014; Gilroy, 2001; Horowitz, 2000; Schaeverbeke, 1996; Nijs, 2002).

SEARCH STRATEGY AND SELECTION CRITERIA

This is comprehensive and selective review of the scientific literature about urogenital mycoplasmas. For the literature review we screened PubMed using the search terms "*Ureaplasma*" and "*Mycoplasma*".

INFECTIONS CAUSED BY *UREAPLASMA* SPP.

*Ureaplasm*a spp. strains were first isolated in a patient with NGU (*Nongonococcal Urethritis*). Over the next years, studies have confirmed their role in this group of patients, as well as in man with prostatitis, urolithiasis, women with PID (*Pelvic Inflammatory Diseases*), BV (*Bacterial Vaginosis*), in the pathological course of pregnancy (inflammation of the membranes premature rupture of the membranes, preterm labor), low birth weight, postpartum fever and infertility. The difficulty in unambiguous assessment of their pathogenic role is the high frequency of *Ureaplasma* spp. isolation also in the study groups without symptoms (Taylor-Robinson, 1996; Schelonka, 2007; Kokkayl, 2015). The incidence of *Ureaplasma* spp. in the genitourinary tract of sexually active young people can be 40% or even 80% (Cassell, 1993; Kokkayl, 2015)

Within a few years after the distinctions of the species *U. urealyticum* and *U. parvum*, it was possible to determine that the presence of *U. parvum* is usually a colonization. The infections are mainly caused by strains of *U. urealyticum*, and the frequency of isolation of this species significantly increases in patients with NGU and NCNGU (*Non-Chlamydial, Nongonococcal Urethritis*) (Yoshida, 2005; Ondondo, 2010). In men, *U. urealyticum* may be involved in the development of urethritis, as well as epididymitis and prostatitis (Horner, 2001; Wetmore, 2011). In women, infections of this etiology refer to the occurrence of PID and BV (Taylor-Robinson, 1996; Taylor, 2013). The possible contribution of ureaplasmas to infertility in both women and men also seems to be important (Dhawan, 2012; Huang, 2016). Moreover, their participation in complications during pregnancy, including low birth weight of newborns, postpartum endometritis, inflammation of the membranes, miscarriages, premature births, perinatal morbidity in newborns (mainly premature babies), including bacteremia, meningitis, chronic and severe pneumonia that can lead to death, or bronchopulmonary dysplasia (BPD) was also discussed (Viscardi, 2014).

Ureaplasma spp. rarely cause severe infections in immunocompetent individuals, although they pose a risk to immunocompromised patients. Among urogenital mycoplasmas, *U. urealyticum* species is most often described as the etiological agent of severe infections, also extragenital location. Reports of peritonitis (Yager, 2010), arthritis (Roerdink, 2016; George, 2015), brain abscess (Deetjen, 2014) and infections in

transplant patients caused of *Ureaplasma urealyticum* etiology have been published. Geissdorfer et al. described the case of a 38-year-old man after kidney transplantation who had meningitis of *U. urealyticum* etiology a few weeks after the surgery (Geissdorfer, 2008). Data of lung transplant patients who developed secondary hyperammonaemia due to the production of urease by this type of bacteria have also been published (Bharat, 2015). Similar cases of secondary hyperammonaemia in lung transplant patients with *U. parvum* etiology have also been described (Bharat, 2015; Fernandez, 2017). Extragenital infections of *U. urealyticum* in adults are rare and mostly affect people with immunodeficiency. Lucke et al. described a patient with deep infection of the sternum wound after aortic valve replacement (Lucke, 2010). García-de-la-Fuente et al. presented a patient with postoperative mediastinitis, pleurisy and pericarditis of the same etiology, which eventually led to septic shock, multi-organ failure and death (García-de-la-Fuente, 2008). All described clinical situations, often complicated and fatal infections of *U. urealyticum* etiology in patients with immunodeficiency or without risk factors indicate the pathogenic potential of *Ureaplasma* spp. and the importance of diagnostics in this direction.

INFECTIONS CAUSED BY *MYCOPLASMA* SPP.

Among the genus *Mycoplasma*, it's mainly *M. hominis* that is most often isolated from the genitourinary system of sexually active people. This species is detected less frequently than strains of *Ureaplasma*, but more often than *M. genitalium*. *M. hominis* is important in the development of BV (Hartmann, 2009), infertility (Huang, 2016), PID, cervicitis, and puerperal fever (Leli, 2017; Haggerty, 2011). In men, colonization of the genitourinary tract in most cases does not cause symptomatic infections, however, the presence of *M. hominis* in the upper urinary tract may be associated with symptoms of acute infection, such as pyelonephritis. (Imudia, 2008).

M. hominis has also been described as a causative agent of opportunistic extragenital infection, mainly in immunocompromised patients. There have been described cases of postoperative wound infections in patients after kidney transplantation, in whom microbiological and genetic diagnostics for mycoplasmas (and ureaplasmas, because in one of the discussed cases, apart from *M. hominis*, *U. urealyticum* was also detected), it was possible to implement appropriate treatment and quickly improve the patients' condition (Loupy, 2008). In 2016, for the first time a peraortic abscess of this etiology was detected in a patient after heart and lung transplantation (Hagiya, 2017). *Mycoplasma* infections are also a threat to the group of patients after cardiac surgery. Post-operative mediastinitis, which is a classic complication of open-heart surgery, occurs at a rate of 1.3%. In most of these cases, coagulase-negative staphylococci or *Staphylococcus aureus* are detected (46% and 26%, respectively) (Gardlund, 2002). Le Guern et al. reported a patient with nosocomial mediastinitis and osteitis after open heart surgery caused by *M. hominis*. Interestingly, this case involved a 37-year-old man with no additional risk factors (Le Guern, 2015).

M. genitalium was isolated after the 1980s and according to the CDC (*Center for Diseases Control and Prevention*) is now a recognized etiological agent of sexually transmitted infections (STI). It is responsible for approximately 15-20% of NGU cases, 20-25% NCNGU and about 30% of persistent or recurrent urethritis (Workowski,

2015). *M. genitalium* has also been associated with prostatitis and epididymitis, however, less commonly (Manhart, 2007; Ross, 2006). Some data describe the importance of *M. genitalium* in proctitis, mainly in sexually active homosexual men. The incidence of infections in this group without clinical symptoms was 1%-5% and this percentage increased in patients with clinical symptoms of inflammation (additionally infected with HIV) from 8% and up to 20% (Bissessor, 2016; Soni, 2010). Other works also suggest a strong correlation between the coexistence of this pathogen with HIV (Mavedzenge, 2015). Moreover, the participation of *M. genitalium* in the promotion of HIV replication in peripheral blood mononuclear cells *in vitro* was also described (Sasaki, 1993). However, this information is also encountered in works that do not confirm these hypotheses (Manhas, 2009; Gatski, 2011), therefore, there is a continuous need for further research to be able to clearly determine whether the *M. genitalium* may be a significant cofactor in the progression of HIV infection. In women, the presence of *M. genitalium* in the urogenital tract is associated with cervicitis, PID, premature birth or the risk of miscarriage (Bjartling, 2012; McGowin, 2017). The importance of this species in the inflammation of the endometrium, the fallopian tubes and infertility was also indicated (Haggerty, 2011). Svenstrup et al. showed the presence of antibodies against *M. genitalium* antigens in the serum in 17% of women with tubal factor infertility (TFI) and only in 4% without fertility disorders (Svenstrup, 2008). In another study, scientists showed *in vitro* that the presence of *M. genitalium* damages the fallopian tube epithelium, while *M. hominis* did not cause morphological changes. It has also been proven that infection with the *M. genitalium* in women with tubal infertility who have high levels of antibodies to *M. genitalium* with no symptoms of acute or chronic urogenital infection, can cause permanent damage of the fallopian tubes (Baczynska, 2007).

OTHERS UROGENITAL MYCOPLASMAS

Species of other mycoplasmas gained the attention in relation to the role *M. fermentans* of the immunomediator in the AIDS development (Wu, 2014). Since then, *M. fermentans*, *M. penetrans* and *M. pirum* were described primarily in relation to HIV-infected patients. Although, the immunomodulatory role of mycoplasmas in HIV infection has been studied by many authors, there is still a paucity of epidemiological data on their prevalence and the potential role of these microorganisms in infected patients. (Chen, 2015). Ainsworth et al. showed *M. fermentans* in broncho-alveolar lavage fluid in 27% patients with HIV and lower respiratory tract disease. In some cases *M. fermentans* was detected alone (Ainsworth, 2000). It should also be noted, that these bacteria are also detected in non-HIV infected patients. According to published data, the frequency of *M. fermentans* isolation in throat swabs in children with community-acquired pneumonia was approximately 16%, with no other potential pathogens causing pneumonia in two-thirds of them (Cassell, 1994). *M. fermentans* has been also reported as a pathogenic microorganism of chronic inflammatory diseases, such as RA (*rheumatoid arthritis*), chronic fatigue syndrome, fibromyalgia, and neurological diseases, however, this association has been difficult to prove (Gilroy, 2001; Horowitz, 2000; Schaeverbeke, 1996; Nijs, 2002). Therefore, there is a need for continuous research into the pathogenicity of *M. fermentans* strains, as well as other mycoplasma species such as*: M. penetrans* or *M. pirum.*

DIAGNOSTICS

Urogenital mycoplasmas are a unique group of bacteria which, due to high breeding requirements, lack of commercially available tests, small cell size and lack of a cell wall (making Gram staining impossible), is still neglected in routine microbiological testing. Commercial kits for the diagnosis of urogenital mycoplasmas (based on the assessment of the biochemical profile of bacteria) detect *Ureaplasma* spp. strains only without identification to species and *M. hominis*. Therefore, other pathogenic mycoplasma species, including *M. genitalium* in standard culture tests are not detected and required separate diagnostic methods based on molecular biology techniques. The distinction between *U. urealyticum* and *U. parvum*, is also impossible without the use of genetic techniques. Kong et al. for the first time developed a diagnostic scheme with species differentiation on *U. parvum* and *U. urealyticum* based on PCR (*Polymerase Chain Reaction*) (Kong, 2000). Molecular diagnostics based on NAAT (*Nucleic Acid Amplification Tests*) techniques is therefore the right choice for this bacteria detection. The literature provides information on primers that are constructed to identify different species of urogenital mycoplasmas. The FDA (*Food and Drug Administration*) in 2019 approved so far, the first and only genetic test for PCR analysis and detection of *M. genitalium* – Aptima Mycoplasma genitalium assay (CE/IVD AMG). High sensitivity and clinical effectiveness of this test has been confirmed in publications (Unemo, 2018; Gaydos, 2019). However, there is still no accepted test for molecular diagnostics of other *Mycoplasma* and *Ureaplasma* species. Undoubtedly, it would be useful due to the still unclear role of mycoplasmas in the pathogenesis of diseases.

RESISTANCE AND TREATMENT OPTIONS FOR INFECTIONS CAUSED BY UROGENITAL MYCOPLASMAS

Urogenital mycoplasmas often constitute asymptomatic colonization, however in the case of symptomatic infections, antibiotic therapy is necessary. *Mycoplasma* and *Ureaplasma* species don't have a cell wall. This important feature generates their natural resistance to widely used β-lactam antibiotics: penicillins, cephalosporins, as well as glycopeptides. All species in the class *Mollicutes* are also resistant to sulfonamides, trimethoprim, the first-generation of quinolones, and rifampicin (Waites, 2005; Valentine-King, 2019). Ureaplasmas are susceptible to macrolides, but resistant to lincosamides. *M. hominis* is naturally resistant to erythromycin but susceptible to 16-membered macrolides (e.g. josamycin) and clindamycin (Waites, 2005).

Data relating to the treatment of mycoplasmas and ureaplasmas infections indicate three groups of antibiotics: macrolides, fluoroquinolones and tetracyclines (Jensen, 2016; Workowski, 2018; Meygret, 2018). Among the recommendation for sexually transmitted infections (STI) there are no general guidelines for the treatment of mycoplasmal infections other than *M. genitalium*, like *M. hominis* or *U. urealyticum.*

MACROLIDES

The CDC (*Centre of the Diseases Control and Prevention*) from 2015 recommended a single dose of 1g azithromycin – a first-line drug for cervical and urethral infections caused by *M. genitalium* (Workowski, 2015). The scientists from Melbourne (Australia) indicating a decrease in the effectiveness of single dose of azithromycin treatment from 84% (2005-2007) to 69% (2007-2009) (Twin, 2012). Falk et al. comparing two azithromycin treatment regimens – single dose and extended (5 days, 1.5g) therapy,

macrolide resistant of *M. genitalium* were found in 10% and 6.5% patients, respectively (Falk, 2015). Anagrius et al. showed that none of the 77 patients treated with the extended azithromycin regimen showed macrolide resistance (Anagrius, 2013). Therefore, CDC from 2018 indicated that in the case of urethritis and cervicitis caused by *M. genitalium*, a longer course of azithromycin (an initial 500 mg dose followed by 250 mg daily for 4 days) might be more effective than the single dose regimen (Workowski, 2018). According to 2016 "European guideline on *Mycoplasma genitalium* infections" recommended treatment for uncomplicated infection in the absence of macrolide resistance azithromycin 500 mg on day one, then 250 mg on days 2-5 or josamycin 500 mg three times daily for 10 days (Jensen, 2016). Which is supposed to provide a better therapeutic path without causing mutants selection. Treatment of individuals with *M. genitalium* urogenital infection prevents sexual transmission and is likely to reduce the risk of complications, including PID and tubal factor infertility. Erythromycin, is the most commonly used for ureaplasmal infection during pregnancy (Tantengco, 2019). However, the high resistance rate of *Ureaplasma* spp. isolated from pregnant patients was performed (Redelinghuys, 2014). In study by Khosropour from 2015, after treatment with azithromycin, persistent urethritis was more commonly observed in patients infected with *U. urealyticum* than in those infected with *U. parvum* (Khosropour, 2015). Whereas another study performed 100% resistance strains to azithromycin (35 isolates) of *U. parvum* (Kadhim, 2017). For *M. hominis* treatment infections, among macrolides josamycin is indicated by scientists. Data showed, *M. hominis* sensitivity for josamycin rates between 52.8-83% (Meral, 2014; Ozturk, 2019).

FLUOROQUINOLONES

Both CDC and European guideline on *M. genitalium* infections recommended moxifloxacin (4th generation of fluoroquinolones) 400 mg for 7-10 days as a second-line drug for uncomplicated macrolide resistant *M. genitalium* infection. For complicated *M. genitalium* infections (PID, epididymitis) moxifloxacin 400 mg for 14 days was also recommended (Jensen, 2016) Unfortunately, resistance to fluoroquinolones increases steadily. Couldwell et al. reported a failure in treatment of *M. genitalium* infection by moxifloxacin (Couldwell, 2013). According to the data, level of resistance to this antibiotics could be vary in different parts of the world. In Japan, resistance to fluoroquinolones in *M. genitalium* strains seems to be alarming. Kikuchi et al. showed 47.1 % resistant strains to moxifloxacin caused by *par*C mutation (Kikuchi, 2014). Other studies carried out in different countries showed lower percentages of resistant *M. genitalium* strains for moxifloxacin, in 15% patients of sexual health clinics from Sydney (Tagg, 2013) and in 5% from UK (Pond; 2014). The Scandinavian level of potential moxifloxacin resistance of *M. genitalium* is also relatively low. In study showed by Unemo et al. percentage of resistant cases was 10.2% in Sweden, 5.1% in Denmark and 4.1% in Norway (Unemo, 2018).

Fluoroquinolone activity against *Ureaplasma* species is low. However, in view of very limited therapeutic options for ureaplasmal infections studies of their activity are continued. Zhao et al. showed the high resistance of *Ureaplasma* spp. to levofloxacin (3^{th} generation of fluoroquinolones) and moxifloxacin – 47.5% and 39.38%, respectively (Zhao; 2020).

TETRACYCLINES

Among tetracyclines, data showed activity of doxycycline for urogenital mycoplasma infections. In European recommendation, doxycycline is a third-line treatment for persistent *M. genitalium* infection (after azithromycin and fluoroquinolones) in dose 100 mg two times daily for 14 days, however, according to Jensen et al. the patients should be informed about the poor eradication rate and accept to comply with advice regarding sexual abstinence or condom use (Jensen, 2016). Moreover, some publications indicate that in the event of fluoroquinolones therapy failure to which *M. genitalium* and other mycoplasmas strains may be resistant, eradication was obtained only after use of minocycline – semi-synthetic tetracycline (Terada, 2012; Bissessor, 2015; Deguchi, 2017). Another authors showed prevalence *Ureaplasma* spp. and *M. hominis* species resistant to tetracycline in the years 2010-2015 in France and indicated resistance ratio 7.5% and 14.8%, respectively (Meygret, 2018). Bayraktar et al. showed *M. hominis* strains with 100% sensitivity to doxycycline (Bayraktar, 2010). Vargovic et al. reported sensitivities of *M. hominis* to doxycycline (100%) and tetracycline (83.8%) (Vargovic, 2013). Another data performed *M. hominis* also high sensitivity rates, > 90% for doxycycline, > 80% for tetracycline and > 60% for minocycline (Meral, 2014; Ozturk, 2019).

NEW THERAPEUTIC OPTIONS

The mechanisms of natural and increasingly observed acquired resistance of urogenital mycoplasmas induces scientists to seek new treatment for these infections. Data showed activity of solithromycin – a fourth-generation macrolide. *In vitro* studies performed greater activity against *Ureaplasma* spp. isolates compared other antibiotics such as azithromycin, telithromycin, doxycycline, levofloxacin and linezolid, which incidentally proved ineffective against this group of bacteria. The MIC range of solithromycin was ≥ 0.004-0.063 μg/ml for *U. urealyticum* and ≥ 0.002-0.31 μg/ml for *U. parvum*. Moreover, high activity against *M. hominis* and *M. genitalium* strains was also performed (Waites, 2009; Furfaro, 2015; Jensen, 2014).

An organic chemical compound of natural origin, an aminocyclitol antibiotic used in the treatment of gonorrhea – spectinomycin, may become a promising drug for *M. genitalium* MDR (*Multi Drug Resistance*) infections. Falk and Jensen presented studies demonstrating success of urethritis therapy caused by macrolides – resistant *M. genitalium* strains. However, further research is required to determine the appropriate treatment regimen for this medicine (Falk, 2017).

CONCLUSION

In summary, urogenital mycoplasma infections considerated as a risk factor in urogenital infections. *Mycoplasma* spp. and *Ureaplasma* spp. rarely cause severe infections, however, postoperative extragenital infections have been described. There is no guidelines available for mycoplasma infections other than *M. genitalium* etiology. The limitations in the choice of therapy resulting from natural resistance and growing resistance to used antibiotics lead to the search for new, effective therapeutic options.

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Prostate biopsy, its clinical significance and infectious complications in the era of increasing antibiotic resistance

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ABSTRACT

Prostate cancer (PCa) is the most common malignant neoplasm among men. The increasing incidence seems to be most influenced by the aging of the population, as this neoplasm remains in a close correlation between the incidence and the age of the patient. Other risk factors for PCa were also detected, including genetic predisposition, racial/ethnic background and geographical area. Thanks to the progress of diagnostic methods in medicine, especially the introduction of Prostate Specific Antigen (PSA) testing and the modern diagnostic imaging methods such as multiparametric magnetic resonance imaging (mpMRI) into diagnostic schemes, it has become possible to offer patients better diagnostic and also therapeutic procedures. Currently the only method of confirming the diagnosis of prostate cancer is histopathological examination of prostatic tissue obtained during a prostate biopsy. Both systemic and targeted biopsies are performed via transrectal (TRUSB) or transperineal (TPUSB) approach. Despite the lower number of infectious complications, transperineal biopsy is performed less frequently due to its lower availability and difficulty in performing. Like any surgical procedure, prostate biopsy can cause complications like urogenital infections, including urosepsis, bleeding and acute urinary retention (AUR). The increasing antibiotic resistance of microorganisms, urges continuous improvement of prophylaxis and antibiotic regimens prior to the procedure. In our own experience in years 2017-2020 the cumulative 137 incidences of UTI with urosepsis was noted among which 5 patients developed urosepsis after the prostate biopsy 5/137 (3.65%). During the analyzed time period, 2.802 TRUSBs were performed. The cumulative incidence of urosepsis as a complication of TRUSB was thus relatively low 5/2802 (0.18%) In our analysis, the etiological factor of urosepsis was *E. coli* in 5/5 (100%) and in 1/5 (20%) it was the ESBL positive strain. Interestingly, all strains were QR defined as resistant to ciprofloxacin.

In this paper is described the role of prostate biopsy in the clinical diagnosis of PCa, the development of prostate biopsy technique in XX and XXI century and the challenges we face with the increasing antibiotic resistance of microorganisms, causing complications after prostate biopsy.

INTRODUCTION

Prostate cancer (PCa) is the second most commonly diagnosed cancer in men, accounting for 15% of all cancers diagnosed worldwide (Ferlay, 2015). The incidence of prostate cancer is closely related to the patient's age. The prevalence of PCa at age < 30 years does not exceed 5%, increasing to a prevalence of 59% by age > 79 years (Bell, 2015). Ethnic background and family history of PCa incidence are associated with an increased PCa prevalence suggesting a genetic predisposition (Jansson, 2012). But only a small subpopulation of men with PCa have true hereditary disease. This is defined as

three or more affected relatives or at least two relatives who have developed early-onset PCa (< 55 years) (Hemminki, 2012). Germline mutations in genes such as BRCA1/2, NBS1, CHEK2 and HOXB13 have been associated with an increased risk of PCa and targeted genomic analysis of these genes could offer options to identify patients at higher risk of developing PCa (Lynch, 2016; Ewing, 2012). Despite numerous studies on the influence of environmental factors on the PCa development, no specific preventive or dietary measures are recommended to reduce the risk of developing prostate cancer. Prostate biopsy and obtained samples for histopathologic analysis used as diagnostic method for PCa are very important for classification of patients into the low, intermediate or high risk group.

BIOPSY INDICATION

The indications for a biopsy are a suspicious digital rectal examination (DRE), high Prostate Specific Antigen (PSA) concentration, suspicious imaging and repeat biopsy in case of active surveillance (AS) (Ross, 2010; Roobol, 2013). PSA is a specific protein produced by prostate gland cells, both normal and cancerous. It found mostly in semen, but a small amount is also found in blood (Bosco, 2001). Generally PSA level < 4 ng/ml is considered normal, however it should be related to the patients age, clinical status and the dynamic of the growth (Mottet, 2020). Most PSA in the blood is bound to serum proteins (e.g. alpha-1-antichymotrypsin) while small amount called free PSA (fPSA) is not protein-bound (Catalona, 1997; Mikolajczyk 2002; Naya, 2004). In clinical practice it is important to distinguish ratio between a free- and total PSA (tPSA), which is defined by the PSA index (fPSA/tPSA). It was described in medical literature that prostate cancer was detected in men (PSA 4-10 ng/mL) by biopsy in 56% of men with f/t PSA < 0.10, but in only 8% with f/t PSA > 0.25 ng/mL. PSA measure should be done every 2 years in patient at risk to fall PCa meaning men > 50 years of age, men > 45 years of age with a family history of PCa or of African descent, men carrying BRCA2 mutations > 40 years of age. Testing PSA level could be postponed up to 8 to 10 years in those not at risk with an initial PSA < 1 ng/mL at 40 years and a PSA < 2 ng/mL at 60 years of age and a negative family history (Mottet, 2020). Correct interpretation of obtained PSA level is extremely important to avoid over-diagnosing or over-treatment and often other diagnostic tools e.g. multiparametric magnetic resonance imaging (mpMRI) are required.

Recently, an abnormal (PIRADS ≥ 3) mpMRI result was used as an indication for prostate biopsy (Weinreb, 2016). Random systematic transrectal ultrasound guided prostate biopsy (TRUSPB) has been the preferred method for years. This method consisted of taking sections of prostate according to the scheme adopted in a given center-usually by taking 6 sections from both lobes of the prostate. The procedure is performed under ultrasound control in local anaesthesia. With the inclusion of mpMRI in the prostate cancer diagnostic scheme, it became clear that TRUSB was not a sufficient diagnostic tool. Advances in mpMRI have allowed for MRI-targeted biopsies of suspicious imaging findings called fusion biopsy (Siddiqui, 2015; Johnson, 2019). Studies have shown that MRI-targeted biopsies result in a higher rate of detection of high-grade cancers than systematic biopsy (Ahmed, 2017; Rouvière, 2019).

The effectiveness of mpMRI in the diagnosis of PCa has been confirmed in PRECISION and PROMISE trials. In addition, several more or less equivalent techniques are introduced to perform targeted biopsies. Despite TR, ultrasonographically guided, 12-core systematic biopsy being the most common method for the initial diagnosis and grading of PCa the superiority of fusion biopsy in detecting clinically significant PCa is undeniable and confirmed in numerous studies (Ahdoot, 2020). Of course, performing such biopsies requires significant financial outlays for the purchase of appropriate equipment and highly trained personnel, which makes TRUSPB still the most popular diagnostic method of PCa.

COMPLICATIONS

Regardless of the technique of the procedure, each patient requires preparation for a biopsy. The patient should be discontinued from anticoagulants and, if they cannot be completely discontinued, switched to low molecular weight heparins (LMWH). The correct state of the patient's blood coagulation is checked during the examination of Prothrombin time (PT) activated partial thromboplastin time (APTT) and International normalized ratio of prothrombin time of blood coagulation (INR). Both reduce the risk of clinically significant bleeding (haematuria, rectal bleeding) – the most frequent complication of TRUSB, although it usually is not clinically significant.

Infectious complications are another major complications associated with the prostate biopsy. During TRUSB, the prostatic tissue is punctured through the rectal wall, which may result in translocation of the rectal bacteria into the prostate. The European Association of Urology strongly recommends antimicrobial prophylaxis prior to the TRUSB. However the choice of regimens and duration of prophylaxis is debatable (Isen, 1999; Kapoor, 1998; Meyer, 1987; Lista, 2014; Liss, 2017). Fluoroquinolones have been traditionally used for antibiotic prophylaxis. However, overuse of fluoroquinolones leads to promoting antibiotic-resistant bacterial strains resulted in an increase in post-biopsy infections worldwide (Liss, 2017; Roberts, 2014). Due to the increase in fluoroquinolone resistance, alternative prophylactic agents such as fosfomycin trometamole, co-trimoxazole, second/third-generation of cephalosporins were analyzed and targeted antimicrobial treatment based on rectal swab was suggested (Caskurlu, 2015). Not only the type of antibiotic prophylaxis used, but also its duration are currently under consideration. A single dose antibiotic prophylaxis with less than 24 hours "antibacterial effect" is clearly inferior to a 3-day prophylaxis. In contrast, a full 1-day prophylaxis was comparable to a 3-day prophylaxis in analyzed sources (Chazan, 2010). While the available Cochrane review of 2011 suggests a one-day prophylaxis with a single agent (Zani, 2011), a recent systematic analysis has pointed towards an augmented antimicrobial therapy (Walker, 2016). Apart from the antibiotic prophylaxis, rectal cleansing with povidone – iodine prior to TR prostate biopsy is strongly recommended. Recently, due to avoiding the translocation of the abundant rectal bacterial microflora the transperineal approach gains increasing favour. Although there are no large multicenter studies on the superiority of TP biopsy over TR biopsy, individual studies show a lower incidence of infectious complications, including sepsis in the case of TP access (Pepdjonovic, 2017; Wadhwa, 2017). However, in Poland, the availability of equipment needed to perform this type of biopsy is low. Moreover, performing this

type of biopsy requires specialized training. The time and cost of implementing this type of change allow us to believe that TR access will dominate in urology practice for a long time.

ANTIBIOTIC RESISTANCE

Despite the measures taken to reduce infectious complications associated with prostate biopsy, it is estimated that the frequency of infectious complications may increase. Despite well known factors increasing the risk of infectious complications such as: renal failure, diabetes mellitus, current indwelling catheter etc, increasing antibiotic resistance of microorganisms remains a serious issue (Taylor, 2013; Zowawi, 2015; Song, 2014). Especially increasing quinolone resistance (QR) and the presence of extended-spectrum beta-lactamase (ESBL)-producing *Enterobacteriaceae* have been emphasized. Mechanisms of QR include two categories of mutation and acquisition of resistance-conferring genes. Mutations in one or both drug target enzymes (DNA gyrase and DNA topoisomerase IV) reduce drug binding to the enzyme-DNA complex (Yoshida, 1990). Other mutations occur in genes that control the expression of native efflux pumps localized in the bacterial membrane (Yamane, 2007). Mutations of both types can accumulate with producing highly resistant strains. Resistance to quinolones can be mediated by plasmids as discovered inadvertently while studying β-lactam resistance produced by a multiresistance plasmid on transfer to a porin-deficient strain of *Klebsiella pneumonia* (Yamane, 2007; Martínez-Martínez, 1998). Resistance genes acquired on plasmids can promote the selection of mutational high-level resistance. Plasmids with these mechanisms often encode additional antimicrobial resistances and can transfer multidrug resistance that includes quinolones (Jacoby, 1991).

Enterobacteriaceae acquire easily the ability to produce extended-spectrum ß-lactamases (ESBLs) through plasmid-mediated mechanisms. ESBLs are enzymes that hydrolyze ß-lactam antibiotics which confer multidrug resistance to broad-spectrum ß-lactam antibiotics, including third-generation cephalosporins (e.g., cefotaxime, ceftriaxone, ceftazidime) commonly used in UTIs which limits options for appropriate drug therapy (Jacoby, 1991). Carbapenems are regarded as the treatment of choice for ESBL-producing pathogens, causing infections. However, the emergence of strains resistant to carbapenems require more and more attention for treatment regimens for infections caused by these pathogens and increased supervision over the proper course of antibiotic schemes (Vardakas, 2012). Increasing prevalence of infections caused by multi-drug-resistant microorganisms has become an emerging public health concern. The prevalence of QR in rectal flora in 2010 was 10.6% (Batura, 2010) compared to 25% in a study from 2015 (Liss, 2015). Korkmaz et al. calculated quinolone resistance in pathogens from rectal swab as 27% (Korkmaz, 2020). Tigen et al. reported the prevalence of ESBL-producing strains in rectal samples as 18% (Tigen, 2014). In study conducted by Korkmaz et al. the prevalence of ESBL positive pathogens in the rectal swabs of 99 patients was 19.3% (Korkmaz, 2020). In 2011 Steensels et al. demonstrated the prevalence of the ciprofloxacin-resistant bacterial strains in rectal swabs as high as 22% of the patients undergone the TRbx. The incidence of infectious complications was 3%, all of them caused by fluoroquinolone-resistant *E.coli* (Steensels, 2012). Among Gram negative bacteria *E. coli* and *K. pneumoniae* are the most commonly reported ESBL-

producing pathogens. This mechanism of resistance makes bacteria resistant to almost all cephalosporins (Bennett, 2014; Pitout, 2010). The frequency of QR together with ESBL in *E.coli* ranges from 50% to 100% (Pitout, 2010). The extended-spectrum beta-lactams and quinolone co-resistance phenomenon may be attributed to the wide use of quinolones and beta-lactam agents. The ease of plasmid transfer of the ESBL resistance mechanism gene also contributes to the increase in the above phenomenon (Jacoby, 1991). Despite the knowledge that the overuse of antibiotic treatment results in the build-up of drug resistance, the authors note the scarcity of data on the length of time this phenomenon persists regarding how long the microflora maintains such resistance after antibiotics are discontinued (Yagci, 2009).

OUR OWN EXPERIENCES

We have recently conducted a retrospective study on risk factors and management of urosepsis in hospitalized patients with urinary tract dysfunctions. This study was a chart review of electronic health records conducted at a 86-bed Specialized in Urology Hospital in Katowice (Silesian region, Southern Poland). The study included adult patients (18 years or older) admitted to the Urology ward as suspected of urosepsis, between January 2017 and June 2020. Patients were initially identified by screening the electronic health records for positive blood culture, obtained by testing with a VITEK 2 instrument (bioMérieux Vitek Systems Inc). The electronic health records of selected initially identified patients were further reviewed by study investigators to determine the urosepsis symptoms. To achieve this goal we have analyzed patients admitted to the Urology ward from the urologic ER in years 2017-2020, with the suspected urosepsis. The inclusion criteria for the analysis were: diagnosed UTI joint with bacteremia, defined as blood culture positive patients with clinical urinary tract dysfunction and the suspicion of the urosepsis, based on the clinical feature (qSOFA score > 2) at the admission. Patient characteristics for data collection and further statistical analysis were determined. Demographic information, including age and sex were also collected. Presence of urologic comorbidities, length of hospitalization, duration of antibiotic treatment, time of appropriate antibiotic therapy, further hospitalization, continuation of antibiotic therapy after discharge were evaluated. The cumulative 137 incidences of UTI with urosepsis was noted at the study period. In the course of the statistical analysis, we identified, among others, a group of patients who developed urosepsis after the prostate biopsy 5/137 (3.65%). Therefore, we decided to analyze the frequency of urosepsis as a complication of prostate biopsy, taking into account the frequency of the above procedure. During the analyzed time period, 2.802 TRUSBs were performed. The cumulative incidence of urosepsis as a complication of TRUSB was thus relatively low 5/2802 (0.18%) significantly lower than that described in the literature by other authors 9.4% (Shahait D, 2016). In the analyzed patients the symptoms (fever, chills, weakness, deterioration of well-being) occurred 1.6 days (1-2) after the biopsy. In our analysis, the etiological factor of urosepsis was *E. coli* in 5/5 (100%) and in 1/5 (20%) it was the ESBL positive strain. Interestingly, all strains were QR defined as resistant to ciprofloxacin. Apart from the ESBL positive strain, sensitive only to aminoglycosides and carbapenems, the remaining strains showed sensitivity to beta-lactams and aminoglycosides. All analyzed patients were treated with ciprofloxacin in a dose of 2 x 500 mg daily, at least 48 h (48-72 h) before biopsy. The mean hospitalization time was 7.8 days

(5-11), and each patient required Foley catheter placement due to difficult micturition without acute urinary retention. Of course the above study had a number of limitations. First of all it was a single-center study therefore, the results may not be generalized to other institutions and regions. Moreover it was based on data obtained retrospectively, which may result in underestimating our cohort. We analyzed only patients admitted to the ward with a positive blood culture result. Given that our study was retrospective, there is the possibility of incomplete data for vital signs and symptoms, comorbidities and former medical history with the antibiotic in-take history. Moreover, our population is very modest. Despite so many limitations, we believe that our experiences will constitute the basis for further research in the future.

DISCUSSION AND CONCLUSIONS

In recent years, there has been a significant development of techniques for detecting PCa. Historically indications for prostate biopsy were suspicious digital rectal examination (DRE) and/or high Prostate Specific Antigen (PSA) concentration. Although still present, they have been supplemented with an abnormal image of the prostate on imaging examination (mpMRI) and the need to monitor the patient with recognized PCa during AS.

At present, numerous studies on strategies to reduce the incidence of infectious complications related to TRUSB are ongoing. Not only the impact of the duration but also the type of antibiotic prophylaxis is studied. In addition to the above, also infectious complications depend on the access used (TR vs TP), type of needle used, needle disinfection during the procedure, etc. Despite the lower number of infectious complications, transperineal biopsy is performed less frequently due to its lower availability and difficulty in performing. Due to the costs associated with the purchase of new equipment and the time needed to conduct appropriate training, the TRUSB will most likely continue to dominate.

Learning the appropriate strategies for preparing the patient for the examination will significantly reduce the frequency of infectious complications. The main problem in preventing infectious complications may be the increasing antibiotic-resistance of microorganisms. Quinolone-resistant strains can develop co-resistance to multiple antimicrobial agents.

In particular, there appears to be a steady rise in ESBL production among quinolone-resistant strains of the *Enterobacteriaceae*. This is due not only to the ease of antibiotic resistance genes transmission by plasmid transfer, but above all from the abuse and misuse of antibiotic therapy.

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Phototherapy in selected dermatological diseases – literature review

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ABSTRACT

Phototherapy is the main treatment method in a number of skin diseases, such as psoriasis, vitiligo, atopic dermatitis, cutaneous T-cell lymphomas, graft versus host disease and scleroderma. It includes UVA irradiation, which is absorbed by chromophores in the deep layers of the skin. UVA radiation can be introduced in treatment with fucomarin derivatives - psoralens including 5-, 8-methoxypsoralen and trimethylpsoralen. UVB radiation penetrates into the epidermal layer and has a significant ability to cause erythema. In order to establish a safe dose of irradiation, the minimum erythema dose (MED), the minimum phototoxic dose (MPD) and the minimum melanogenesis dose (MMD) are determined. Photodynamic diagnosis includes the detection of neoplastic lesions of the skin at early stages. The changes are visualized by providing porphyrin precursors that induce the accumulation of protoporphyrin IX in pathological cells exposed to UV radiation. Photodynamic diagnosis is also used to determine the cancer border, the site for a biopsy control, as well as to monitor the effects of photodynamic therapy during repetitive therapy.

INTRODUCTION

Phototherapy, otherwise known as light therapy, is a branch of physical therapy that employs natural or artificial light sources in the treatment of dermatoses. Back in ancient times, ultraviolet radiation (UV) on the human body was used to treat vitiligo and psoriasis.

The exponential development of phototherapy has been recorded since 1903, when Niels Ryberg Finsen received the Nobel Prize in Medicine in acknowledgement of his work in treating diseases, in particular *lupus vulgaris*, using concentrated light rays (Singer, 2018; Duarte, 2006). The mechanism applied is based on the interaction between UV radiation and the skin, while the effectiveness of treatment depends on the wavelength used (the longer the wavelength, the greater the ability to penetrate the skin), the type of radiation and the patient's individual sensitivity to UV radiation (Singer, 2018; Duarte, 2006); Bowszyc-Dmochowska, 2006). The effects of UV also depend on the size of a single dose, the number of doses (single, multiple and chronic exposure) and the time interval between individual exposures. Before the initiation of therapy, attention should be paid to the patient's age and the area of irradiated body surface (Wolska, 2007; Bowszyc-Dmochowska, 2006).

Ultraviolet radiation is part of the electromagnetic spectrum; it has greater energy than visible light, oscillating between 200 nm and 400 nm. It is divided into three main subranges (bands): UVA, UVB and UVC, which are presented in Table 1. The UVA subrange is divided into: UVA1 and UVA-2, while the UVB sub-range into: broad band UVB (BB UVB) and narrow band UVB (NB-UVB) (Singer, 2018; Duarte, 2006; Wolska, 2007; Bowszyc-Dmochowska, 2006).

Table 1. Bands of UV radiation

Type of radiation	Wavelength [nm]
UVA	320-400
UVA-1	340-400
UVA-2	320-340
UVB	280-320
BB-UVB	290-320
NB-UVB	311
UVC	200-290

UVA radiation is a long wave, of which > 50% penetrates into the reticular and papillary layers, acting more effectively, among others, on fibroblasts, dendritic cells, vascular endothelial cells and the intercellular matrix (Wolska, 2007).

About 90% of UVB radiation, also known as erythematous radiation, is absorbed in the epidermis, where it affects keratinocytes, Langerhans cells and melanocytes. (Wolska, 2007). The penetration depth of UV radiation is proportional to wavelength. With higher UV radiation, the erythematogenic effect decreases, therefore UVB radiation causes erythema at lower radiation doses than longer UVA waves.

UVC radiation is a short wave which is completely absorbed by the ozone layer. It is characterized by the strongest ability to be absorbed by DNA; therefore, it leads to the damage thereof. Due to its bactericidal effects, it is used in dermatological practice to disinfect rooms. However, it is not applicable in phototherapy, therefore it will not be discussed in the following chapters (Duarte, 2006; Wolska, 2007).

Erythema, pigmentation and thickening of the epidermis are defense mechanisms against higher doses of UV radiation. The tendency to erythema or tan after exposure to UV radiation categorizes phototypes, also known as Fitzpatrick skin types, characterized in table 2. People with light skin suffer the biggest burns from exposure to sunlight, while people with darker skin colors are usually less likely to burn, and a tan is much more common (Christensen, 2012).

Table 2. Division of the skin due to skin reactions after exposure to sunlight according to Fitzpatrick (Wulf, 2010)

Skin type	Skin color	Skin reaction after exposure to sunlight
I	White	Always burns, does not tan
II	White	Often burns, sometimes tans
III	Olive, creamy	Occasionally burns, usually tans
IV	Light brown	Rarely burns, always tans
V	Brown	Almost never burns, always tans
VI	Black	Never burns, always tans

Immediate pigment darkening and *immediate pigmentation dose* resulting from the skin's reaction to UVA radiation, is associated with a dose of about 10-30 J/cm^2 at

a wavelength of 330-460 nm, which appears within a few minutes and disappears within a maximum of two hours after exposure. Immediate pigment darkening in the case of natural sunlight occurs in the afternoon and during psoralen therapy in combination with UVA radiation (PUVA, or *psoralen plus UVA*).

Persistent pigment darkening appears at least 24 hours after exposure to UVA and UVB radiation. Persistent pigment darkening occurs within 3-5 days of exposure to UVA. UVB-induced pigmentation is a delayed tanning process that precedes the appearance of erythema on the skin.

UV radiation is used in the treatment of many skin conditions, but it can cause acute and chronic effects, i.e., skin burns, photoaging and photocarcinogenesis. Acute side effects include gastrointestinal disturbances, headache, dizziness, insomnia, depression, erythema, epidermal hyperplasia, onycholysis, sublingual hematoma, tachycardia, and hypertrichosis. Sunburns are mainly caused by UVB radiation, which penetrates into the epidermis, and stimulates the production and release of prostaglandins, leukotriene, histamine, interleukin 1 and tumor necrosis factor α (TNF-α).

Chronic symptoms include: carcinogenesis, photoaging, actinic keratosis, basal cell carcinoma (BCC), squamous cell carcinoma (SCC), melanoma malignum (MM), cataracts, xerosis, changes in skin pigmentation, formation of lentigo and immunosuppression (Duarte, 2006; Wolska, 2007; Valejo Coelho, 2016; Christensen, 2012).

Photoaging is caused by exposure to UVB and UVA radiation. Manifestations of skin photoaging include lower skin elasticity, reduced wound healing capacity, the formation of wrinkles, dyspigmentation, dry and rough skin, and actinic keratosis. UVA penetrates deeply into the dermis and leads to the production of reactive oxygen species (ROS) that damage DNA, proteins and lipids. Moreover, ROS trigger a cytokine cascade, which leads to photoaging of the skin (Duarte, 2006; Valejo Coelho, 2016; Christensen, 2012).

In selecting a treatment, it is important to remember about the immense benefits and potential complications of phototherapy, which is the main treatment method in many dermatoses, such as psoriasis, vitiligo, atopic dermatitis, cutaneous T-cell lymphomas, graft-versus-host disease and scleroderma.

The further portion of this paper shall describe the diagnostic options and skin disorders in which phototherapy is a routine treatment.

SEARCH STRATEGY AND SELECTION CRITERIA

The purpose of this paper is to collect up-to-date knowledge on the Phototherapy in selected dermatological diseases. The following databases were used in the study: PubMed (NCBI), Google Scholar, Web of Science and Scopus; and the following key words were used: phototherapy, photodynamic diagnosis, UV radiation, PUVA, UVA-1, UVB, epidemiology, pathogenesis, diagnosis, treatment and prognosis.

SELECTED METHODS OF IRRADIATION USED IN PHOTOTHERAPY

UVB

UVB radiation is absorbed by chromophores found in the upper layers of the skin, mainly in the epidermis, resulting in the formation of photoproducts, i.e., molecules that undergo structural modifications under the influence of radiation. UVB-induced photoproducts are cyclobutane pyrimidine dimers, and to a lesser extent (6-4) – photoproducts. Pyrimidine dimers induce apoptosis, immunosuppression and carcinogenesis. UVB radiation generates ROS and is able to directly activate apoptosis-related molecules such as CD95 and TRAIL receptors. It also leads to the release of pro-inflammatory cytokines, such as interleukins 1, 6, 8 and TNF-α, which are responsible for radiation burns (Berneburg, 2013)

UVB phototherapy distinguishes between broad band UVB and narrow band UVB, which covers a different wavelength range, described in Table 1.

The main indications for UVB therapy are psoriasis, atopic dermatitis, graft versus host disease (GVHD), vitiligo, cutaneous T-cell lymphoma (CTCL), lichen planus and hives (Christensen, 2012; Adamski, 2008).

BROAD BAND UVB

Broad band UVB covers wavelengths between 290 and 320 nm. This type of irradiation was initially used in treating atopic dermatitis and has been used since the 1970s. The first step in therapy with broad band UVB is to establish the MED and begin irradiation at a dose of 70% MED, which should then be increased approximately every 2-3 treatments depending on the patient's response. The procedure is usually performed three times a week. Identifying the MED is very important due to a high risk of acute erythematous reactions while applying this type of therapy (Wolska, 2007; Adamski, 2008).

Due to the high potential of erythema and the relatively low efficiency, broad band UVB radiation has been replaced with narrow band UVB irradiation, which is more effective (Ortiz-Salvador, 2017).

NARROW BAND UVB

Narrow band UVB covers a wavelength of 311 nm. This approach was implemented in the 1980s. Such therapy is more effective, leads to faster remissions than irradiation with broad band UVB and does not cause acute erythema, therefore it is used much more frequently than broad band UVB. Moreover, there are no contraindications to conducting narrow band UVB therapy in pregnant women and children. When combined with topical agents such as calcipotriol or retinoids, the effectiveness of this method in the treatment of skin diseases is enhanced (Patrizi, 2015; Christensen, 2012).

Similarly to broad band UVB, the minimal erythema dose should be determined before the onset of therapy, and increased every 1-2 treatments by about 100 mJ/cm^2. Treatments should be repeated three times a week.

Absolute contraindications for UVB radiation therapy are parchment skin (*xeroderma pigmentosum)* and systemic lupus (Wolska, 2007).

UVA

UVA radiation covers wavelengths from 320 to 400 nm. It is a long wave absorbed by the deeper layers of the skin and the dermis. UVA radiation is poorly absorbed by DNA and, by producing ROS, it induces endogenous chromophores. It affects dendritic cells in skin, inflammatory cells and fibroblasts. It also impacts mast cells, granulocytes and collagen fibers. UVA therapy carries the risk of damaging DNA, proteins and lipids, which can lead to skin photoaging and carcinogenesis. Exposure to UVA radiation has elevated significantly due to the popularity of tanning beds that use UVA rays to tan the skin (Berneburg, 2013; Adamski, 2008; Ortiz-Salvador, 2017).

UVA-1

In 1981 there was a development of lamps emitting radiation in a narrower spectrum covering 340-400 nm, in which it is possible to reduce the time of light exposure (Berneburg, 2013; Patrizi, 2015). Similarly as broad band UVA, it induces the production of ROS, CD4+ lymphocytes responsible for apoptosis, otherwise known as helper T cells. Contrary to the apoptosis observed in cells irradiated with UVB or PUVA, in UVA-1 apoptosis is not mediated by protein synthesis only in the presence of ROS. This effect is called direct apoptosis. The production of ROS induces the expression of *Fas*-ligand cytokines on the surface of a T cell exposed to UVA-1 radiation, which, after rebinding to *Fas,* is responsible for the process of T cell death (Ortiz-Salvador, 2017; Mang, 2005; Sage, 2012)

Irradiation involves the use of small, medium and high doses of UVA-1 radiation, amounting to 20 J/cm^2, 30-60 J/cm^2 and 130-150 J/cm^2. The start of therapy should be preceded by determining the minimal tanning dose (MTD) by irradiating six skin areas with increasing doses of UVA-1 and taking a reading after 24 hours. MTD is intended to assess the degree of patient reactivity to UVA-1, but it is not used to determine the first dose of irradiation. Treatment with UVA-1 is usually repeated 5 times a week, and the exposure time per session ranges from 10 minutes to 1 hour (Wolska, 2007; Ortiz-Salvador, 2017; Mang, 2005).

The positive effect of UVA-1 irradiation was observed in the treatment of atopic dermatitis, scleroderma, lichen sclerosus, GVHD, urticaria pigmentosa, CTCL, and pruritus (Christensen, 2012; Mang, 2005).

Contraindications to the use of this irradiation method are age below 18 years, use of medications with photosensitizing properties and skin cancers (Wolska, 2007).

The issue associated with UVA-1 irradiation are lamps that are characterized by high price, require more space and appropriate ventilation machines, which makes them unaffordable for some centers. The high temperature generated by the lamps determines the need to install specific cooling systems (Ortiz-Salvador, 2017).

PUVA

PUVA is based on the combination of UVA radiation with photosensitizing agents – psoralens. It was introduced in the 1970s and is also referred to as photochemotherapy. PUVA therapy uses psoralen as an artificial chromophore to increase the effectiveness

of UVA radiation. Psoralens are derivatives of fucomarin. The most commonly used psoralens in dermatology are 8-methoxypsoralen (8-MOP), 5-methoxypsoralen (5-MOP) and trimethylpsoralen (TMP), the structure of which is shown in figure 1. Orally administered psoralens are metabolized in the liver and peak blood levels are reached within one to three hours. 8-MOP and 5-MOP are natural psoralens, unlike TMP, which is produced synthetically (Singer, 2018; Duarte, 2006). Characteristics of psoralens are presented in table 3.

Table 3. General characteristics of psoralens used in PUVA therapy based on the source (Wolska, 2007; Wolska, 2006; Prabhu, 2014)

Psoralen	Phototoxic action	Dose	Time between administration of the medication and exposure	Main use
8-MOP	Strong	0.6 mg/kg	1 hour	Orally
				PUVA soak
				PUVA bath
				Cream application
5-MOP	Weaker	1.2 - 1.5 mg/kg	2 hours	Orally
TMP	The weakest	0.08-0.33 mg/l	During therapy	PUVA - bath

Psoralen is first absorbed by the DNA of keratinocytes. Next, after exposure to UVA radiation, it binds covalently to the nucleobase to form monoadducts. As a result of UVA re-excitation, psoralen can bind to a second pyrimidine base on the opposite strand of DNA, resulting in interstrand crosslinks which severely damage DNA. Repair of damage requires the cell to delay replication by arresting the cell cycle so as to extend the time of repair mechanisms. However, if the DNA damage is too extensive, the cell initiates apoptosis. Reparation caused by the formation of monoadducts occurs much more frequently than reparation due to the combination of both strands of DNA.

In the PUVA method, we can observe an increase in interleukin-2 and interferon γ (IFN-γ) and a decrease in interleukin 4, 5 and 10. This phenomenon increases the activity of Th1 lymphocytes. By interacting with membrane lipids, PUVA may influence gene expression. Moreover, PUVA therapy involves the induction of ROS, which indirectly damage the DNA structure, leading to the induction of matrix metalloproteinases (Singer, 2018; Berneburg, 2013).

In PUVA, the patient takes psoralen by mouth, and then he is irradiated with UVA. There are two protocols for the application of PUVA therapy, characterized in table 4.

Table 4. Characteristics of PUVA methods based on the source (Wolska, 2006; Prabhu, 2014)

Method	Factor which represents the dose of UVA	Increasing UVA doses	Frequency of treatments
American	Skin type	0.5 - 1.5 J/cm^2	2-3 times a week
European	Establishment of MPD	0.5 - 5 J/cm^2	4 times a week

Indications for PUVA therapy include psoriasis, vitiligo, urticaria pigmentosa, atopic dermatitis, nodular prurigo, alopecia areata, lichen planus, scleroderma, GVHD, light eruption and CTCL (Singer, 2018; Wolska, 2007)

This method of therapy should not be used in people who are hypersensitive to solar radiation, pregnant and breastfeeding women, patients with liver or kidney damage, people suffering from epilepsy and cancer, children under 10 years of age and people taking immunosuppressive drugs (Adamski, 2008; Prabhu, 2014).

During the procedure, the eyes should be protected with UV blocking glasses (Prabhu, 2014).

In addition to system PUVA, we also distinguish *PUVA-bath*, *PUVA-soak* and *PUVA-cream*, which will be discussed later in this paper.

PUVA BATH

Bath PUVA is a modification of the system PUVA initiated in Scandinavian countries. Bath PUVA treatment can involve reduced UVA doses due to the higher concentration of psoralens which affect the skin. In this method, the patient is bathed in a psoralen solution. The psoralens used are 8-MOP and TMP, and their concentrations are respectively 0.5-4.6 mg/l for 8-MOP and 0.08-0.33 mg/l for TMP. TMP concentration is lower than 8-MOP due to higher phototoxicity when applied topically. The bath is kept at a temperature of 30-37°C and lasts about 15-20 minutes. When this time expires, the patient is exposed to UVA rays. The initial dose of UVA is determined in the same way as in system PUVA, and the treatment is performed 3 times a week. Remission of lesions are observed after 12-15 treatments. Maintenance treatment should be introduced for 2-3 months after achieving remission with a reduction of treatments to initially 2 and then 1 per week. PUVA baths generate fewer side effects than system PUVA by eliminating digestive system ailments after oral administration of psoralen; however, they may cause an increased burning sensation of the skin during the procedure and are more time-consuming than system PUVA (Wolska, 2007; Duarte, 2006; Berneburg, 2013; Pai, 2015).

LOCAL PUVA

Local PUVA is a method limited to the therapy of selected parts of the body, most often the hands and feet. The procedure can be performed in two ways. The first is PUVA soak, which involves soaking hands and feet in a psoralen solution for 20 minutes, followed by irradiating these areas with UVA rays after 30 minutes. Treatments are repeated 3 or 4 times a week, with the initial dose of UVA at 1-2 J/cm^2 and in increments of 0.5 J/cm^2. The second method known as PUVA-cream consists in the application of creams or ointments containing most often 0.0006% 8-MOP, and then after 30-60 minutes irradiation of these areas (Berneburg, 2013; Pai, 2015; Ortiz-Salvador, 2017).

SKIN DISEASES IN WHICH PHOTOTHERAPY WAS APPLIED

PSORIASIS

Psoriasis is an immune-mediated chronic inflammatory disease, which mainly affects the skin and joints. It occurs in about 2-3% of the population. Pathogenesis is associated with abnormal interactions between innate immunity, T cells, and keratinocytes.

Cells of the immune system release pro-inflammatory factors, leading to uncontrolled activation of the innate and acquired immune systems, including the NF-κB signaling pathway and differentiation of helper T cells towards Th1 and Th17 cells (Zhang, 2018; Hemne, 2017).

The disease occurs in representatives of both sexes and can appear at any stage of life. There are two types of psoriasis on the basis of the age at which the first symptoms occurred, as shown in table 5.

Table 5. Differences between type I and type II psoriasis based on the source (Hemne, 2017; Wolska, 2006).

Type I psoriasis	Type II psoriasis
Onset of symptoms before the age of 40 years (most often at the age of 16 in women and 22 in men)	The onset of symptoms after the age of 50 years
Peak onset at 16–22 years of age	Peak onset around age 60 years
Presence of HLA antigen - Cw6	Lack of HLA - Cw6 antigen
More severe course of disease	Milder course of disease
Occurrence of streptococcal infections	Lack of streptococcal infections

In addition to the physical dimensions of the disease most often manifested as skin eruption (fig. 1), psoriasis also has extensive emotional and psychosocial effects on patients. This may result in stigmatization, poor self-esteem and increased stress, which in turn affects social functioning and interpersonal relationships **(**Hemne, 2017).

Psoriasis is a genetic condition. If one of the parents has psoriasis, their children have a 10 percent chance of getting symptoms. If both parents have psoriasis, the risk is 50 percent (Hemne, 2017).

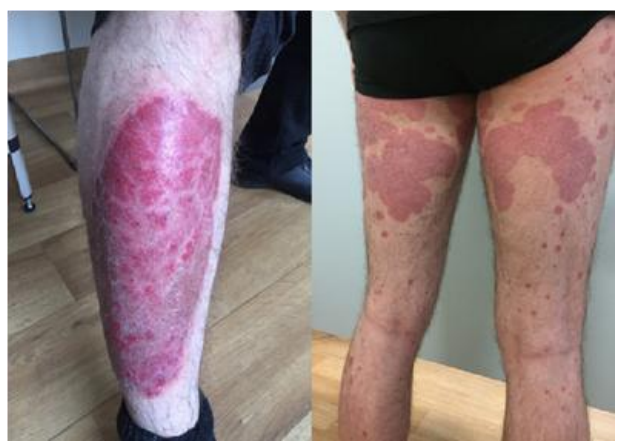

Figure 1. Patients with skin lesions characteristic of psoriasis, which involve the legs

The triggering factors are skin injuries and cuts, streptococcal infections, chronic stress, taking medications such as lithium, beta-blockers and interferon, and corticosteroids. The disease is more frequent and more severe in people infected with the human

immunodeficiency virus. Moreover, excessive alcohol consumption and smoking exacerbate psoriasis and worsen the patient's condition (Wolska, 2006).

Psoriasis is very diverse and can take different forms, each of which has different features that are listed in table 6.

Table 6. Characteristics of the types of psoriasis based on the source **(**Hemne, 2017)

Type of psoriasis	Clinical picture	Occurrence
Plaque psoriasis	Plaque psoriasis is characterized by inflammatory red lesions covered with a silvery-white scales.	It usually occurs on the elbows, knees, the scalp and lower back.
Eruptive psoriasis	It appears as tiny red spots on the skin and often begins in childhood or adolescence.	It usually appears on the torso and limbs.
Inverse psoriasis	Red smooth and shimmering lesions	It affects the armpits, groin, area under the breasts, around the genitals and buttocks.
Pustular psoriasis	Pustular psoriasis is characterized by white bumps filled with non-infectious pus surrounded by reddened skin.	Exemplary locations include the hands and feet.
Psoriatic erythroderma	It is characterized by periodic, extensive, fiery skin redness. Skin erythema and peeling are often accompanied by severe itching and pain.	It occurs on most surfaces of the body
Arthropatic psoriasis	It causes pain, stiffness and swelling in and around the joints.	It often occurs in the hands and feet

The treatment of psoriasis with UV rays began in the 1920s. The PUVA method is of the greatest importance; thanks to photochemotherapy, skin lesions can disappear in a short time. A well-established method of treatment is UVB irradiation.

PUVA penetrates deeper into the tissue than UVB and is more effective, especially in hand and foot psoriasis and eczema, which is due to the thicker skin present in these anatomical sites. PUVA also provides a longer remission period than UVB (Kim, 2017).

Patients with nail psoriasis usually respond well to PUVA, i.e., there is a 70% response rate after 3-4 months of treatment. Improvement is usually observed after 6-10 treatments, and 20-30 treatments are required for complete improvement (Nguyen, 2009).

There have been reports of a lower effectiveness in regards with complete remission with PUVA treatment in erythrodermic psoriasis and pustular psoriasis (Duarte, 2006).

Common side effects of PUVA therapy used in the treatment of psoriasis include nausea, headache and dizziness, burning, itching, and photosensitivity. To avoid nausea, the dose can be divided and administered with food 15 minutes before surgery. In addition, antiemetics such as trimethobenzamide or promethazine can be used; the medication

can be taken 1 hour before consuming psoralen. Squamous cell carcinoma and melanoma are significantly more frequent in fair-skinned people who have undergone at least 200-250 treatment sessions. People with fair complexion are recommended to undergo a maximum of 200 PUVA treatments (Nguyen, 2009).

The results of treatment of psoriasis with PUVA baths are comparable to the effectiveness of oral administration of psoralens, and these outcomes are obtained with the same number of treatments for both therapies. Remission with PUVA baths requires lower doses of UVA (Wolska, 2007)

Narrow band UVB is more effective than broad band UVB radiation in the treatment of psoriasis. UVB therapy that targets psoriatic lesions should be performed at least three times a week. If UVB phototherapy is performed in fewer sessions than recommended, the rate of clinical improvement is significantly reduced. That said, narrow band UVB treatment requires higher radiation doses to achieve minimal erythema and therefore requires longer treatment times compared to broad band UVB. Narrow band UVB is well tolerated by pregnant women and children, and is considered safer; it also has fewer side effects than PUVA therapy (Nguyen, 2009).

Patients with plaque psoriasis require at least 20-30 treatments with UVB rays to produce significant improvements. Patients should be treated for approximately 3 months and then followed up with maintenance treatment, which consists of treatments performed 1-2 times a week, which may vary from several months to several years, depending on the patient's response and the course of the disease (Nguyen, 2009).

VITILIGO

Vitiligo is an acquired, chronic depigmenting disorder of the skin resulting from the selective destruction of melanocytes. The prevalence of vitiligo is 0.5-1% of the population. The highest incidence was recorded in India, Mexico and Japan. Adults and children of both sexes are at equal risk of developing the disease. The risk of developing vitiligo for first-degree relatives is 6–8% (Nguyen, 2009; Richmond, 2017)

Vitiligo can be divided into two main forms: non-segmental and segmental, which are characterized in Table 7.

Table 7. Characteristics of non-segmental vitiligo and segmental vitiligo based on the source (Speeckaert, 2017; Richmond, 2017)

	Non-segmental vitiligo	Segmental vitiligo
Age of onset	Most often between 10 and 30 years of age	Before the age of 30 years
Prevalence	85-90%	10-15%
Depigmentation area	On both sides of the body	On one side of the body
Course of illness	Chronic with the possibility of progression	Fast course and stabilization after 1-2 years

Skin lesions in people suffering from vitiligo are mainly observed on the face, hands and wrists (Ghafouriani, 2014). Vitiligo, as in the case of the previously described psoriasis, can affect the psychological and the physical. More than 1/3 of affected patients experienced depressive symptoms (Nguyen, 2009).

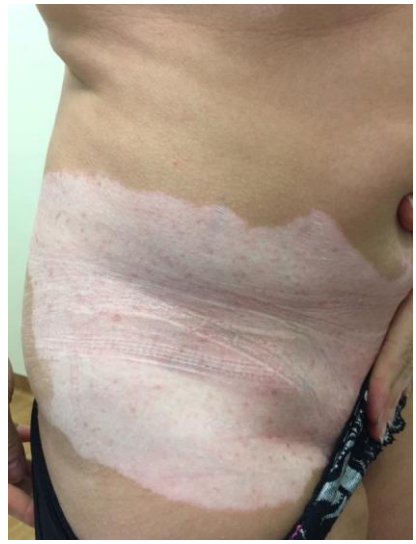

Figure 2. Patient with a skin lesion characteristic of vitiligo

There are currently several treatments for vitiligo, including topical and systemic corticosteroids, PUVA photochemotherapy, and narrow band UVB irradiation. Since the second half of the last century, PUVA has been the most popular form of phototherapy in patients with vitiligo. However, in recent years it has been gradually replaced by narrow band UVB, which has shown greater effectiveness, higher re-pigmentation results and fewer side effects than PUVA in studies (Iwanowski 2018; Esmat, 2017).

The core difference between PUVA treatment in patients with psoriasis and vitiligo is the need for prolonged treatment of 150-200 sessions and difficulty in obtaining a complete or nearly complete response, whereby the therapy itself can last up to several years. The results are much faster with 5-MOP than in 8-MOP, and improvement is noticeable after about 80 treatments, while in the case of 8-MOP only after 140 treatments. The best repigmentation results with PUVA is achieved in the face, trunk and limbs. It is difficult to re-pigment the fingers and toes and the genital area, and in 50-100% of cases the pigment is lost again (Wolska, 2007; Prabhu, 2014).

Topical PUVA may be a suitable treatment option for patients with localized vitiligo. It is safer than oral PUVA due to the lower cumulative dose of UVA and the lack of systemic absorption of psoralen. Contrary to orally administered PUVA, it is considered safe in children over 2 years of age (Esmat, 2017).

Narrow band UVB is used more often due to better remission efficiency, greater safety and the possibility of applying this method in pregnant women and children. In the case of vitiligo, MED is 35% lower compared to normal skin. Generally, less than 100 treatments are required to obtain satisfactory results, although re-pigmentation is possible only after approximately 180 treatments (Wolska, 2007; Nguyen, 2009).

ATOPIC DERMATITIS

Atopic dermatitis, also known as atopic eczema or childhood eczema, is one of the most common inflammatory skin diseases characterized by a chronic and recurrent course. This disease has a genetic background and affects patients of all ages; however, it is much more common in children. It is characterized by eczematous skin lesions and itching, in addition, there may appear urticaria and many other allergic disorders, which are manifested in the upper or lower respiratory tract, the eyes and the gastrointestinal tract. The disease is often associated with elevated concentrations of total immunoglobulin E and usually begins in infancy. Acute inflammation of the limb and face areas is a common feature in infants, while children and adults experience the domination of symptoms of chronic inflammation in the elbow and knee flexions. While the pathogenesis is currently under discussion, several researchers have documented the major role of defective epidermal barrier function in inducing disease, with marked epidermal hyperplasia and Th2 and Th22 immune activation that can progress to Th1 in chronic stages. Over the past three decades, the incidence of atopic dermatitis in developed countries has tripled (Ortiz-Salvador, 2017; Patrizi, 2015; Prezzano, 2017; Rodenbeck, 2016; Fernández-Guarino, 2016).

Treatment of atopic dermatitis involves various forms of phototherapy, including broad band UVB, narrow band UVB, UVA, UVA-1, and PUVA. Phototherapy is considered as a second-line therapy for the treatment of atopic dermatitis, especially in adults (Rodenbeck, 2016; Fernández-Guarino, 2016).

Narrow band UVB may be more often preferred to broad band UVB because it does not cause an acute erythematous reaction. There may occur a burning sensation and significant dryness of the skin, therefore moisturizing preparations are essential. Narrow band UVB is considered a treatment that results in long-term improvement of atopic dermatitis and, very importantly, it is safe in children. A satisfactory result is obtained after about 12 weeks of treatments applied 2-3 times a week.

Systemic PUVA is indicated in the acute phase of atopic dermatitis in severe forms with extensive skin involvement, but is used when all other treatments have failed. In order to achieve remission of lesions, at least 30 to 50 procedures should be performed (Duarte, 2006; Wolska, 2007).

Local PUVA is indicated in localized eczema lesions involving the hands and feet (Duarte, 2006).

Treatment with high doses of UVA-1 radiation, using multiple applications over a short period of time may be an alternative to corticosteroids. Treatments are most often performed five times a week, but it is not recommended for adolescents and children under the age of 18 years. The cycle should not exceed 10-15 treatments. Treatment regimens using UVA-1 at an average dose are 3-5 sessions per week for 3-8 weeks. Treatment time can vary from 10 minutes to 1 hour per session (Duarte, 2006; Wolska, 2007; Berneburg, 2013).

CUTANEOUS T-CELL LYMPHOMAS (CTCL)

CTCL is a heterogeneous group of lymphoproliferative disorders characterized by the accumulation of malignant T cells in the skin, which accounts for approximately 75% of all primary cutaneous lymphomas. The most common subtypes are mycosis fungoides, Sézary syndrome, primary cutaneous anaplastic large cell lymphoma, and lymphomatoid papulosis. These subtypes account for approximately 95% of T-cell lymphomas. Due to the expression of factors such as the cutaneous lymphocyte antigen and the chemokine receptor, early clinical stages of CTCL are present in the epidermis, with infiltrates especially along the basal layer and the adjacent layer of Langerhans cells (photo 8). Women are more likely to be affected than men (Wollina, 2012; Desimone, 2015). Phototherapy is recommended as a monotherapy in patients with early fungal granulomas and in combination with systemic therapies, in early refractory or advanced disease. The choice of narrow band UVB or PUVA as initial therapy may be dictated by patient preferences or availability. UVA exhibits deeper skin penetration than UVB, and patients with follicle-stimulating disease or darker skin may experience greater benefits from choosing PUVA therapy (Tarabadkar, 2019). PUVA was the first type of phototherapy used in the treatment of CTCL and continues to be the treatment of choice. PUVA is effective in early mycosis fungoides and in lymphomatoid papulosis, but is deprived of effectiveness in the tumor stage and in the treatment of Sezary syndrome. Patients with skin phototypes I and II exhibit the greatest response to PUVA therapy. PUVA treatment takes place three sessions per week and the average total treatment time is three to six weeks.

Bath PUVA therapy can be used to treat patients with lesions on the hands and feet. Maintenance therapy is recommended to extend the duration of remission (Tarabadkar, 2019).

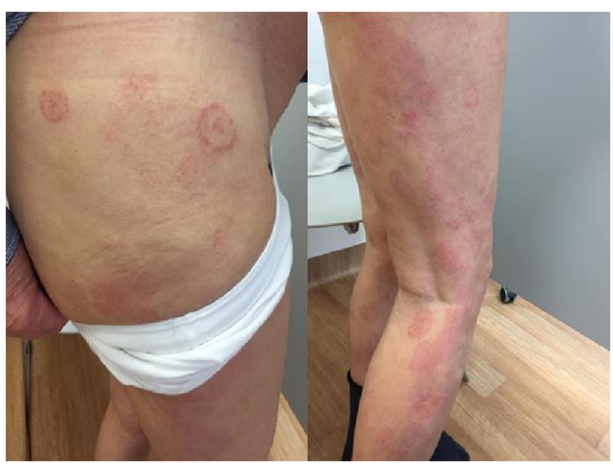

Figure 3. Patient with erythematous lesions and infiltrates caused by CTCL

UVA-1 penetrates the dermis and requires no additional psoralen, which results in lower phototoxicity compared to PUVA. Contrary to PUVA therapy, it is possible to achieve remission in erythrodermatous lesions. Irradiation is performed 5 times a week,

and a favorable result is observed after about 16-20 treatments. Considering the low toxicity of UVA-1 therapy, it may be a safe alternative or complement to other therapies (Tarabadkar, 2019; Wolska, 2007).

GRAFT-VERSUS-HOST DISEASE (GVHD)

GVHD is a major complication following allogeneic hematopoietic cell transplant. It can also appear as a complication after solid organ transplantation or secondary to blood transfusion. GVHD is divided into acute and chronic forms, which differ in terms of both the clinical picture and the risk of long-term consequences (Shi, 2018)

Acute GVHD usually manifests as an erythematous rash in the form of bumps that appear less than 100 days after transplantation. Other features of acute GVHD include acral erythema and erythroderma, or generalized exfoliative dermatitis with possible blistering and skin peeling. Lesions associated with acute GVHD include generalized erythematous papulosquamous lesions, ichthyosis, and pruritus (Tarabadkar, 2019; Shi, 2018).

Chronic forms of GVHD include lichen-type GVHD and scleroderma-type GVHD. Lichen-type lesions appear as individual and confluent papules, and lichen planus-like plaques. In children, this morphology is rare. Sclerodermic lesions usually appear later in the course of chronic GVHD and develop in 15-20% of people (Bowszyc-Dmochowska, 2006)

Other systems and organs frequently affected by GVHD include the gastrointestinal and hepatic systems, as well as the hematopoietic, musculoskeletal, ocular, and lung systems. While GVHD is generally less common among children than in adults, the incidence of GVHD in children increases due to more frequent use of stem cells from unrelated donors (Shi, 2018).

Rapid diagnosis and treatment of GVHD in pediatric patients is very important as chronic GVHD can have severe and progressive life-long consequences for the skin and other organs.

Phototherapy is indicated in both acute and chronic GVHD in lichen-type and scleroderma-type forms. PUVA phototherapy was the first method used in this disease. Other forms of irradiation with favorable results are UVA-1 and UVB radiation therapy (Duarte, 2006).

In the case of oral psoralen plus UV-A (PUVA) therapy, the treatments are repeated 3 or 4 times a week and the therapy lasts from 4 to 8 weeks. The effectiveness of PUVA in lichen-type lesions is 80-100%, unfortunately a much weaker therapeutic effect is obtained in scleroderma-like lesions. When it comes to the treatment of acute GVHD, PUVA alleviates or resolves skin lesions, but it should be remembered that this form of therapy does not prevent the development of a chronic form of GVHD (Wolska, 2007).

UVA-1 therapy is used in both forms of the disease. An over 50% efficacy was found in the treatment of UVA-1 in both lichen-type and scleroderma-type GVHD. In the case of acute GVHD, the results showed a ratio of 70%. The methodology includes sessions 3 to 5 times a week. As UVA radiation is able to penetrate the dermis, it is

believed that UVA-based therapies are a more potent treatment for scleroderma-like lesions (Garbutcheon-Singh, 2015).

Narrow band UVB therapy is performed 2 to 5 times a week. Research of the chronic form revealed that the effectiveness of therapy was 75%. For the acute form, studies have shown over 50% of patients who achieved complete clearance (Garbutcheon-Singh, 2015).

SCLERODERMA

Scleroderma is a chronic disease of connective tissue characterized by the production and deposition of collagen, resulting in fibrosis and vascular changes. Apart from the skin, dermatosis can also attack the joints and internal organs, which is why there are two types of scleroderma: localized, which includes only skin lesions, and systemic, which affects the skin and internal organs. The skin lesions in all types of scleroderma show identical histological characteristics. Pathogenesis includes damage to endothelial cells that leads to their apoptosis and reduction of vascular density, which are associated with inflammatory cell infiltration (Keyal, 2017; Hassani, 2016).

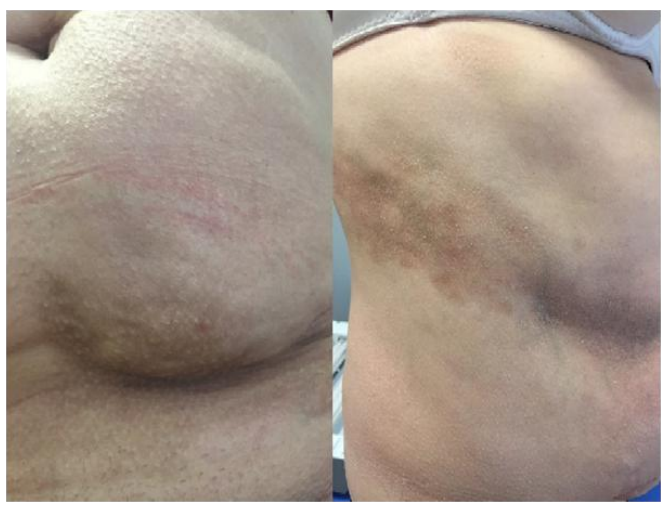

Figure 4. Characteristic lesions in patients with scleroderma of the abdomen and chest

Initially, the most common therapy for scleroderma using phototherapy modalities was PUVA, but due to lower risk of side effects and phototoxic reactions, it is replaced by UVA-1 therapy (Keyal, 2017). Current evidence suggests that the effects obtained with UVA-1 therapy are dose dependent. Better results are seen with high doses of UVA-1 of 80 - 120 J/cm^{2,} compared with low and medium irradiation doses of respectively20-40 J/cm^2 and 40-80 J/cm^2. Nevertheless, in many cases, obtaining favorable results does not require high doses, since a dose of 20-40 J/cm^2 is already sufficient; it can inhibit collagen synthesis, initiate collagenase activity and reduce cellular infiltration. This results in softening of hardened skin observed in a clinical setting. The therapy can be used in all Fitzpatrick skin types, with similar results. Most often, treatments are repeated 3-5 times a week and include about 40 sessions. Based on the current

research, it should be concluded that UVA-1 irradiation is an effective method in the treatment of localized scleroderma. However, as for systemic scleroderma, the results are less optimal and UVA-1 irradiation should not be treated as a first-line therapy (Keyal, 2017; Hassani, 2016; Gambichler, 2018).

CONCLUSION

The origins of light therapy can be traced back to ancient times, but the greatest advances have been made in the last century, providing targeted therapies with specific wavelengths, thereby seeking to minimize complications and improve treatment efficacy.

The best results in the treatment of dermatoses are obtained by irradiating the skin with UVA-1 rays, narrow band UVB and applying PUVA therapy.

Medical diagnosis before beginning treatment is very important due to the individual sensitivity of patients' skin to UV rays. Even though performing diagnostic tests with UV rays is time-consuming, it allows to adjust a safe dose of irradiation, thus eliminating burns and acute skin reactions. Photodynamic diagnostics enables successful detection of neoplastic changes in the early stages.

The effectiveness, ease of treatment, outcomes visible after a small number of therapeutic sessions, and a relatively side effect and safety profile make phototherapy the preferred method of treating many skin diseases.

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Biologic treatment of psoriasis in the COVID-19 era

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ABSTRACT

Psoriasis is a chronic, immune-mediated inflammatory disease. It primarily affects the skin but can damage other tissues and have a detrimental impact on the patient's mental wellbeing. The disease presents itself in diverse ways. There are several types of psoriasis depending on the character and location of lesions. Psoriasis can affect nails and/or joints, which leads to psoriatic arthritis. Hyperproliferation of keratinocytes and a severalfold reduced epidermal turnover time result in the creation of psoriatic lesions. Innate and adaptive immune mechanisms play a crucial role in the pathogenesis of the disease.Psoriasis can be treated with topical medications (such as corticosteroids or vitamin D derivatives), phototherapy with ultraviolet light, or with systemic treatments (comprising of biologic and non-biologic agents). Biologic drugs, available for the treatment of psoriasis since the early 2000s, offer a target-specific approach to it. The invention of these molecules has revolutionized the therapy of psoriasis. The application of these agents, however, leaves the patient in an immunocompromised state.In light of the ongoing COVID-19 pandemic, further administration of biologic drugs could come into question. Currently some patients consider biologic treatment discontinuation and are often unsure if they can be vaccinated while on biologics. Thus it should be considered whether it is safe to continue systemic treatment of psoriasis. Do patients with psoriasis have a higher risk of SARS-CoV-2 infection? Can they safely receive a COVID-19 vaccine during biologic treatment? In this review, we present background information on psoriasis and summarize the current state of knowledge to answer these questions.

INTRODUCTION

Psoriasis is a common, chronic, inflammatory and psychologically debilitating disease of the skin that can begin at any age. Its prevalence tends to increase with distance from the equator. In adults, psoriasis is observed from 0,91% (United States) to 8,5% (Norway) of the population. Children are less often affected and the prevalence varies from 0% (Taiwan) to 2,1% (Italy). There is no apparent gender predilection of the disease (Parisi, et al. 2013). From 2008 to 2015, more than 1 million patients in Poland received dermatologic care due to psoriasis. The prevalence of the disease was estimated to be 2,99% in the Polish population (Borzęcki, 2018).

PATHOGENESIS OF PSORIASIS

Psoriasis belongs to the group of immune-mediated inflammatory diseases, in which dysregulation of specific inflammatory pathways causes the malfunction of other systems. The genetic factors contribute to the pathogenesis of the disease to a large extent. The heritability of psoriasis was estimated to be 68% (Lønnberg, 2013). The PSORS1 locus, located on the 6th chromosome in the MHC region, accounts for 35 to 50% of genetic susceptibility for psoriasis and is associated with the early onset of the disease (Allen, 2005).

Many features can trigger psoriasis in people with a genetic predisposition. Mechanical stress – epidermal lesions, air pollution, ultraviolet exposure, smoking and alcohol

consumption, infections (especially streptococcal), drugs (e.g., imiquimod, beta blockers, terbinafine) are the extrinsic risk factors. Certain comorbidities can be associated with the incidence or flares of psoriasis and these are obesity, dyslipidemia, diabetes mellitus and hypertension – which can be perceived as intrinsic risk factors for the disease (Kamiya, 2019).

The development of psoriasis can be divided into two phases: initiation and maintenance. Initiating the disease process could be attributed to triggered keratinocytes, which secrete antimicrobial peptides such as psoriasin, beta-defensins and cathelicidins (Takahashi, 2020). Out of many functions, these molecules can bind self-DNA and enhance activation of plasmacytoid dendritic cells (pDC) – which appear to be crucial to the initiation of psoriasis. The activated pDC secrete large quantities of interferon-α (IFN-α) which in turn promotes the development of conventional and inflammatory DC, as well as the release of interleukins (IL): IL-23, IL-12, tumor necrosis factor alpha (TNF-α) and other cytokines, leading to the differentiation of T cells (Wang, 2020). Th1, Th17 and Th22 lymphocytes subpopulations emerge and secrete IFN-γ, IL-17 and IL-22. This handful of cytokines strongly stimulates keratinocytes' hyperproliferation, thus epidermal turnover time decreases more than fivefold (to approximately 9,8 days) leading to the characteristic hallmark of the disease (Zhang, 2015). It has been known for a long time that T lymphocytes play a key role in psoriasis (Valdimarsson, 1986) and its pathogenesis can be described as T-cell mediated.

PSORIASIS: CLINICAL ASPECTS

Clinical presentation depends on the type of psoriasis. Plaque psoriasis (or psoriasis vulgaris) is the most prevalent type (concerns about 90% of affected patients). Other variants include pustular, inverse, guttate, and erythrodermic psoriasis. Although an uncommon type, erythrodermic psoriasis requires emergency care, as in this variant, at least 75% of the body is erythematous and inflamed. These patients need close observation due to impaired thermoregulation, fluid abnormalities and risk of septic complications (Singh, 2016).

Plaque psoriasis presents with erythematous patches (differing from 1 to 10 centimeters in diameter) with sharply defined margins, usually covered with fine, silvery scales. The lesions occur mainly on the scalp, extensor surfaces (knees, elbows) and gluteal cleft but can appear anywhere on the body surface. If the palms and soles are affected, the patients experience painful and thick plaques that impair their mobility (Armstrong, 2020). A feeling of chronic pruritus may accompany the disease.

Diagnosis of plaque psoriasis is based on physical examination, yet in some cases, a skin biopsy may be needed to confirm the diagnosis. There are specific clinical signs of plaque psoriasis.The Koebner phenomenon describes the development of new lesions in the irritated areas of healthy skin (e.g., by scratch, pressure or bump). Such triggered lesions typically appear 10 to 20 days after the trauma (Ahad, 2015). Also, pinpoint bleeding is observed that occurs after removing (e.g., by scraping with a spatula) a psoriatic scale. This phenomenon, called the Auspitz sign, results from damaging tortuous capillaries located beneath the thin layer of the immature epidermis.There is one more sign revealed on scratching a psoriatic lesion – the candle-grease sign. It

refers to the greasy appearance of scales collected on the surface of the sharp tool used to scratching the plaque. It is important that while all these signs may aid in making a diagnosis, none of them alone can confirm it.

One more feature that a clinician should be aware of is nail psoriasis. It is estimated to affect up to 50% of patients with psoriasis. The pathologies concern nail matrix, nail bed as well as nail plate, and symptoms like onycholysis, nail pitting or discoloration, subungual hyperkeratosis and splinter hemorrhages can be observed (Tan, 2012). Since nail psoriasis can often be overlooked and lead to a deterioration of a patient's quality of life, the medical professional should always pay attention to this area during the treatment.

Some patients with psoriasis develop psoriatic arthritis (PsA). A meta-analysis revealed a prevalence of PsA of 19,7% in patients with psoriasis and 24,6% in patients with moderate to severe disease (Alinaghi, 2019). PsA may present with pain, stiffness and effusions in the affected joints. Nail lesions and enthesitis are also symptoms characteristic of PsA.According to another meta-analysis, up to 15,5% of patients with psoriasis seen by dermatologists may have undiagnosed PsA (Villani, 2015). As untreated PsA may lead to irreversible joint destruction, a particular focus on this clinical entity should be maintained.

Psoriasis is known to affect the patients' mental wellbeing. The psoriasis-related disability in the field of physical and mental functioning was found to be comparable to that disability observed in other major diseases like cancer, arthritis, heart disease, diabetes, hypertension and depression (Rapp, 1999).Psoriasis evokes social stigmatization and often makes it harder to get employed. The prevalence of psychiatric disorders is higher among patients with psoriasis than in healthy persons. In this group, the odds ratio (OR) for depression was found to be 1,49 and OR for stress-related disorders was 1,41 compared to the healthy control group (Schmitt, 2010).The psychiatric morbidity was revealed to be a strong predictor of poor adherence to dermatologic treatment. On the other hand, compliance with therapy was strongly associated with complete patient satisfaction (Renzi, 2002). Better mental health leads to improved treatment adherence, which in turn increases patient satisfaction. Hence it is crucial for the medical professional to be aware of patients' possible psychiatric disorders and to aid them accordingly if needed.

OVERVIEW OF THERAPEUTIC OPTIONS

Psoriasis is a chronic, recurrent disease that cannot be cured. Therefore the target is achieving remission and controlling the disease. An array of therapeutic options is huge and the approach differs according to the clinical presentation. Regardless of offering treatment, it is important to educate the patient about the disease and suggest considering membership in an organization like National Psoriasis Foundation.

Topical treatment describes medications applied to the skin. It often serves as the first line of therapy in a newly diagnosed person but can also be combined with other treatment courses. Topicals are available in many formulations (e.g., ointments, lotions, creams or solutions) and exert their effects through various mechanisms. The most

important types are corticosteroids, vitamin D analogues, tars, dithranol, emollients and keratolytics (Chiricozzi, 2017).

Another option is the use of ultraviolet (UV) light and this approach is known as phototherapy. UV exerts antiproliferative and anti-inflammatory effects, which may aid in cases of insufficient topical treatment. Phototherapy is usually performed when at least 10% of body surface area is involved. Common side effects include erythema, pruritus, blisters and edema. However, the risk for carcinogenesis is also increased. Therefore patients should be carefully qualified for this kind of therapy (Zhang, 2018).

For moderate to severe psoriasis, systemic treatment may be required. It entails greater risk for adverse effects but offers better efficacy than previously mentioned procedures. Systemic agents can target the immune system (immunosuppressants and immunomodulators) as well as keratinocytes' hyperproliferation.The conventional non-biologic drugs (e.g., methotrexate, cyclosporin or retinoids like acitretin) have a long history in the treatment of psoriasis. These systemic drugs are less expensive than biologic ones but act not selectively.

BIOLOGIC DRUGS IN THE TREATMENT OF PSORIASIS

There is no doubt that the creation of biologic drugs paved the way for the target-specific treatment of psoriasis. Biologics are complex molecules engineered using living organisms (e.g., bacteria). They include monoclonal antibodies and receptor-fusion proteins administered subcutaneously or intravenously, interfering with specific targets and directly altering their function. There are several possible targets for biologics in psoriasis and to date, there are 11 registered agents which inhibit TNF-α, IL-12, IL-17 or IL-23 and related pathways.A meta-analysis confirmed that biologic treatments were associated with higher chances of achieving a 90% reduction in psoriasis area and severity index (PASI) score than conventional or small-molecule systemic treatments (Sbidian, 2017).Yet nothing comes without a price. Administering a biologic agent increases the direct costs of treatment by 3 to 5-fold on average. The annual cost of treating psoriasis was found to be between $2,077 and $13,322 per patient-year. This analysis was based on data from 5 European countries (Burgos-Pol, 2016).However, as the patent for a biologic drug expires (not shorter than eight years from the authorization of a medication), its biosimilar molecules can be created. A biosimilar should be highly similar to the reference molecule and have no clinically meaningful differences (in terms of safety and effectiveness). These drugs are typically 25 to 30% less expensive than original biologic agents. Nevertheless, their efficacy and safety should be carefully assessed, as not only financial factors should affect clinical decisions (Ruiz-Villaverde, 2021).

Application of immunosuppressive drugs brings satisfactory reduction of inflammation in the skin but blocking the immune system, which is responsible for defense against infections and tackling cancerous cells, may cause adverse events.While administering biologic drugs increases the risk of contracting a serious infection, psoriasis itself is independently associated with a greater vulnerability to them (Rademaker, 2019). The course of biologic treatment should only be started when all serious infections have been resolved. Screening for latent *mycobacterium tuberculosis* infection is of particular importance in patients who are candidates for biological therapy. There is an increased

risk of reactivation of tuberculosis (TB) under immunosuppressive treatment (especially with TNF-α inhibitors) and the prognosis for such cases is worse than for the new infections (due to a higher risk of fulminant course of the TB) (Carrascosa, 2018); thus quantiFERON-TB tests became almost a standard diagnostic procedure before treatment. Latent TB infection was reported to concern between 5 and 29% of patients with psoriasis, depending on the region (Gisondi, 2014).

INSIGHT ON BIOLOGIC THERAPY OF PSORIASIS IN THE COVID-19 ERA

In general, psoriasis was found to be associated with a small increase in the risk of serious infections, leading to hospitalization, as compared to the population without psoriasis (Yiu, 2021). However, currently available data indicate that the incidence of SARS-CoV-2 infections and clinical outcomes of COVID-19 in patients with psoriasis are similar to the rest of the population (Gelfand, 2021). At the same time, risk factors for poor outcomes of COVID-19 (e.g., obesity, diabetes mellitus and chronic heart, kidney or lung diseases) are known comorbidities occurring in psoriasis. Nonetheless, current guidelines indicate that treatments for psoriasis do not change the risk of infection or the worse COVID-19 outcome in a meaningful way (Gelfand, 2021). In a cohort study of 6501 patients with chronic plaque psoriasis receiving biologic therapy in Northern Italy, no adverse effects of biologics on COVID-19 outcomes (in terms of hospitalizations and deaths) were reported (Gisondi, 2021). Another study assessed the impact of TNF-α inhibitors and/or methotrexate on hospitalization and mortality due to a SARS-CoV-2 infection. The probability for these events was not significantly different when the group receiving treatment was compared to the rest of the infected people (Yousaf, 2021). In fact, it has been suggested that TNF-α and IL-17 inhibitors may be of some benefit to the treatment of COVID-19. The use of these biologic agents could prevent the cytokine storm (in which IL-17 takes part) and multi-organ failure (Kamiya, 2021). IL-17 antibodies were found to downregulate the expression of angiotensin-converting enzyme 2 (ACE2) in the skin; thus, potential SARS-CoV-2 infection through psoriatic lesions could be diminished or prevented by IL-17 inhibitors (Xu, 2021). Most importantly, the Task Force on behalf of the National Psoriasis Foundation advises that: "it is recommended that patients who are not infected with SARS-CoV-2 continue their biologic or oral therapies for psoriasis and/or psoriatic arthritis in most cases" (Gelfand, 2021).

Table 1. List of currently approved biologics for the treatment of psoriasis

Name	Type	Target	Application	Year of approval to treat psoriasis*
Etanercept	TNF-α receptor fused with Fc fragment of IgG1	TNF-α inhibitors	Sc	2004
Infliximab	Chimeric mouse-human IgG1 mAb		Iv	2006
Adalimumab	Human IgG1 mAb		Sc	2008
Certolizumab	Humanized Fab' fragment of IgG1 mAb conjugated with PEG		Sc	2018

Ustekinumab	Human IgG1 mAb	IL-12/IL-23 inhibitor	Sc	2009
Secukinumab	Human IgG1 mAb	IL-17 inhibitors	Sc	2015
Ixekizumab	Humanized IgG4 mAb		Sc	2016
Brodalumab	Human IgG2 mAb	IL-17 receptor inhibitor	Sc	2017
Guselkumab	Human IgG1 mAb	IL-23 inhibitors	Sc	2017
Tildrakizumab	Humanized IgG1 mAb		Sc	2018
Risankizumab	Humanized IgG1 mAb		Sc	2019

*by U.S. Food and Drug Administration
Applied shortcuts: Fab' – fragment antigen-binding, Fc – fragment crystallizable, IgG – immunoglobulin G, Iv – intravenously, mAb – monoclonal antibody, PEG – polyethylene glycol, Sc – subcutaneously

However, patients sometimes discontinue treatment on their own due to the lack of safety and COVID-19 related concerns. In a study of 178 psoriatic patients, it was found that 6 % (11 patients, 8 of whom were on biologics) discontinued their treatment during lockdown (Sacchelli, 2020). It appears that the level of the patient's knowledge of the current pandemic is connected to the potential decision of cessation of the therapy. Results from a questionnaire performed on Italian dermatological patients treated with biologics indicate that more patients with lower scholarity and less knowledge of COVID-19 have thought to autonomously discontinue or modify their treatment (Bragazzi, 2020).

Suspending systemic treatment may cause psoriatic flares and deteriorate the patient's condition. Cessation of therapy could result in loss of response during re-treatment and even production of anti-drug antibodies (Lebwohl, 2020). It is hence crucial to supply patients with trustworthy information on their therapy and pandemic to prevent biologics discontinuation.

Vaccines nowadays can consist of live, attenuated pathogens or not and then be regarded as non-live vaccines, which can include killed, inactivated pathogens, toxins or genetic material. It is generally recognized that attenuated vaccines are contraindicated during immunosuppressive treatment, which should be suspended in advance when administering such vaccination is required. On the contrary, patients on immunosuppressive therapies can receive non-live vaccines without interruption of treatment. However, it has been found that conventional systemic drugs (e.g., methotrexate or cyclosporine) are associated with decreasing the vaccine-triggered antibody production, thus impairing the vaccination efficacy. Of note, biologic systemic treatments are not associated with lowering the protective antibody titers, probably due to their selectivity in immunomodulatory effects (Chiricozzi, 2020).

Above all, the current National Psoriasis Foundation recommendation is that: "systemic medications for psoriasis or psoriatic arthritis are not a contraindication to any currently available COVID-19 vaccines (be they mRNA-based or adenovirus vectored vaccine)".

It is further recommended that patients do not cease their systemic treatment in most cases when receiving the mRNA vaccine. Similarly, a continuation of systemic treatment is recommended in most cases of receiving adenovirus vectored vaccine, with the exception of methotrexate in some patients (Gelfand, 2021).

At the same time, patients with psoriasis on biologics do not know (36,6% of respondents of a recent electronic survey) or are unsure (26,6%) about the difference between attenuated and inactivated vaccines. Around 66,9% of questioned patients were unsure whether it was possible for them to get the inactivated vaccine during a biologic treatment (Le, 2021).

Some encouraging case reports of patients with psoriasis on biologics who received COVID-19 vaccination have already been announced (Damiani, 2021). As the body of evidence is growing and the effectiveness of vaccines in this group is being further established, the role of dermatologists could be to assure their patients and strongly recommend "getting a jab".

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