The role of Interleukin-7 serum levels as a biological marker in breast cancer

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This dissertation was made at the Department of Oncology, University Hospital Center Zagreb, Croatia and Department of Thoracic Surgery in University Clinical Center of Kosovo in Prishtina, Kosovo.

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LIST OF ABBREVIATIONS

Akt	Protein kinase B (serine/threonine-specific protein kinase)
AJCC	American Joint Committee on Cancer System
ALL	Acute Lymphoblastic Leukemia
ASCO	American Society of Clinical Oncology
AUC	Area under the Curve
BC	Breast cancer
CA15-3	Cancer Antigen 15-3
CEA	Carcinoembryonic Antigen
CDH1	CDH1 gene
CD274	CD274 gene
CISH	Chromogenic in situ hybridization
CNS	Central nervous system
CTLA4	Cytotoxic T-Lymphocyte Associated Protein 4
DCs	Dentritic cells
EIBC	Early invasive breast cancer
EMT	Epithelial mesenchymal transition
EGF	Epidermal growth factor
ER	Estrogen receptor
ENCR	European Network of Cancer Registries

FISH	Fluorescent in situ hybridization
IARC	International Research Agency
IDC	Invasive ductal carcinoma
IFN- y	Interferon gamma
ILC	Invasive lobular carcinoma
IL-7	Interleukin-7
IL-7R	Interleukin-7 receptor
ILs	Interleukins
JAK-1	Janus kinases-1
JAK-3	Janus kinases-3
LVI	Lympho-vascular invasion
MDCS	Myeloid-derived suppression cells
MHC	Major Histocompatibility Complex
NST	Non special type
NCI	National Cancer Institute
PD-L1	Programmed death-ligand 1
PDCD1	Programmed Cell Death Protein 1
PI3K	Phosphoinositide 3 kinase
PI3K/Akt	Phosphoinositide 3-kinaze/protein kinase B
PNI	Perineural invasion
PR	Progesterone receptor
SLNB	Sentinel lymph node biopsy

SEER	Surveillance, Epidemiology, and End Results
TNM	Tumor node metastasis
Treg	T regulatory cells
TSLP	Thymic stromal lymphopoietin
TSLPR	Thymic stromal lymphopoietin receptor
UHCZ	University Hospital Center Zagreb

1. INTRODUCTION AND BACKGROUND FOR THE PROPOSED RESEARCH

The word cancer indicates a group of diseases caused by the uncontrolled change and proliferation of cells in the body. In most cases, cancer develops as a mass in an organ from its cells and it is named as a tumor of that organ. Breast cancer (BC) develops from breast tissue, which consists of glands for milk production or lobules, and ducts, which make the connection between the lobules and the nipple. In addition, the composition of the breast includes connective, adipose, vascular, and lymphatic tissue (1).

Breast development begins in intrauterine life, then at puberty and later during the lactation period. In the girls around the age of adolescence, the first signs of breast formation begin. Estrogen, which begins to be secreted by the ovaries, affects the growth of the breasts and the development of the duct system. At the time when ovulation and the menstrual cycle begin, the maturation of the breasts begins with the formation of secretory glands (2).

1.1. The structure of the breast

The breast is an organ that is anatomically located on the outside of the thoracic wall between ribs II and VI, from the sternal line to the axillary line. For clinical reasons, the breast is divided into 4 quadrants: lower inner, lower outer, upper inner, upper outer. BC usually develops in the upper outer quadrant. The breast is made up of 15-20 lobes, and each lobe is surrounded by connective and adipose tissue, and it consists of many lobules. The lobules are also named ductal-lobular units and are the basic structural unit of the breast. This structure is surrounded by epithelial cells. The lobules are divided into dozens of alveoli, the unit where milk is produced. Milk flows from the alveoli into the ducts. The ducts flow into larger ducts (10-15), and each of them meets a lobe, connecting it to the nipple. The most common BC originates from the epithelial cells. About 85% of breast cancers originate from ductal cells (ductal carcinoma) (3).

The rest originates from lobules (lobular carcinoma) (4). Other tumors are rare and originate from connective tissue. The breast also contains blood and lymph vessels. Most of the breast lymph vessels drain into the axillary lymph nodes, while some of them in the infraclavicular and supraclavicular lymph nodes. Cancer cells can penetrate the blood and the lymph vessels and spread through them to other parts of the body.

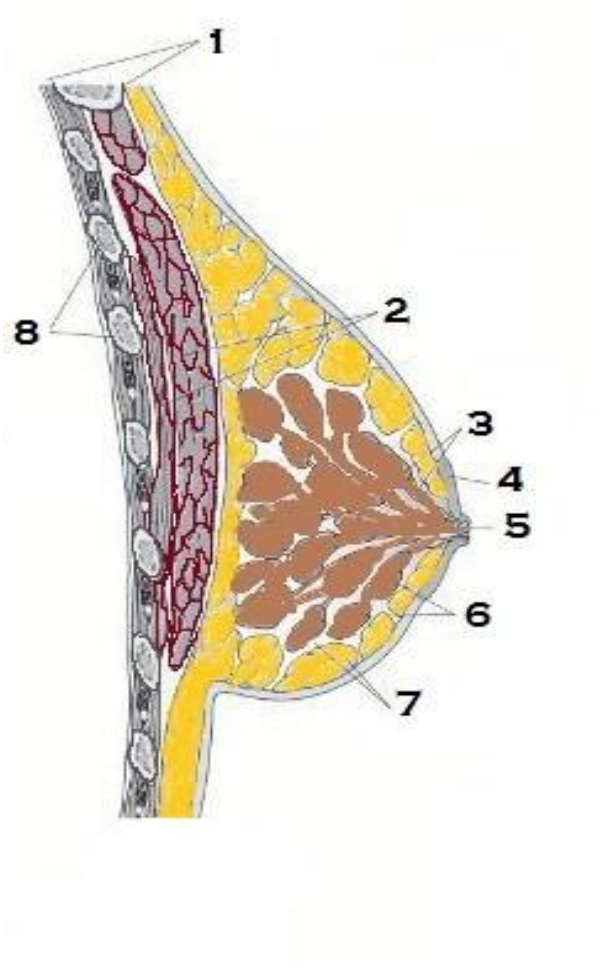

Figure 1. Anatomy of the breast: 1-chest wall, 2-muscle, 3-ducts, 4-areola, 5-nipple, 6-lobules, 7-stroma, 8-ribs. The image modified from: American Cancer Society (5).

1.2. Epidemiology

BC is the most common malignant pathology in women with about 32% of all malignant tumors affecting them. BC is also responsible for 15% of deaths from malignant tumors in women worldwide. In 2020, 2.261.419 new cases were registered and there were 684.996 worldwide deaths caused by this disease (6). While the incidence of BC is in rise in both development end developing countries, the number of deaths in developed countries is declining, whereas in the developing countries is in rise.

The global incidence of BC has increased from year to year by about 3%. This has been seen in the last four decades, and this trend seems to continue (7). All over the world, the burden of cancer, including BC on women, is increasing regardless of the level of economic development of different countries, due to the general growth and aging of the population. The female gender constitutes 49.5% of the worldwide population in general, while it constitutes most of the population over the age of 60 (8). However, the incidence is higher in developed countries than in developing and underdeveloped countries, due to the increased presence of risk factors and easier access to mammography and other diagnostic methods. It is important that, in developed countries, the diagnosis of BC is made at an early stage, enabling a more effective treatment and longer life expectancy than in developing countries, where they are usually diagnosed at more advanced stages and consequently have a worse prognosis (9). The data from the previous study have also shown that, in women living in Asia, BC occurs at a younger age, the vast majority at the age of 40-50 years, while in the mentioned places the peak is around the age of 60-70 years (10). Tumor biology also varies between different ethnicities. Therefore, in African and African American women the triple negative BC type dominates, also a higher percentage of metastatic disease and a higher percentage of poor-differentiated tumor is observed, which affects the poor prognosis of these patients (11).

BC is a disease that usually affects women, and it is relatively uncommon in men. The female: male ratio is 100:1. The incidence of BC in men has remained unchanged for decades, except for Africa, where an increase in incidence has been observed recently, for reasons still unclear. The

risk of developing BC varies depending on age groups. For example, from 0–39 years old is 1 in 229 or 0.44%, in the age group 40–59 years old it is 1 in 24 or 4.14%, while in the age group 60–70 years old it is 1 in 13 or 7.53% (12). Caucasian women have a higher rate of BC compared to African Americans, although these differences are not apparent before age 50 (13).

In terms of the incidence of BC in women internationally, this varies significantly. The United States and the Northern Europe lead with the largest number of new cases, followed by Southern and Eastern Europe with South America, and the lowest incidence is in Asia (14). The incidence of BC is higher in women with a higher socio-economic level, and this has more to do with lifestyle. The left breast is usually affected more often than the right one, while the most common localization of the disease is in the upper outer quadrant and the retroareolar region.

1.3. Risk factors

Many risk factors, such as genetic, environmental, hormonal factors are associated with development of the BC.

Genetic factors. Hereditary forms occur in only 5-7% of all the cases of BC. Mutation in *BRCA1, BRCA2* and *p53* genes increases the risk for developing BC. The *BRCA1* gene is located on chromosome 17. This gene is quite broad and complex with more than 1000 different mutations detected so far. Mutations in *BRCA1* are inherited in an autosomal dominant manner and are associated with an increased risk of breast, ovarian, and to a lesser extent prostate cancer. Women with the mutation in *BRCA1* gene during their lifetime risk developing BC at 56-85%. The *BRCA2* gene is located on chromosome 13 and it is longer and more complex than *BRCA1*. Mutations in the *BRCA2* gene have been linked to an increased incidence of BC in both women and men. *BRCA1* and *BRCA2* are jointly responsible for familial breast and ovarian cancer (15).

Family history. The relative risk of developing BC in a women with a positive first-degree family history (mother, daughter, sister) is 1, 7. In premenopausal women, this risk is 3 times higher, while in postmenopausal women it is 1, 5. The diagnosis of some special breast conditions, such as ductal hyperplasia, lobular and ductal atypical hyperplasia, lobular carcinoma

in situ and ductal carcinoma in situ, called also as "*Proliferative breast diseases",* is associated with increased risk for later development of invasive BC (16).

Personal history of cancer. If a woman has a personal history of BC, this poses a risk factor for developing another BC. This risk increases by 1% each subsequent year from the moment of diagnosis (17).

Reproductive factors. The onset of menarche before the age of 12 is associated with moderate increase in the development of BC. When menopause occurs before the age of 30, it reduces the risk by about 2 times compared to women who enter menopause after the age of 55. The first pregnancy before the age of 30 seems to have a protective effect against BC, while when the first pregnancy occurs after the age of 30, or there is an absence of births, it is associated with an increased risk. It is also thought that lactation protects against the development of BC (18).

Exposure to radiation. There has been an increase in the incidence of BC in the survivors of the atomic bomb blast in Japan after a period of 15-20 years (19).

Estrogen intake. Epidemiological data suggest a strong link between plasma estrogen levels and BC risk (20).

Alcohol and diets. Moderate alcohol intake appears to increase modestly the risk of BC. Women who consume large amounts of red meat or high-fat foods in their diet have a higher risk of developing BC (21).

Obesity. Obesity seems to be a risk factor for developing BC, especially in postmenopausal women. It is estimated that about 20% of cases of BC are attributed to risk factors such as obesity, physical inactivity, and alcohol use (22).

1.4. Signs and symptoms

Signs and symptoms that can occur in BC are mass in the breast or axilla, thickening or swelling of a part of the breast, bulging of a part of the breast, red or cracked skin, pulling of the nipple from the inside or pain in the area around it, leakage from the nipple (23).

The increase in the number of malignant pathologies detected in asymptomatic persons is attributed to the use of mammography for screening. Mammography findings that suggest malignant nature are the presence of mass, asymmetry, micro calcifications, and architectural changes. Further, these data should be evaluated by ultrasonography and in rare cases by magnetic resonance. The most common complaint, 65-75% of cases, is the breast mass. Inflammatory BC is particularly aggressive and accounts for about 5% of cases with BC. The breast looks red and wrinkled, with oedematous skin (orange peel). Ultrasonography can help to distinguish between mastitis and abscess with inflammatory carcinoma. Paget's disease is associated with intraductal carcinoma developed in the terminal ducts of the breast. It can present as an eczematous change in the nipple, breast mass or haemorrhagic discharge from the nipple. Often the diagnosis can be made through cytology.

1.5. Diagnosis

The diagnosis of BC is based on clinical examination, imaging (mammography, ultrasound), and needle biopsy. Mammography is used as a routine method to detect BC in women who have obvious signs and symptoms (24). Diagnostic mammography can help determine these signs and symptoms whether they are related to BC. Diagnostic mammography uses X-rays in more detail compared to screening mammography and at the same time, it requires a longer time for its realization (25). The effectiveness of mammography in diagnosing BC depends on the size of the tumor and the density of breast tissue (26). Therefore, by starting from the latter, it follows that mammography is used more in women over the age of 50 due to the lower density of breast tissue. Recently mammography techniques have advanced and now 3D mammography or

tomosynthesis can also be used. This new mammography technique is up to 28% more accurate in diagnosing BC compared to standard mammography (27).

Ultrasound is useful when the mass is large enough, and the images can be further evaluated for other abnormalities. Through breast ultrasound, the mass can be distinguished in solid, cystic, or fluid containing. It also enables the determination of the size of the mass and the correct localization (28).

Magnetic resonance imaging (MRI) enables the examination of the breast tissue through multiple images, and it is usually used when preliminary examinations are not conclusive. MRI scans breast tissue by taking detailed photographs of areas within the breast and distinguishing between normal and pathological tissue (29).

Biopsy is an invasive method by which a piece of tissue or fluid is taken from a suspicious site, to be examined under a microscope, and then other tests are done in order to verify the presence or absence of malignant cells. Biopsy is the only diagnostic procedure that definitively determines whether a suspicious area is cancerous. There are different types of biopsies: fine-needle aspiration, core-needle aspiration, and surgical biopsy. Several factors determine the type of biopsy, i.e., appearance of mass, size, and localization (30).

In addition to clinical and imaging examination, two tumor markers are already in use (CEA and CA15-3). The investigation for the ideal tumor marker that will be used for the diagnosis, prognosis or monitoring of the therapy is continuing. The ideal tumor marker needs to be tumor specific, measurable without invasive methods, with high sensitivity and specificity (31).

Pathological report

For an invasive carcinoma, the histopathological report should contain details, such as histological type of tumor, tumor size, histological grade, hormonal receptors status, Progesteron (PR) and Estrogen (ER), Her2 status, involvement of lymph nodes, perineural and lymphovascular invasion as well as surgical margins. These data are necessary to establish an adequate treatment for the patient (32). Based on the latest edition of the WHO classification, BC is divided into 19

subtypes (33). The most common types of BC are Non special type (NST) which constitutes about 70-75%, Lobular invasive carcinoma 10-15 %, while the tumors that appear less frequently are Mixed type, and more rarely Ductal carcinoma in situ, Lobular carcinoma in situ, Medullary carcinoma, Tubular carcinoma, Mucinous carcinoma (Colloid), Paget disease of the breast, and Angiosarcoma (34).

The histological grade is made according to the system of Elston and Ellis-modified Scarff-Bloom-Richardson (35), and it is based on the proportion of cancer cells, which are in tubular formation, anisocaryosis and the number of mitoses. The final grade is marked with G1, G2 or G3. The histological grade indicates the potential aggressiveness of the tumor and it is an important prognostic factor. In all invasive BC, it is necessary to determine the ER, PR and Her2 receptors status (36, 37, 38) as a predictive factor to help determine the therapy. These are determined through immunohistochemistry as a routine diagnostic procedure. According to the American Society of Clinical Oncology (ASCO), if nuclear staining occurs in >1% of tumor cells it is considered a positive hormone (ER and PR) (39). Her2 can be positive, negative, or equivocal. In cases where it results equivocal, additional analysis by fluorescent in situ hybridization or chromogenic in situ hybridization is used to determine the status of Her2, whether it is positive or negative. Ki67 is used to determine cell proliferation and it is a predictive factor for determining chemosensitivity, especially for BC ER-positive and Her2 negative. The determination of perineural and lymphovascular invasion is also of great importance, because a correlation between these factors, lymph node metastases, and local recurrence, has been observed (40).

Lymph node evaluation is done through sentinel lymph node biopsy (SLNB) or through complete axillary dissection. Through SLNB, it is determined if there are any pathological lymph nodes even when they are clinically negative, and this is done to avoid unnecessary axillary dissection. Axillary lymph node dissection is a surgical procedure through which the metastatic lymph nodes confirmed by SLNB or clinically pathological lymph nodes are removed from the axillary region. The results of these tests help to determine more accurately the treatment of the patient as well as the prognosis of the disease (41).

1.6. Staging and prognosis

Tumor staging is based on the American Joint Committee on Cancer System (AJCC) (42), also known as TNM classification system, which is based on tumor size, lymph node status, and the presence of distant metastases.

Prognostic factors include tumor type, disease stage, tumor grade, lymphovascular invasion, age. Patients who are diagnosed with BC before 35 years old have potentially more aggressive disease than the ones in older age (43). The detection of the disease at an earlier stage through the widespread use of mammography has made patients have a better prognosis. The histological type also has prognostic importance, for example, some special types such as tubular, cribriform, and mucinous, have a better prognosis. Whereas some other types like pleiomorphic lobular carcinoma, high grade metaplastic carcinoma and micropapillary carcinoma have worse prognosis. Molecular surrogate subtypes of invasive BC according to the expression of formerly mentioned molecular markers, namely Luminal A-like, Luminal B/Her2 negative-like, Luminal B/Her2 positive-like, Her2 positive type and Triple negative, represent five groups in which the prognosis differs significantly from each other and help to determine the most specific therapy for a particular patient (38, 44).

1.7. Treatment

BC, as the most common malignant disease in women, is very heterogeneous at the molecular level. In the last two decades, much importance has been given to this heterogeneity to select biological therapy, which will be more specific, and with fewer side effects on the patient. Early BC, which is considered as such when it is localized only in the breast or even when it has involved axillary lymph nodes, is now considered curable in up to 70-80% of cases, thanks to the advancement of multimodal treatment. The whole concept of BC treatment is divided into two main pillars: locoregional and systemic treatment. This is largely based on the clinical-pathological and molecular characteristics of the tumor. In clinical practice, the classification of five surrogate subtypes based on histological and molecular characteristics is now used (45). It is

important that treatment by specialized multidisciplinary teams has given good results in survival and quality of life in patients with localized and metastatic BC.

Localized breast cancer

For localized and non-metastatic BC, the standard treatment is surgery. However, most of the patients should be treated with systemic therapy (endocrine therapy, chemotherapy, target therapy). In the patients with large tumor or locally advanced disease, systemic therapy may be applied prior to surgery (neoadjuvant therapy). Tumor type, tumor size, degree of disease progression and many immunohistochemical biomarkers which are also used as prognostic factors participate in the definition of systemic therapy (adjuvant therapy). Patients with positive ER and/or PR receptors should be treated with endocrine therapy, while the ones with high risk factors should be treated with chemotherapy (46). There are already many guidelines based on the prognostic values of MammaPrint and OncotypeDx, based on which treatment is planned (47, 48, 49).

Surgery

The surgery of the primary tumor remains the main method of treating BC. Historically, mastectomy has been the standard method, but in recent decades, breast conservation has become the primary method, when it can be applied. In studies performed on young women <40 years old with BC, breast conserving surgery combined with adjuvant radiotherapy has showed the same overall survival rate as the ones who have undergone total mastectomy (50). When, from oncological point of view, mastectomy is indicated, breast reconstruction can be done in patients who want to do it. Surgery can be applied as the first method or after neoadjuvant therapy, depending on the size of the tumor and the relationship with the breast, tumor biology and the decision of the patient. Axillary evaluation is preferably performed by SLNB, given the morbidity that can be caused by open axillary dissection (51).

Radiation therapy

The radiation therapy after surgery is used in early BC patients with lymph nodes involved or after partial breast surgery. The dose of radiotherapy to the whole breast is (45 – 50) Gy, divided in (1.8 – 2.0) Gy fractions. An additional dose of (10 – 16) Gy is delivered to the boost. The boost volume includes the surgical bed with a (1.5 – 2) cm margin. This treatment procedure has enabled the increase of overall survival and has provided a local control by reducing the rate of local recurrence (52).

Systemic therapy

Systemic therapy (endocrine or chemotherapy) has been shown to be very effective in early BC by significantly reducing mortality (53, 54, 55, 56). Indications for systemic treatment are based on clinical-pathological characteristics, tumor biology, molecular type, and risk of recurrence. Chemotherapy can be applied after the surgery (adjuvant therapy), or before the surgery (neoadjuvant therapy) to reduce the size of the tumor and at the same time enable a breast conserving surgery. Giving neoadjuvant chemotherapy also enables the evaluation of the effectiveness of the therapy. In luminal early BC with positive ER and/or PR receptors, endocrine therapy is a standard treatment, which should continue for at least 5 years. Tamoxifen is used as standard endocrine therapy in premenopausal women. The analogue of gonadotropin-releasing hormone (GnRH), which makes ovarian suppression, is added (57, 58). In postmenopausal women, Tamoxifen or an aromatase inhibitor as monotherapy is standard treatment. In BC Her2 positive patients, treatment with anti-Her2 therapy is standard of care (59, 60). In cases with triple negative BC, chemotherapy is the standard treatment. The combination between anthracyclines and taxanes has been shown to be the most successful (61).

Advanced breast cancer

Advanced BC is called when the disease is localized but inoperable or metastatic.

Surgery

In the metastatic BC, at the time of diagnosis, surgery is not used as a routine. Several clinical studies have been performed and it has been observed that breast surgery in metastatic disease has not given any benefit compared to non-operated patients (62, 63, 64). Therefore, the issue of surgical intervention remains optional, and it should be considered in each patient separately (65). Therefore, in cases where there is a significant regression of metastases after the treatment with systemic therapy, surgery of the primary tumor may be an option. Palliative surgery can be used in case of indication.

Radiation therapy

Radiation therapy in metastatic disease can be used only for palliative purposes, to treat painful bone metastases, brain, and soft tissue metastases. In the palliative treatment of bone metastases, several modalities of doses and fractions have been proposed, but the most recommended dose is (20 – 24) Gy in 4 or 6 fractions, but cases and their treatment must be highly individualized. The standard treatment of the multiple brain metastases is Whole-Brain radiation therapy (WBRT) with a dose of 30 Gy delivered in 10 daily fractions. Stereotactic radiosurgery as a highly precise beams of radiation is recommended to the patients with one and three brain metastases, in combination with WBRT (52).

Systemic therapy

Systemic therapy in metastatic disease is determined based on the tumor biology. Biopsy and the determination of the status of hormonal receptors (especially ER and Her2, while PR in this case is less relevant), are necessary. For all the types of luminal-like metastatic BC, the endocrine therapy should be used as first line, and these drugs should be continued until disease

progression (62, 66). Combination of the chemotherapy should be reserved for cases of rapid disease progression (62). If the patient has been previously treated with anthracycline and taxane as adjuvant chemotherapy, then Capecitabine, Vinorelbine, or Eribulin are recommended. Anthracyclines and taxanes can be reused in patients with a treatment-free interval of more than one year (67). In the patients with Her2-positive regardless of hormonal receptors, anti-Her2 agents should be used until disease progression (68, 69). The first line of choice is trastuzumab with pertuzumab in combination with Docetaxel, Paclitaxel or Capecitabine (70). For patients with triple negative BC the use of platinum agents has given good results (71). In addition, the use of PARP inhibitors (olaparib or talazoparib) has shown to increase progression-free survival (PFS) and improve quality of life (72). Recently the use of immunotherapy (pembrolizumab or atezolizumab) has shown good results by increasing PFS (73, 74).

Recent studies in the field of molecular biology and immunology have provided impressive data, based on which future therapy for BC will be decided.

1.8. Screening

BC screening is defined as the evaluation of women without signs and symptoms to detect BC in its early stage. The most effective and widely used method worldwide is mammography screening. Other methods used for screening are breast self-examination, clinical breast examination, digital breast tomosynthesis, ultrasonography, magnetic resonance imaging of the breast and identification of various genetic oncogenes (75). The main advantages of BC screening programs are early diagnosis, classification, and prevention of risk factors, and timely treatment to reduce morbidity. Screening has also reduced the overall mortality rate by about 20%. The main disadvantages of BC screening are overdiagnosis, high cost, ionizing radiation, recommendation of false positive biopsies, false negative results. Most of the countries in the World recommend starting screening at the age of 50 and continuing until 74 years old (76). However, some countries, based on the higher risk of their populations being affected by BC, recommend beginning screening at 40.

1.9. Epidemiology of the breast cancer in Croatia

Like in Europe, breast cancer (BC) is the most common malignant tumor among women in Croatia; one-quarter of newly diagnosed women with cancer have BC (77). In 2016, 2735 new cases of BC were registered among Croatian women (126.6 per 100,000 people) with 990 deaths (45.8 per 100,000 people), making it the most common cause of death for women diagnosed with cancer in Croatia (77). A constant increase in the incidence of BC was evident with 2303 women diagnosed in 2005 and 2748 women in 2015. However, with the stabilization of the mortality trend in recent years, the number of cases in 2012 was 1033, whereas it dropped to 990 cases in 2016 (77, 78, 79, 80). Compared to other European countries, Croatia has a medium-high incidence and high mortality due to BC (81). Some progress has been made in the last 15 years regarding the treatment of BC and cancer survival rates in Croatia. However, the statistics remain below the average for EU member states.

Since 2006, when the National Program of Breast Cancer Early Detection (Mamma) was first implemented in Croatia, 60-70% of newly diagnosed women (50-69 years old) with BC were the localized stage. However, prior to the start of Mamma, this value was 40%. The positive effect of the implementation of the screening program of BC is also evident in the 2017 data, which demonstrate a 15-20% reduction in the mortality rate from BC. A significant achievement has been observed in the last two years. Specifically, a decrease in mortality from BC was noted in all age groups with the largest decline in the older age groups of 70 years and older.

According to the results of the CONCORD-3 study, which analyzed individual records of 37.5 million patients diagnosed with one of 18 cancers from 322 population-based registries in 71 countries, Croatia is in the lower half of the European rankings for BC 5-year survival (79%) (82). In comparison, according to the EUROCARE-5 study, the 5-year BC survival rate for women diagnosed between 2000 and 2007 in Croatia was 76.3%, whereas the average survival rate in European countries was 81.8% (83). According to the aforementioned data, over the last 15 years, there has been progress in terms of cancer survival in Croatia; however, there is still room for improvement.

1.10. Epidemiology of the breast cancer in Kosovo

In contrast, data in relation to the situation with BC in Kosovo are limited. Centralized oncology services in Kosovo started for the first time in 2011. Until this time, oncology patients have been partially treated in various clinics and surgery departments of the University Clinical Center of Kosovo (UCCK) in Prishtina. In 2011, the National Program for Cancer Control in Kosovo was prepared by the National Board for Cancer Control in Kosovo. Based on this program, since 2012, the registration of the new cases of cancer in the relevant application form by health personnel throughout Kosovo has begun (84).

The data from the Kosovo Agency of Statistics-Health Statistics show a steady annual increase in new BC cases. During the period of 2012 to 2018, there was a continuous increase in the number of new cases ranging from 207 in 2012 to 444 new cases in 2018 (85). Notably, in 2019, there were 376 new registered cases (20.9 per 100000 people). These figures in Kosovo can be attributed to the worldwide increase in incidence as well as improved reporting, better means of detection, and mammography program, which was established in 2014, has played an important role in reaching out to patients living in remote areas. According to Kosovo data, only approximately 40-50% of cases are diagnosed in the early stages of the disease regardless of age (86). However, data on the survival and mortality rates of BC patients in Kosovo are not available.

1.11. The immune system and cancer

The idea that the immune system helps fight cancer is very old and dates back more than a century, when in 1909 the German scientist Paul Ehrlich stated that the incidence of cancer would be greater if the human immune system was weak, and that immune system cannot recognize and damage malignant cells. Meanwhile, various scientists have dealt with cancer and the immune system, but as a separate field, it began to develop in the 1950s. It took several decades to develop the concept of immune surveillance. Thus in 2001, it was reported that in genetically modified mice with weakened immune system components, there was a higher incidence of cancer (87). This theory was later supported by several studies in humans. In patients with

HIV/AIDS in whom the immune system has been compromised, there were more frequent cases of some rarer types of cancer, such as Kaposi's sarcoma (88). In addition, in patients who had undergone various organ transplants, treated with immunosuppressive therapy to prevent the rejection of the transplanted organ, cases of cancer that were more frequent were observed (89, 90, 91).

The external environment that surrounds us contains many types of pathogens, such as bacteria, viruses, fungi. The main role of the immune system is to protect the body from these pathogens, but it also plays a very important role in protecting against cancer. The immune system is divided into two main pillars: the innate immune system, which protects from the birth, and it is always active, and the adaptive immune system, which is a defence that must be prepared and therefore requires a certain time. The two components interact with each other and, as a result, they protect the organism from various pathological agents as well as against cancer (92).

Innate immune system is the first line of defence that operates based on three components: mechanical, chemical, and cellular protection. The mechanical part consists of the skin, which prevents the passage of various pathogens inside the body. The hairs on the nose prevent various particles from entering through the respiratory tract. Chemicals such as acidic substances secreted by the skin, mucus secreted by the epithelial cells of the respiratory tract, gastric juice, tears and saliva contain an enzyme called lysozyme, which destroys a large number of bacteria. All these constitute the chemical component. These two components, mechanical and chemical, protect the organism largely from different pathogens, but a number of pathogens still pass inside the organism. In this case, the third component is activated, which consists of innate immune cells and includes macrophages, dendritic cells, neutrophils, and natural killer cells (93). These cells carry out the defence by swallowing pathogens, with the process called phagocytosis, as well as the release of some inflammatory molecules that alert the body to the presence of external pathogens. Innate immune system recognizes different pathogens through different substances (lipopolysaccharides and peptidoglycans) which are found in the cell wall of bacteria, double-stranded DNA in some viruses, etc (94, 95).

Adapted immune system: As long as the innate immune system is present from the birth and it is constantly active, the adaptive immune system needs time to develop. It does not exist from the birth but it is created in time, after the body is faced with different agents (95). The adaptive immune system has three components: diversity, specificity, and memory (96). It can be said that it is characterized by *diversity* due to the possibility that it must recognize any foreign molecule in countless targets. It is *specific* because it detects and protects the body from specific pathogens, for example a specific virus. Moreover, the fact that it distinguishes between components of our body and foreign agents is particularly important. The adaptive immune system has *memory,* which means that if the body is once exposed to a pathogenic agent, then the immune system will memorize it and, in case of re-encounter, it will react very quickly, within a few hours. The fact that in old age we have weaker adaptive immunity is one reason why malignant diseases are more common. The adaptive immune system is made up of specialized white blood cells called lymphocytes, which born in the bone marrow and after their maturation pass into the bloodstream (97). T lymphocytes perform part of their maturation in the Thymus. After the maturation, T and B lymphocytes pass to the lymph nodes and other lymphoid organs. A characteristic of the T and B lymphocytes is that they could generate almost endless new receptors for the identification of pathogens. They accomplish this through genetic regeneration and DNA resynthesize. T and B lymphocytes, in addition to the receptors they have on their surfaces, also secrete various chemicals that enable communication between them and other immune cells. These chemicals are called *cytokines* (98).

The inflammatory process is very important for the protection of the body from external pathogens and cancer. In this case, both the innate and the adaptive immune system interact with each other. The moment a foreign agent enters the body, it is immediately surrounded by immune cells, and the cells of the innate immune system, including macrophages and neutrophils, are the first to arrive. Inflammation also plays an important role in cancer. During the tumor growth, it causes disruption of the structure of the organ and in this case stimulates the immune response. In addition to directly damaging cancer cells, another function of innate immune system cells is to present this information to the cells of the adaptive immune system, a process called *antigen presentation* (99). These antigens are recognized by the respective T cell

receptors. Just as the immune system responds with humoral and cellular immune responses to external pathogens, it is thought to react to cancer cells by recognizing them as foreign. Given that the immune system is not perfect, then the onset of cancer occurs, but the focus of future studies is precisely on strengthening the immune system in the fight against cancer.

1.12. Interleukins

Inflammation plays a key role in the immune system and in protection against various pathogens, including viruses, bacteria, and it is thought to mediate tumor onset and development, angiogenesis, and metastasis (100). Oncogenic changes such as hypoxia, cytokines, chemokines, and other factors are attractive to inflammatory cells. Inflammation of the tumor microenvironment includes infiltrating immune cells and activated fibroblasts, which secrete chemokine and other growth factors, which act on the tumor (101).

Interleukins (ILs) are a group of naturally proteins whose role is to mediate the communication between cells. Under normal conditions ILs are secreted by cells of the immune system to recognize and localize a foreign cell and bind to it through receptors on the cell surface. This induces a cascade signal within the cell through which change cell's behavior having impact in cell growth, differentiation, and motility (102). In addition, their role is important in stimulating immune responses, as well as inflammation (103). ILs were discovered in the 1970, and at that time, it was thought that ILs were made by leukocytes and act on the other leukocytes. For this reason, they have been called *Interleukins*. Because ILs are involved in immune processes, firstly it was thought that their function was only in modulation of immune response. Now it is known that this is an important role of ILs, but it is also known that ILs are produced and interact with several cells that are not included in immune processes (104). Thus, the role of ILs in the human body is much greater than what was thought initially.

In tumor genesis, interleukins directly stimulate stromal cells at the tumor site, causing the cytotoxic effector cells to recognize the tumor cells. Many ILs are present in the tumor microenvironment and interact with various biomolecules, such as cancer stem cells, markers of

epithelial mesenchymal transition (EMT) (105). The involvement of ILs in tumor-promoting mechanisms such as DNA methylation, immunoassay, immunosuppression, and inflammation-induced carcinogenesis serves as a platform for the future research into an effective anti-cancer immunotherapy.

As the tumor grows, it tends to evade the recognition by the immune system and create a microenvironment suitable for further progression. Down-regulation of MHC expression makes them insensitive to T cells (106). On the other hand, tumors can secrete various substances that inhibit the response of T cells, such as, immunosuppressive cytokines: TGF-β and IL-10 as well as some enzymes that catabolize amino acids that are essential for T cell function, such as: arginase, indoleamine-2,3-dioxygenase, IDO (107, 108, 109). In the tumor microenvironment, the immune cells, which are tolerant towards tumors such as exhausted1 cytotoxic T lymphocytes, macrophages, and T helper cell type 2, as well as more myeloid-derived suppression cells (MDCS) and T regulatory cells (Treg), responsible for inhibition of effector immune responses are often found (110). New advances in cancer immunotherapy rely on the use of cytokines to create a more controlled in vitro environment for the normal development of antitumor T cells.

Many ILs participate in tumor development and progression, and thus determining the immunogenic phenotype of tumors. It is now clear that there is an interaction between immune and tumor cells in the tumor microenvironment. This interaction between immune and tumor cells goes through three stages (111). In the first one, the immune cells destroy the malignant cells. The ones that survive this phase enter the second on. During the second stage, immune system cells and tumor cells coexist in the tumor microenvironment. At this stage the cells of the immune system, although they cannot destroy the tumor cells, nevertheless do not allow them to multiply and metastasize. In the third stage, immunologically sculpted tumors begin to grow progressively creating a suitable and immunosuppressive tumor microenvironment. The results of recent studies have confirmed the fact that in the absence of adaptive immunity, the production of IFN-γ NK cells stimulates Macrophages M1, which play the role of important effectors in the cancer editing process (112, 113, 114). Based on the data, some ILs such as IL-17, IL-1 β and IL-6, are involved in key mechanisms of tumor genesis. IL-4, IL-6, IL17A, and IL32 β are

involved in several EMT pathways contributing specifically to the metastasis process (115, 116, 117, 118). The role of many other ILs in tumor genesis is also very important for further studies, to develop the diagnosis and new immunotherapeutic tools in cancer. For this reason, better understanding of the signaling pathways used and the interaction between ILs and TME, stem cells, microRNA, mesenchyme epithelial transition, DNA methylation, immunoassay, is of particular importance.

1.13. Interleukin 7

Interleukin-7 (IL-7) is a particularly important cytokine to the adaptive immune system. It supports the development of lymphocytes in the thymus, the organogenesis of the lymph nodes, and ensures the maintenance of activated T cells in the secondary lymphoid organs (119, 120).

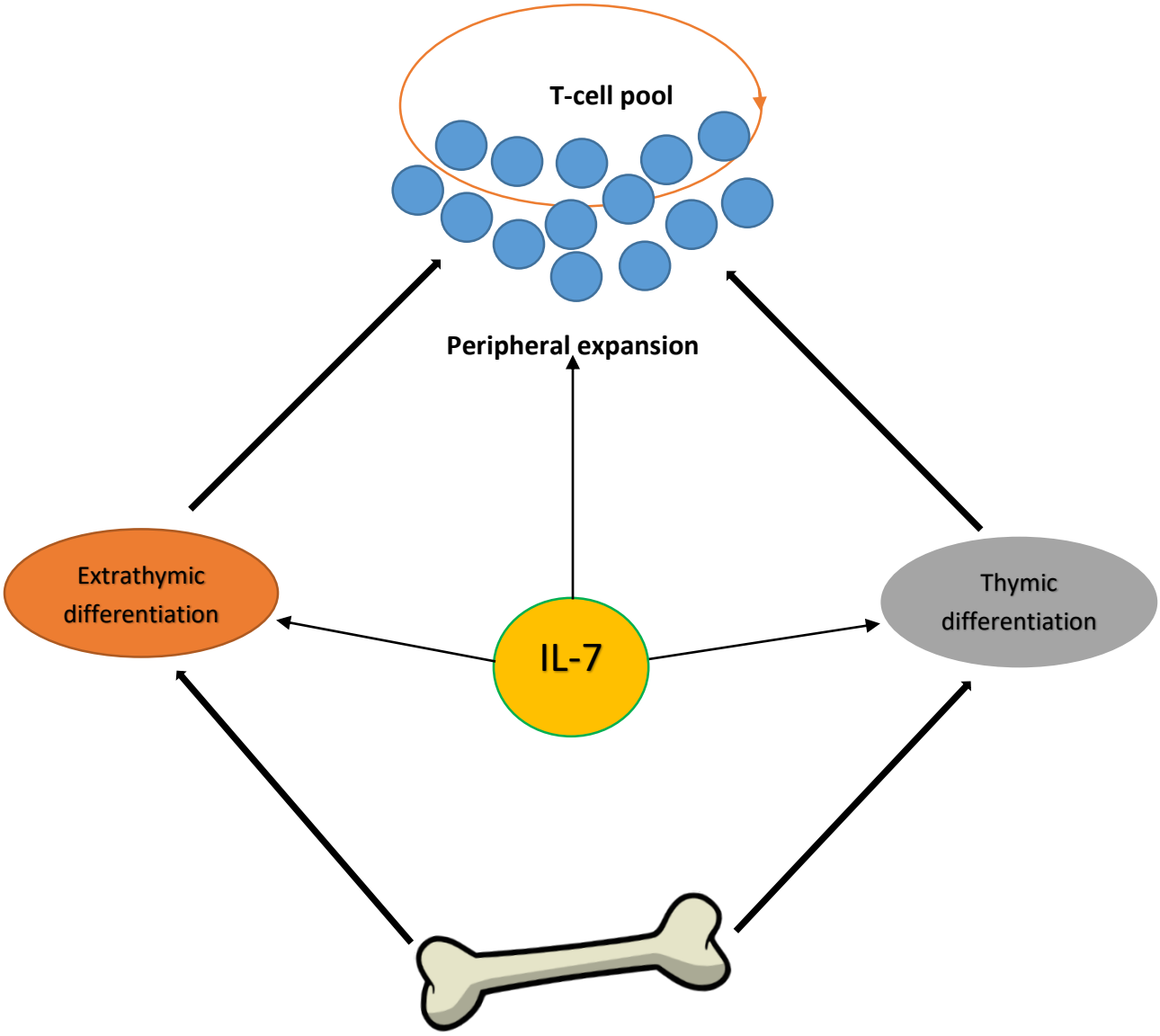

Figure 2. IL-7 has the main role in the regeneration of T cells in the Thymus as well as in the peripheral expansion of the Mature T cells.

IL-7 is produced by non-hematopoietic cells, such as fibroblastic cells of the bone marrow and lymphoid organs, epithelial cells in the thymus, prostate, and intestine (121, 122, 123), as well as from keratinocytes of the skin (124). IL-7 transcripts and proteins are also found in the liver tissue of adults, produced by lymphoid cells. The production of IL-7 by dendritic cells of the immune system has also been observed (125). On the other hand, evidence show that IL-7 can be produced by stroma of tumor cells (126).

The human IL-7 gene locus is 72 kb long, and it is located on chromosome 8q12-13. It encodes a protein of 177 amino acids with a molecular weight of 20kDa (127). Under physiological conditions in the human body, IL-7 is found in very limited amounts, while the stromal cells produce IL-7 in approximately constant amounts, unaffected by external stimuli (128). In diseases associated with lymphopenia, such as HIV infections, idiopathic $CD4^{+}$T lymphopenia, autoimmune diseases, an increase in circulating IL-7 levels has been observed (129). The immune system that prevents the development of cancer in the human body depends on the amount of T lymphocytes, especially CD8+, T cells. This amount of T lymphocytes in the human body is maintained in a dynamic equilibrium. Antigen-specific T effector cells, upon completion of their mission, die. The creation of new T cells fills their absence. IL-7 does this through thymopoiesis and proliferation of homeostasis. Most of the T lymphocytes are created in the thymus, and IL-7 is also needed for their development. The association between mutation in IL-7Rα and severe immunodeficiency syndrome combined with T lymphocyte deficiency has been established.

In the secondary lymphoid organs, the adaptive immune response against tumors occurs, especially in the drainage of the lymph nodes of the pathological sites. These represent the microenvironments suitable for the activation of specific antigen lymphocytes. Interactions between hematopoietic and stromal cells in secondary lymphoid organs are essential for the functioning of immune cells (130). Fibroblastic reticular cells in secondary lymphoid organs secrete the chemokine CCL19 and CCL21, which attract T cells and DCs, and at the same time facilitate their migration organizing a complex network of extracellular collagens (131, 132, 133).

An insufficient amount of IL-7 in secondary lymphoid organs may be the reason for the lower survival of activated T lymphocytes and at the same time weakening immunosuppression of cancer.

IL-7 acts through the IL-7 receptor (IL-7R) which consist of two chains: Alfa (α) and Gama (ϒ). The ϒ chain is found in all the types of hematopoietic cells, while α chain is mainly expressed in lymphocytes, enabling the development of T and B lymphocytes, respectively naive and memory T cells (134). IL-7 action is realized through these signaling pathways: Janus kinase/STAT (Jak-Stat) and phosphatidyl-inositol-3-kinase (PI3K-Akt) and the Ras/Raf signaling cascade (135).

Through these pathways they have impact in development, survival, proliferation, differentiation, and maturity of the immune cells such as: T-lymphocytes, B-lymphocytes as well as natural killer cells. IL-7, IL-7Rα and γc form a complex that play a significant role in the extracellular matrix, remodeling, development, and homeostasis of T and B cells. IL-7Rα also can reacts with thymic stromal lymphopoietin (TSLP) and its receptor (TSLPR), activating the TSLP pathway and resulting in proliferation of humans T and dentritic cells, and further B cell development in mice. Low-simulation of the IL-7 pathway indicated by mutations in the IL-7Rα ectodomain inhibits T and B cell development, and in this away have impact on severe combined immunodeficiency. On the other hand, the overstimulation of this pathway causes allergic rhinitis, autoimmunity, heart disease, and proliferation of cancers (136).

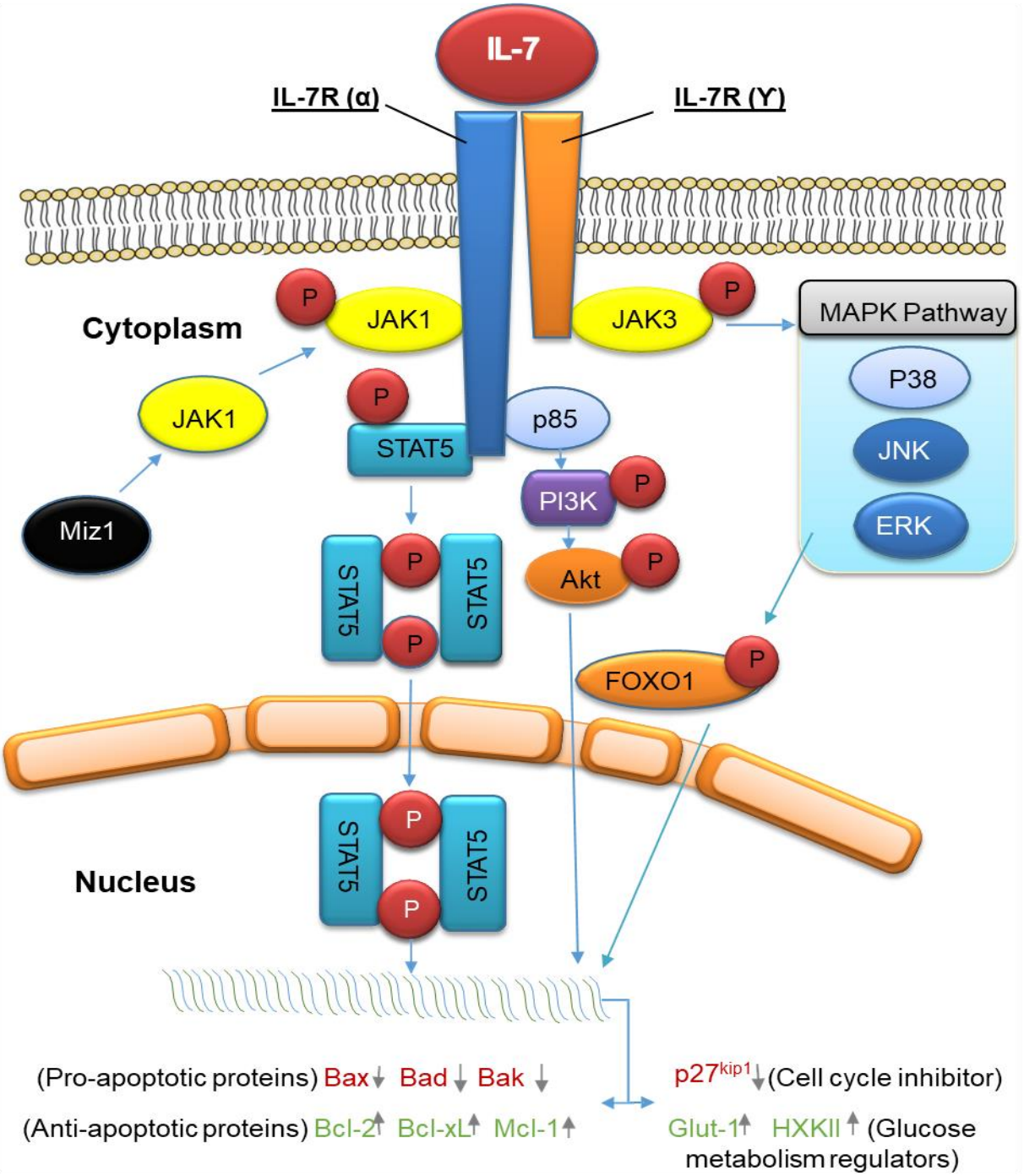

Figure 3. IL-7 receptor signaling pathway. IL-7 participates in the regulation of pro-apoptotic and anti-apoptotic genes. Increased concentration of IL-7, increases the expression of anti-apoptotic proteins (Bcl-xL, Bcl-2, Mcl-1) and decreases the expression of pro-apoptotic proteins (Box, Bad, Bax). Also, it is involved in glucose metabolism in the tumor cell. Thus IL-7 inhibits apoptosis and increases energy in the cell and consequently the growth and proliferation of tumor cells.

IL-7 as an immune regulatory protein, which is largely produced by stromal cells and by cells in inflammatory sites, except its role in the development of B and T cells, as well as in the homeostasis of T cells, it participates in the mediation of many physiological processes as well as in various diseases. There are data showing that overexpression of IL-7 has impact on development and progression of variety of tumors, furthermore IL-7 mRNA it was found in many types of tumors, such as renal, colorectal, and central nervous system (CNS) (137). IL-7 receptor (IL-7R) mRNA was also seen in variety of tumor cells including breast, colon, lung, renal and CNS (138). There is now known that IL-7 induces the proliferation of some variety of cancers, such as leukemia and lymphomas (137).

The increased of aggressiveness and metastasis in carcinomas including BC is associated with EMT as it permits cells to invade surrounding tissues and through the blood stream to enable the establishing metastasis. There is evidence showing that the phosphoinositide 3-kinaze/protein kinase B (PI3K/Akt) signaling pathway is implicated in EMT (139, 140). Now it is known that hyperactivity of Akt accompanied frequently in human cancers and is associated with metastasis process. In addition, activated Akt it was seen to have impact in reduction of cell-cell adhesion, increase cell motility, increasing invasiveness of human squamous cell carcinoma lines. In contrast, inactivated of Akt decrease the potentiality of BC metastasis (141).

In a previews study it was seen that IL-7δ5 play a role in morphological changes and invasion in BC cell lines. IL-7δ5 induce BC cell proliferation and cycle progression of the cells via activation of PI3K/Akt pathway, which is parallel with the up-regulation of cyclin D1 and down regulation of p27 ^{kip1}. Akt activated has been well known as an important regulator of cancer cell EMT and invasion. In the study done by Yang et al. (142), it was found that IL-7δ5 has inductive role in BC cell EMT and invasion through concomitant up-regulation on N-cadherin expression and down-regulation of E-cadherin expression. Thus, the results of the study show that IL-7δ5 induces BC cell lines invasion and metastasis by induction of cell EMT transition. The PI3K/Akt pathway has an important role in tumor genesis through activation of Akt which can regulate the cell

proliferation, apoptosis, angiogenesis, and metastasis through activating the downstream cell receptors of effectors (143).

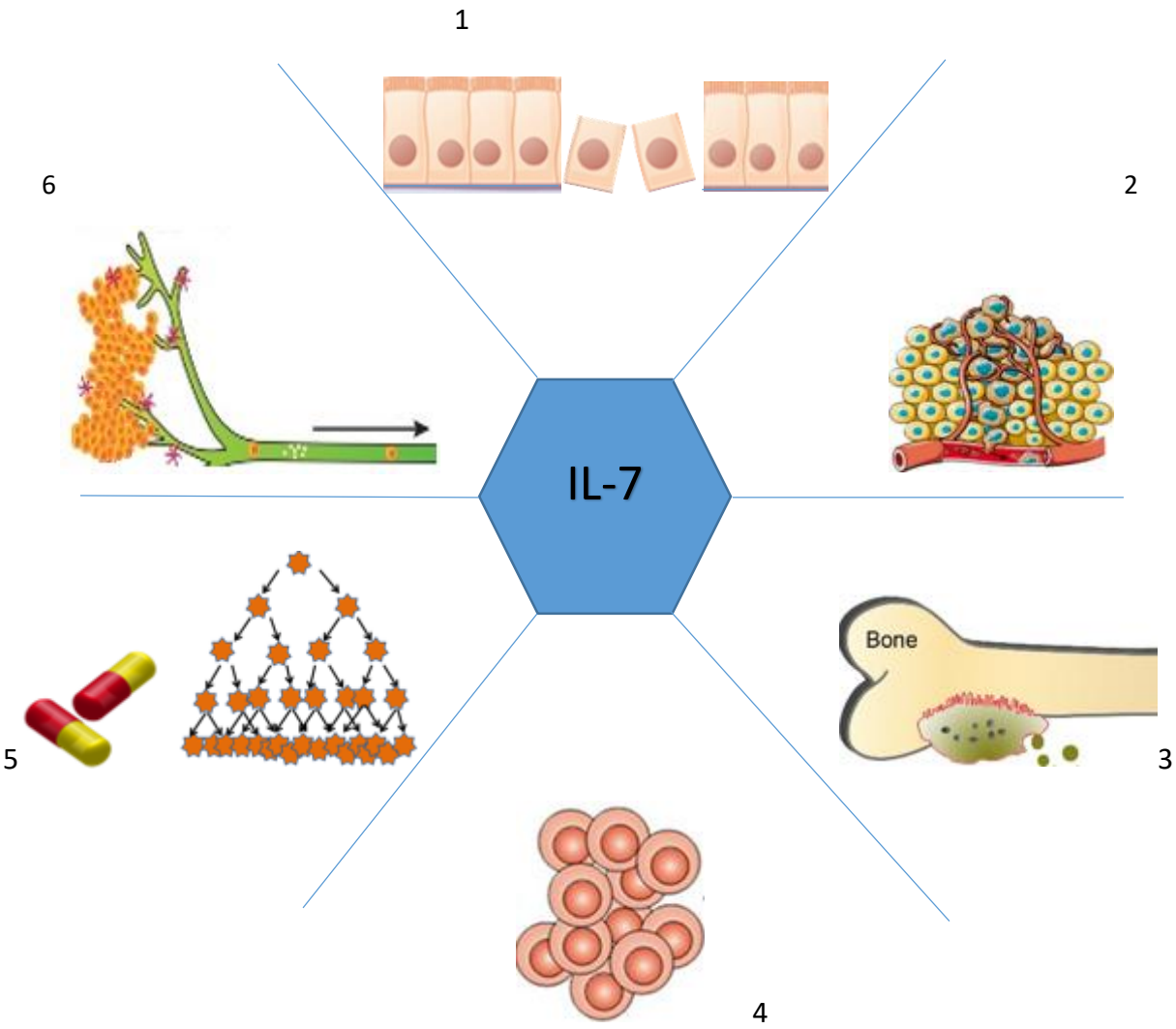

Figure 4. IL-7 has a key role in many processes of the tumor development: 1- epithelial mesenchymal transition (EMT), 2- migration, invasion, and metastasis, 3- osteoclastogenesis in bone metastasis, 4- proliferation, 5- resistance to chemotherapy, 6- lymphangiogenesis.

On the other hand, there are evidence showed that administration of IL-7/IL-7Rα-Fc inhibits tumor growth and prolong survival in lung cancer by induced afferent and efferent antitumor responses (144). Furthermore, under the IL-7 stimulating conditions the cytotoxic T lymphocytes reduce the pulmonary metastatic sarcoma in mice (145).

According to the best data we have, there is only a study done with Iranian patients, where is evaluated the serum IL-7 concentration in the BC patients and healthy control group, and there was not seen any significant differentiation between these two groups. Interestingly there was a higher level of the IL-7 concentration in the healthy control group in comparison with BC patients (107). In the study conducted by Linkov et al., the results showed that serum IL-7 concentration of the patients with squamous cell carcinoma of the head and neck was higher in comparison with the healthy control group (146). In another study done in patients with malignant ovarian tumors, the serum IL-7 concentration was significantly higher then in healthy control group as well as in patients with benign ovarian tumors (147). In the serum of the patients with prostate cancer as well as with colorectal cancer, it was found significantly higher concentration of the IL-7 in comparison with the healthy control group (148, 149).

These controversial data show that more research is needed to evaluate the IL-7 function, to analyze whether it functions as a promoter or inhibitor of tumor growth, but at the same time they highlight the association of IL-7 with tumor development. However, there is a lack of information regarding the correlation between IL-7 serum level and the histopathological characteristics of BC, or for the prognosis of the disease.

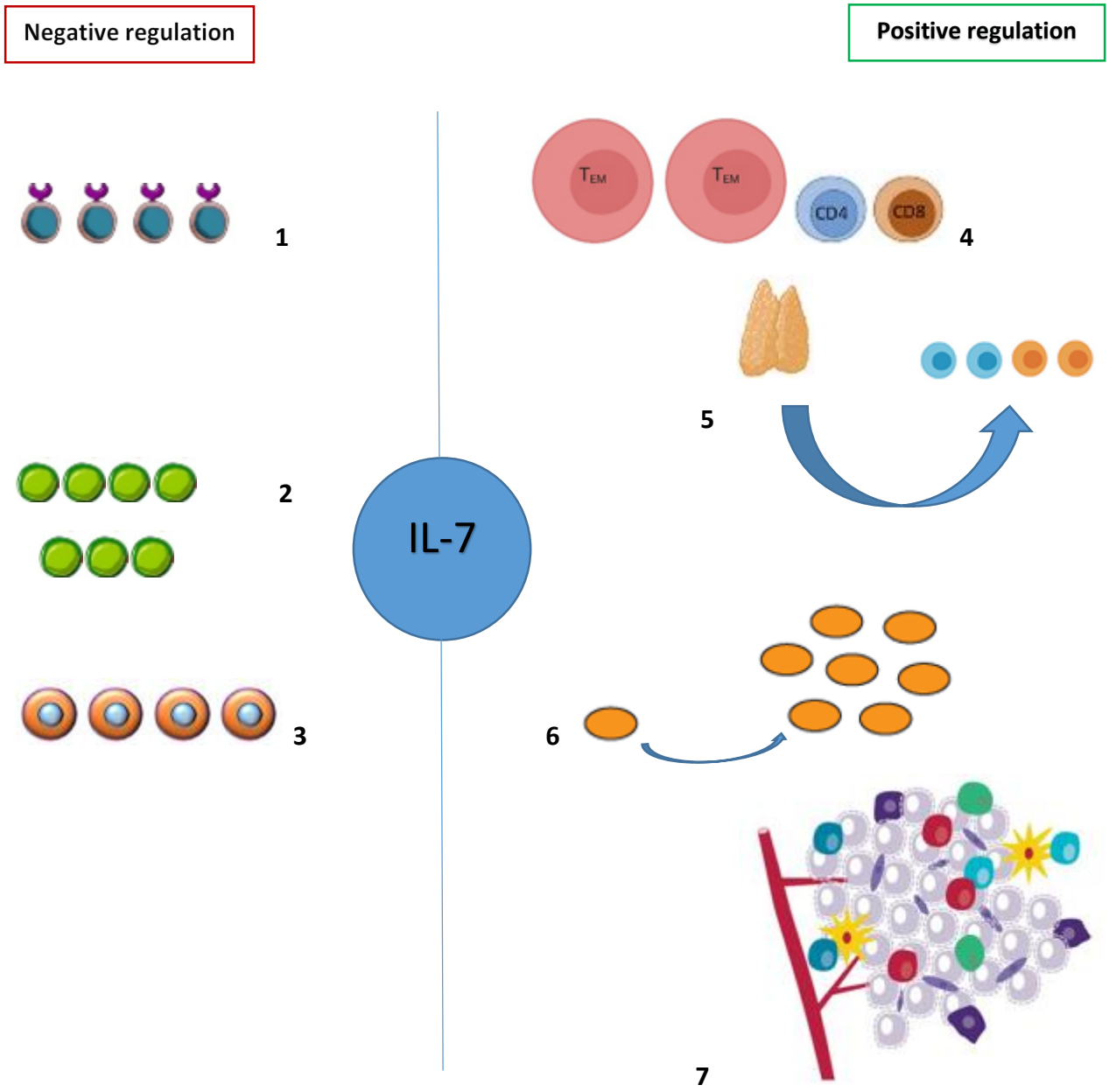

Figure 5. The effects of IL-7 in immunotherapy: IL-7 plays an important role in the survival and function of memory T cells and CTL. Supports immune system regeneration through thymopoiesis and homeostatic expansion of peripheral T cells. Affects tumor regression by increasing the number of effector cells such as: NK, CTL, Th17 and NK.

Negative regulation: 1- (PD-1), 2- (Treg), 3- (TGF-β).

Positive regulation: 4- (Survival of effector and memory T cells), 5- (Thymus), 6- (Homeostatic proliferation), 7- (Immune effector cells infiltration).

2. HYPOTHESIS

- IL-7 serum concentration is higher in the patients diagnosed with early invasive BC in comparison with healthy control group, and positively correlates with the tumor size, poor cell differentiation, lymphovascular and perineural invasion, negative hormone receptors' status, axillary lymph node metastasis, and the high Ki-67 proliferation index.
- In addition, there is no difference in the IL-7 serum concentration between the patients recruited in Croatia and Kosovo, respectively.

3. AIMS AND PURPOSE

3.1. AIMS OF THE RESEARCH

General aim:

- To assess the presence of elevation of the IL-7 serum concentration in the patients diagnosed with early invasive BC in comparison to the healthy control group.

Specific aims:

- To evaluate the correlation between the IL-7 serum concentration in patients with BC and the clinicopathological characteristics of the tumor.
- To evaluate possible differences in clinicopathological characteristics between Croatian and Kosovo subcohorts.
- To evaluate IL-7 serum concentration differences between Croatian and Kosovo subcohorts of the patients diagnosed with early invasive BC.
- To evaluate IL-7 serum concentration among different surrogate subtypes of BC.

3.2. PURPOSE OF THE RESEARCH

The purpose of the research is to confirm that the IL-7 concentration in the serum of BC patients is higher compared to the healthy control group. In the future the IL-7 could be used as a tumor biomarker to complete the BC diagnosis. As a proinflammatory cytokine could be tested as a poor prognostic factor and could help us to stratify risk of recurrence, as well as to be used in follow-up of the patients after the primary oncological treatment.

4. MATERIALS AND METHODOLOGY

4.1. Study population

In this dual center, cross-sectional and case-control study included consecutive female patients with BC which were referred for surgery at the University Hospital Center Zagreb, in Zagreb and University Hospital Center of Kosovo in Prishtina, from January 2018 until June 2019. Both of these university hospitals are located in capital cities and are the largest hospitals in both countries.

Patients have been selected as consecutive among women, aged between 20 -70.

Patients meeting the following criteria were included in this study:

1. Patients confirmed to BC by biopsy.
2. Operable and without distant metastases.
3. Overall good health condition
4. Willingnes to participate

Exclusion criteria were:

1. BC patients previously treated by surgery, chemotherapy, radiation therapy, endocrine therapy, target therapy or immunotherapy.
2. Patients with any other malignant disease at the same time.
3. Patients which were using any immunomodulatory therapy.
4. Patients with any acute or chronic inflammatory disease.
5. Patients with dementia or any other psychological disorders.
6. Patients in fasting state.

The criteria for withdrawal from the study:

1. Worsening of the patient's clinical status
2. Voluntar withdrawal of the patient.

The control group has been composed by women, aged between 20 - 70, without breast tumor, confirmed by ultrasound or mammography in the last three months or any other confirmed malignancies, without any acute or chronic inflammatory disease as well as no receiving immune modulatory therapy.

4.2. Blood samples

Blood samples (5 ml venous blood) have been taken from 100 EIBC patients from Kosovo University Hospital and 113 EIBC patients from Croatian University Hospital, prior to surgery and other oncological treatments, such as chemotherapy, radiation therapy, endocrine therapy or target therapy. On the other hand, the blood samples have been taken also from other 62 healthy participants, which compose the control group, from whom 32 from Kosovo and the other 30 from Croatia.

Blood samples categorized in tubes have been identifiable only by the numbers attributed to participants and have been stored frozen in temperatures of -20°C, in order to conduct the analysis later. There were used “serum tubes” in a volume of 6ml and without any additives. One test tube was used for each patient as well as for the cases of control group. Blood samples of patients from Kosovo, were stored under certain conditions until all were collected, including blood samples from the control group. Then they were transported by express mail (DHL) under specific conditions to the Unit for Pathophysiology and Experimental Oncology in Rebro, Zagreb, and together with samples collected from the patients in the Oncology Department in Rebro, were tested.

4.3. IL-7 determination

In this study, the IL-7 serum concentration has been measured, using "Sandwich" ELISA Immunoenzyme test, human IL-7 antibody, and Platinum ELISA using research tools from eBioscience Inc., located in San Diego, CA USA. The centrifuge force during sample processing was 3000 rpm/15 min. The analysis was done in Clinical Unit for Pathophysiology and Experimental Oncology in Department of Oncology, University Clinical Center Zagreb.

4.4. Histopathological data

Patients underwent partial mastectomy or modified radical mastectomy with axillary lymph node evaluation (axillary dissection or sentinel lymph node biopsy) as their primary treatment and were subsequently assigned to receive adjuvant chemotherapy, endocrine therapy and adjuvant radiotherapy as indicated by tumor biology and disease stage according to guidelines valid at the time (150). Subsequently, histopathological and immunohistochemistry data performed as routine in pathological laboratories, were collected. Tumor size, histological type, Nottingham histological grade, presence or absence of LVI, number of positive lymph nodes and resulting stage of disease were noted. Additionally, data on molecular markers, such as immunohistochemical expression of estrogen receptor (ER), progesterone receptor (PR), amplification of Her-2/neu receptor and proliferation index as measured by Ki-67, were obtained. Tumor staging was based on the 8th American Joint Committee on Cancer System (AJCC) (151). Histological subtyping was determined according to the World Health Organization Classification criteria (152). ER, PR, Her-2 status and Ki-67 proliferation index were interpreted according to the criteria of the American Society of Clinical Oncology (ASCO) and St. Gallen International Expert Consensus (150, 153). According to these recommendations, ER and PR are considered positive if ≥1% of tumor cell nuclei exhibit a positive reaction. According to the same criteria, Her-2/neu expression by immunohistochemistry may range from 0 to 3. If the result is 0 to 1, it is

considered negative. In contrast, if the result is 3, it is considered positive. Cases with a value of 2 are considered equivocal and are subject to fluorescent in situ hybridization (FISH) or chromogenic in situ hybridization (CISH) analysis. Based on the St. Gallen International Expert Consensus criteria, a Ki-67 value less than 20% is considered low, and Ki-67 values greater than or equal to 20% are considered to exhibit a high proliferation index. This value is used to discriminate between luminal A and luminal B surrogate subtypes (150). The St. Gallen Consensus recognizes five molecular surrogate subtypes of invasive BC according to the expression of formerly mentioned molecular markers, namely, Luminal A-like, Luminal B/Her-2 negative-like, Luminal B/Her-2 positive-like, Her-2 positive type and Triple negative (Table 1).

Table 1**.** Molecular surrogate subtypes of invasive breast cancer based on St. Gallen Consensus.

Subtype		ER and/or PgR	HER-2	Ki-67
Luminal A-like	(LumA)	Positive (≥20%)	Negative	<20%
Luminal B/HER2 negative-like	(LumB/HER2 neg.)	Positive (<20%)	Negative	≥20%
Luminal B/HER2 positive-like	(LumB/HER2 pos.)	Positive	Positive	Any
HER-2 positive	Her-2 overexpression	Both (-)	Positive	Any
Triple negative	(TN)	Both (-)	Negative	Any

The data on age and menopausal status have been also obtained from the patients.

IL-7 serum concentration has been initially determined in all the study participants, including patients and the control group. Then it has been evaluated if there has been any difference of IL-7 serum concentrations between EIBC patients and control group. After that, appropriate analyses have been done to see if there is any correlation between IL-7 serum concentration of the patients and histopathological characteristics of the tumor, as well as age and menopausal status. In addition, the potential difference of IL-7 serum concentrations between patients coming from Croatia and Kosovo has been evaluated.

4.5. Ethical consideration

The study has been carried out in compliance with the Code of Ethics of the Helsinki Declaration, as well as the ethical approval from both institutions: Ethics committee of the University Clinical Center of Kosovo (no. 913/2; 30 October 2017), and the University Clinical Center Zagreb (no. 02/21 AG; 02 November 2017). All the participants have been informed about the aim of this study along with safety and security considerations prior to signing the informed consents, as study prerequisites. Moreover, they have been informed about the right to withdraw and ask for their data to be destroyed.

4.5. Statistical analysis

After checking for normality of distribution by the Kolmogorov-Smirnov test, numerical variables have been presented as median and interquartile range. The differences of the distribution of the numerical variables have been analyzed with Mann-Whitney U test and Kruskal-Wallis ANOVA test. Associations between numerical variables have been analyzed as Spearman rank correlation coefficient. The diagnostic accuracy and the optimal cut point value for IL-7 level between two groups have been obtained based on the value of the area under the ROC curve. Categorical variables were compared using chi-square analysis and presented as

frequencies and percentages. The results were calculated as the mean and standard deviation or median and range. Analyses have been performed with statistical software IBM SPSS Statistics for Windows, Version 25.0. (IBM Corp. Armonk, NY). A P-value < 0.05 was considered statistically significant.

5. RESULTS

The IL-7 serum concentration in the EIBC patients has been significantly higher compared to the control cases (P<0.001) and is presented in Table 2 also in Figure 6.

Table 2. The distribution of the IL-7 serum concentration of the breast cancer patients and the control cases.

Variable	Breast cancer patients (N=213)	Control cases (N=62)	Mann-Whitney U test adjusted z=9.23; P<0.001
IL-7 serum concentrations Median (25% - 75%)	61.7pg/ml (24.3 – 152.6)pg/ml	4.6pg/ml (2.7 – 12)pg/ml	

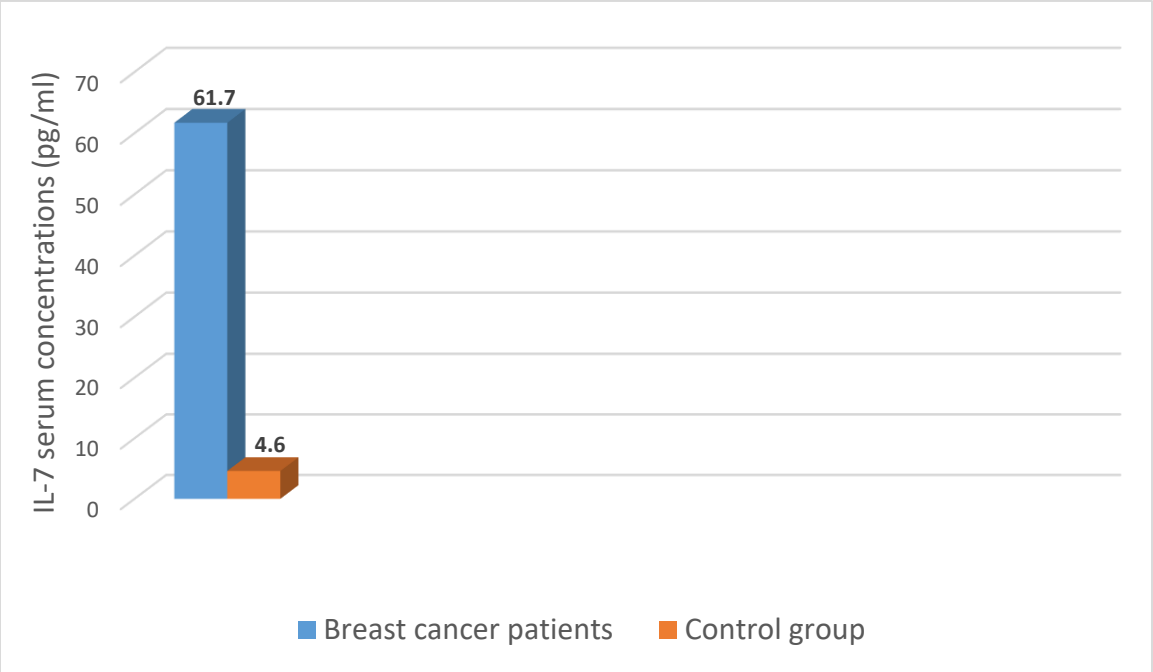

Figure 6. Median IL-7 serum concentrations/BC patients vs. control group.

ROC curve analysis has been applied to analyse diagnostic accuracy of measurements and optimal cut-point values for IL-7 concentration between verified EIBC and control group.

IL-7 concentration 14 shows 73.7% sensitivity and 83.3% specificity for carcinoma, AUC was 0.854 (CI 95% 0.803-0.896) at a cut-off value of 13.4 pg/ml. Figure 7.

IL-7 concentration 99 show 88.9% sensitivity and 90.9% specificity for carcinoma, AUC was 0.942 (CI 95% 0.819-0.991) at a cut-off value of 41.8 pg/ml. Figure 8.

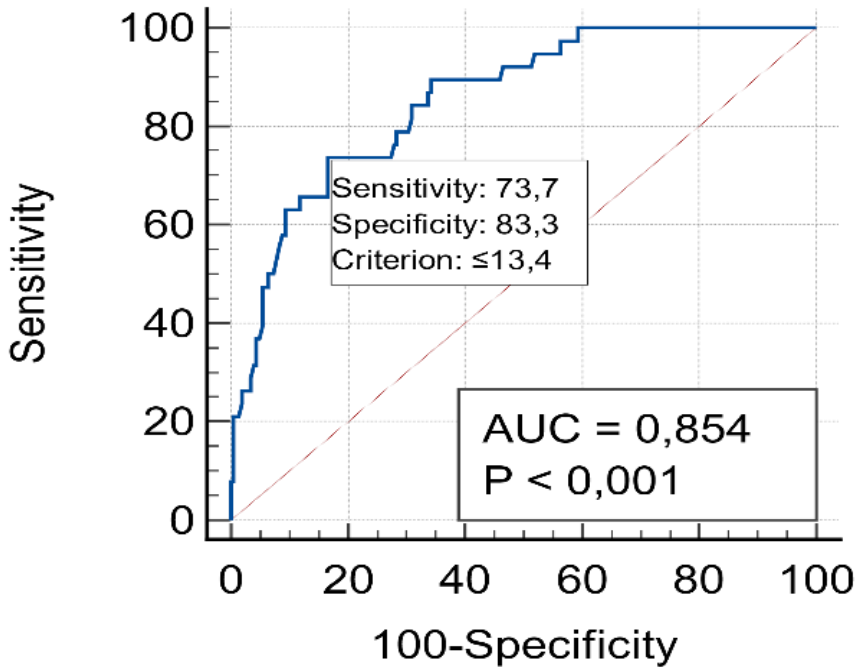

Figure 7. This figure represents the standard ROC curve analysis of all patients and the control group. IL-7 concentration 14 shows 73.7% sensitivity and 83.3% specificity for carcinoma, AUC was 0.854 (CI 95% 0.803-0.896) at a cut-off value of 13.4 pg/ml.

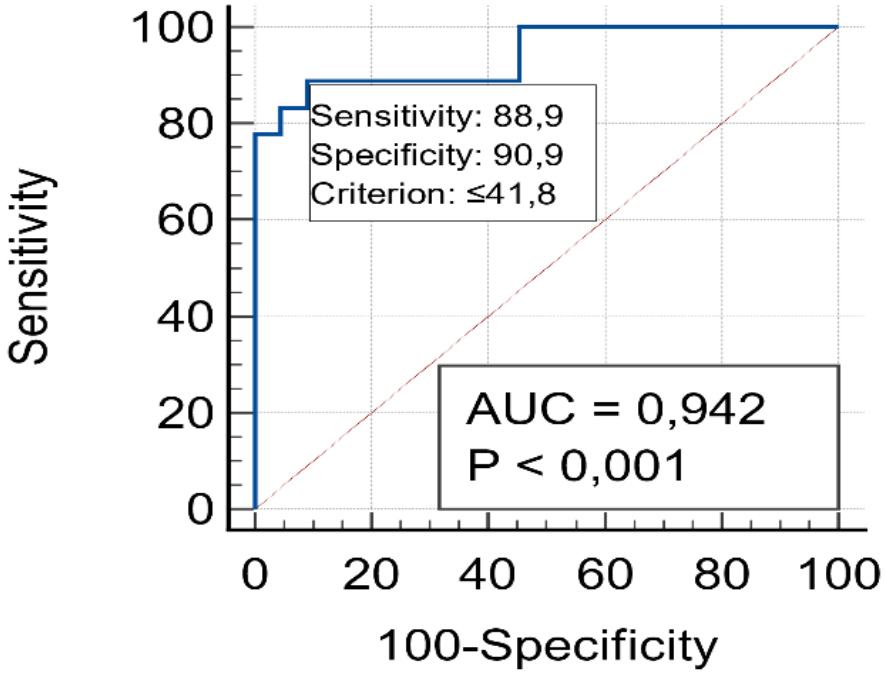

Figure 8. This figure represents the ROC curve analysis of patients with high IL-7 values (≥99 pg/ml). IL-7 concentration 99 show 88.9% sensitivity and 90.9% specificity for carcinoma, AUC was 0.942 (CI 95% 0.819-0.991) at a cut-off value of 41.8 pg/ml.

Regarding the EIBC patients, there have been no correlations between IL-7 serum concentration and tumor size, age, and Ki-67 (%) (*Spearman's ρ = 0.5, P =* 0.397*, Spearman's ρ = 0.5, P =* 0.611 *and Spearman's ρ = 0.3, P =* 0.698*, respectively*) (Table 3).

Table 3. Correlation of the tumor size, age and involved lymph nodes number, and IL-7 serum concentration.

	Spearman's (*ρ)*	P value
Tumor size (mm)/IL-7(pg/ml)	0.057	P=0.397
Age/IL-7(pg/ml)	0.024	P=0.611
Ki-67(%)/IL-7(pg/ml)	0.028	P=0.698

Patients with invasive lobular carcinoma (ILC) seems to have lower IL-7 serum concentration compared to other histological subtypes, and the difference is statistically significant (P=0.043) (Figure 9, Table 4). There have been no significant differences of IL-7 serum concentration among different stages of disease, molecular surrogate subtypes, and histological grade. Immunohistochemical expression or absence of molecular markers, such as PR, ER, amplification of Her-2/neu receptor and proliferation index as measured by Ki-67, as well as LVI and PNI, have been not correlated with IL-7 serum concentration (Table 4).

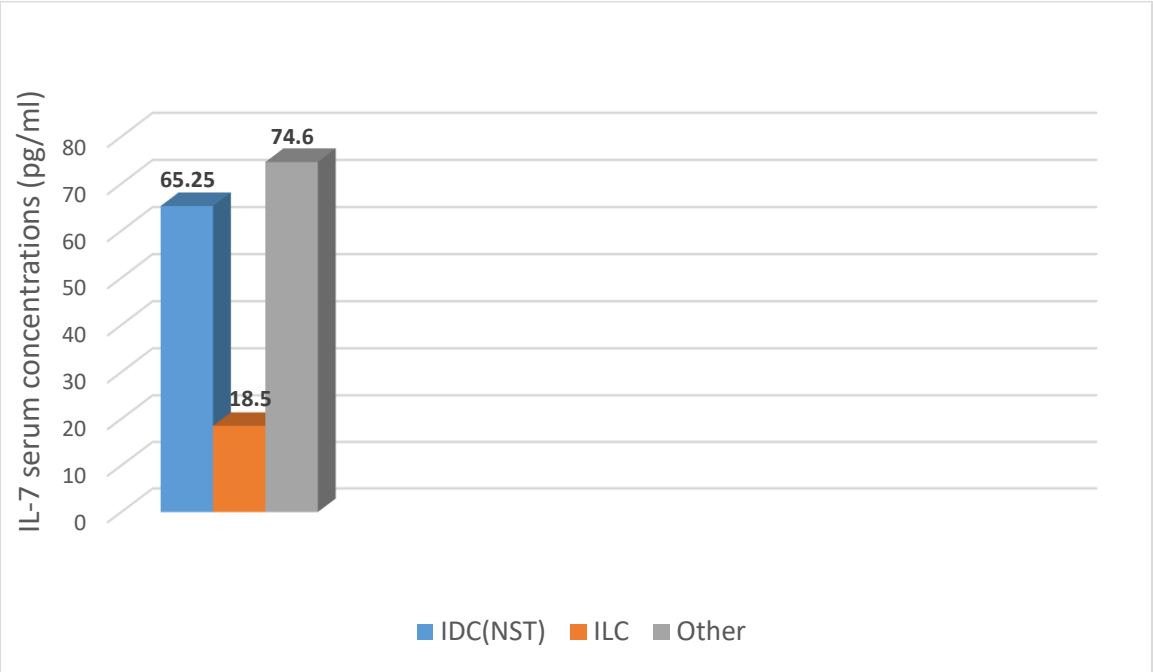

Figure 9. Median IL-7 serum concentrations/histological subtypes.

Table 4. The distribution of the IL-7 serum concentrations according to the tumor characteristics.

Grouping variables	Categories of grouping variables (n)	IL-7/ Median (25% - 75%)	P value
Histological subtype	IDC (NST) (164)	65.25 pg/ml (29.4- 166.0) pg/ml	Kruskal-Wallis ANOVA test value H (2.0) =6.30; P = 0.043
	ILC (20)	18.5 pg/ml (6.9- 150.3) pg/ml	
	Other (28)	74.6 pg/ml (23.4-148.0) pg/ml	

Axillar involvement	Yes (86)	70.9 pg/ml (30.8-166.5) pg/ml	Mann-Whitney adj z=0.94; P=0.348
	No (127)	58.8 pg/ml (22.9-147.0) pg/ml	
T-Stage	T1 (103)	59.0 pg/ml (22.8-144.1) pg/ml	Kruskal-Wallis ANOVA test value H=0.3; P=0.856
	T2 (101)	64.0 pg/ml (25.1-166.7) pg/ml	
	T3 (9)	57.9 pg/ml (26.9-182.0) pg/ml	
Stage	I (77)	52.1 pg/ml (19.2-134.3) pg/ml	Kruskal-Wallis ANOVA test value H=1.72; P=0.419
	II (97)	63.6 pg/ml (26-166.4) pg/ml	
	III (39)	79.9 pg/ml (23.9-169.3) pg/ml	
Grade	I (13)	42.4 pg/ml (11.6-174.1) pg/ml	Kruskal-Wallis ANOVA test value H (2.213)= 1.14; P=0.572
	II (138)	62.4 pg/ml (29.1-166.1) pg/ml	
	III (62)	62.4 pg/ml (16.8-129.2) pg/ml	
ER	Positive (169)	59.0 pg/ml (23.9-159.3) pg/ml	Mann-Whitney adj z=0.53; P=0.593
	Negative (44)	66.1 pg/ml (20.0-168.2) pg/ml	
PR	Positive (65)	40.5 pg/ml (11.7-110.9) pg/ml	Mann-Whitney adj z=0.96; P=0.334
	Negative (148)	66.7 pg/ml (29.9-168.1) pg/ml	
Her-2	Positive (40)	66.8 pg/ml (26.6-140.0) pg/ml	Mann-Whitney adj z=1.42; P=0.672
	Negative (172)	61.1 pg/ml (23.4-166.0) pg/ml	
Molecular surrogate subtypes	LumA (77)	71.1 pg/ml (34.1-183.9) pg/ml	Kruskal-Wallis ANOVA test value H (5.47) 4.0; P=0.247
	LumB/HER2 neg (75)	51.1 pg/ml (18.9-117.3) pg/ml	
	LumB/HER2 pos (20)	52.6 pg/ml (23.9-90.0) pg/ml	
	Her-2 overexpression (19)	96.6 pg/ml (43.9-225.3) pg/ml	
	TN (22)	57.2 pg/ml (17.9-151.0) pg/ml	

LVI	Positive (70)	62.6 pg/ml (34.7-166.3) pg/ml	Mann-Whitney adj z=0.7; P=0.484
	Negative (138)	62.4 pg/ml (23.1-156.0) pg/ml	
PNI	Positive (24)	69.0 pg/ml (37.9-166.8) pg/ml	Mann-Whitney adj z=0.87; P=0.385
	Negative (80)	54.5 pg/ml (22.9-158.1) pg/ml	

There is no difference in the IL-7 serum concentrations between premenopausal and postmenopausal patients, neither between patients coming from Croatia and Kosovo (Table 5).

Table 5. The distribution of the IL-7 serum concentrations in the patients coming from Kosovo and Croatia and according to the menopausal status.

	N	IL-7/ Median (25% - 75%)	P value
Country	Kosovo (100)	66.1 pg/ml (31.5-183.3) pg/ml	Mann-Whitney adj z=1.42; P=0.156
	Croatia (113)	54.7 pg/ml (18.0-134.3) pg/ml	
Postmenopausal	Yes (144)	67.0 pg/ml (25.3-166.4) pg/ml	Mann-Whitney adj z=0.56; P=0.575
	No (69)	52.1 pg/ml (20.3-137.3) pg/ml	

The median age at diagnosis for patients from Kosovo and Croatia was 54 and 57, respectively (P=0.173). The percentage of premenopausal women included in the study was 33% and 37% in Kosovo and Croatia, respectively (P=0.972) (Table 6).

Table 6. Demographics of Kosovo and Croatian cohorts

	Kosovo (n=100)	Croatia (n=113)	P value
Median age (25%-75%)	54 (46.25-62.00)	57 (46.50-57.00)	P=0.173
<40 years	12 (12%)	11 (9.7 %)	P=0.586
≥40 years	88 (88%)	102 (90.3%)	
Menopausal status			P=0.972
Premenopausal	33 (33%)	37 (32.7%)	
Postmenopausal	67 (67%)	76 (67.3%)	

The median tumor size at diagnosis was significantly larger in subjects from Kosovo than in those from Croatia (P=0.013) (Figure 10, Table 7). Additionally, axillary lymph node involvement was significantly increased in Kosovo compared to Croatian patients (47% vs 38%, P=0.047) (Table 7). The number of total and metastatic lymph nodes was also significantly increased in Kosovo patients (P=0.014, respectively P=0.011) (Table 7). The patients were staged using the TNM staging system. Given that some of the substage categories were not represented or had a small number of subjects, groups were merged as stages I, II, and III. Among Croatian patients, 51.3% were stage I. In contrast, only 18% of Kosovo patients were in the same stage. There were 8.8% and 28% stage III patients from the Croatian and Kosovo cohorts, respectively (P=0.013) (Figure 11, Table 7).

The rate of LVI was significantly increased (P=0.012) as well as the expression of PR (P=0.014) in Kosovo patients. Differences were noticed between the two cohorts regarding Her-2/neu amplification status, with higher rate in Kosovo cohort, but this tendency did not reach statistical significance (P=0.073) (Table 7).

The histological types did not significantly differ between the two groups. Invasive breast carcinoma (NST) was the most common type with a frequency of approximately 75% in both countries. Histological grade, Ki-67 proliferation index, ER status and distribution of molecular subtypes were not significantly different when compared in subjects living in Kosovo or Croatia.

Table 7. Comparison of tumor characteristics of the Kosovo and Croatian cohorts

	Kosovo	Croatia	P value
Histological subtype			P=0.078
IBC, NST	76 (76%)	88 (78.6%)	
ILC	6 (6%)	14 (12.5%)	
Mixed type	7 (7%)	6 (5.4%)	
Other	11 (11%)	4 (3.6%)	
Median tumor size (cm)			P=0.013
(25%-75%)	2.8cm (2.2- 3.5)cm	1.6cm (1.1- 2.2)cm	
Axillary involvement			
Yes	47 (47%)	38 (33.6%)	P=0.047
No	53 (53%)	75 (66.4%)	
pN(+) status			
N1	20 (42.1%)	27 (71.1%)	P=0.023
N2	17 (36.2%)	9 (23.7%)	
N3	10 (21.3%)	2 (5.3%)	

Stage I II III	 18 (18%) 54 (54%) 28 (28%)	 58 (51.3%) 45 (39.8%) 10 (8.8%)	P=0.012
Histological grade G1 G2 G3	 3 (3%) 69 (69%) 28 (28%)	 10 (8.8%) 69 (61.1%) 34 (30.1%)	P=0.168
Ki-67 (%) (Median, 25%-75%)	 20 (10-30)	 22 (12-35)	P=0.134
Estrogen Receptor status ER(+) ER(-)	 77 (77%) 23 (23%)	 92 (81.4%) 21 (18.6%)	P=0.418
Progesteron Receptor status PR(+) PR(-)	 16 (16%) 84 (84%)	 45 (39.8%) 68 (60.2%)	P=0.014
Her-2/neu amplification status Her-2 (+) Her-2 (-)	 24 (24%) 76 (76%)	 16 (14.2%) 97 (85.8%)	P=0.073
LVI status Positive Negative	 52 (52.5%) 47 (47.5%)	 18 (25.7%) 91 (65.9%)	P=0.012
Total Lymph nodes obtained (Median, 25%-75%)	 13 (9-18)	 8.5 (4.75-13.25)	P=0.014
Metastatic lymph nodes (Median, 25%-75%)	4 (2-8)	2 (1-4.25)	P=0.011
Molecular subtypes			P=0.403

Lum A	34 (34%)	43 (38.1%)	
Lum B/HER2 neg	31 (31%)	44 (38.9%)	
Lum B/HER2 pos	12 (12%)	8 (7.1%)	
HER2 positive	12 (12%)	8 (7.1%)	
Triple negative	12 (12%)	18 (8.8%)	

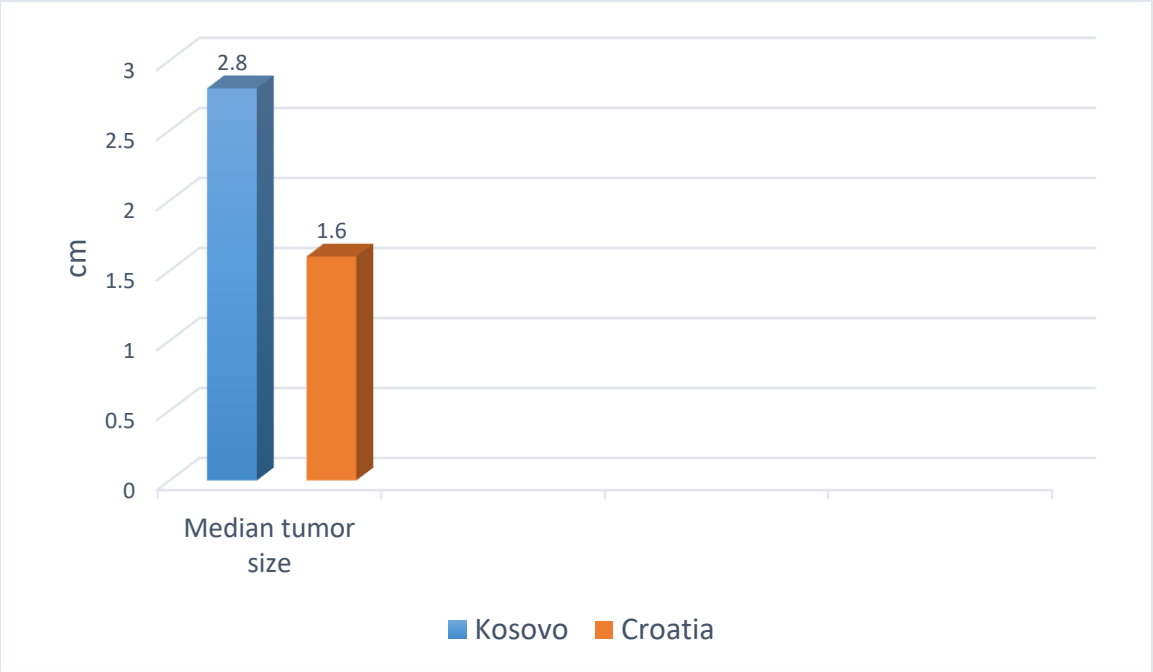

Figure 10. Median tumor size (cm) at the time of diagnosis: Kosovo/Croatia.

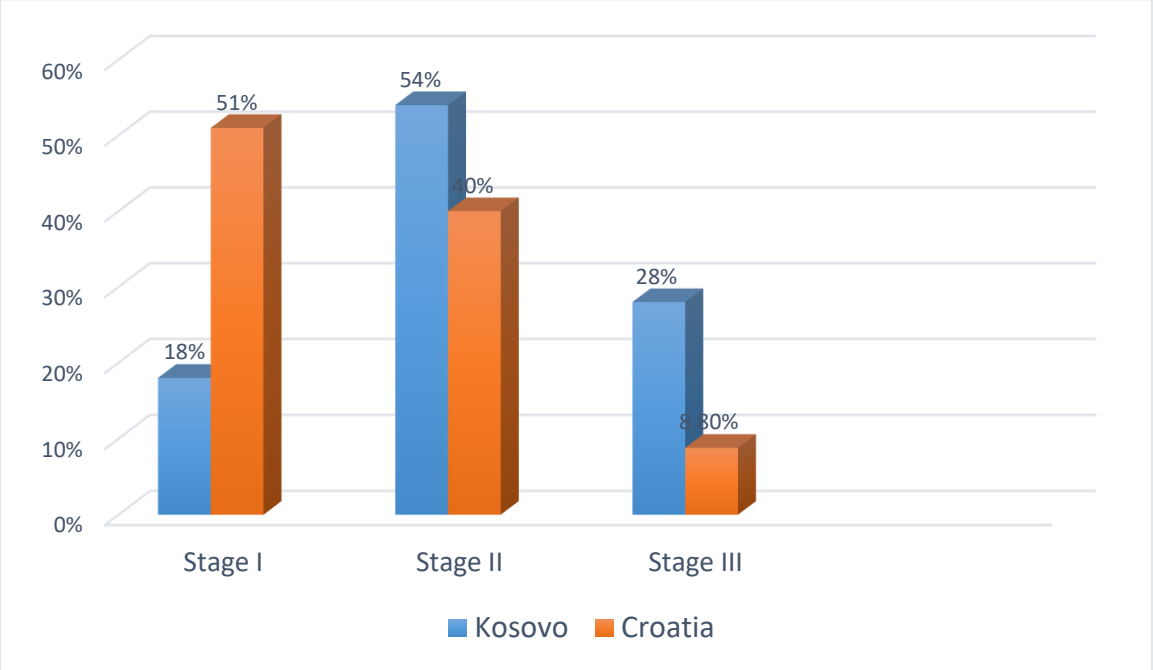

Figure 11. Breast cancer stage distribution of Kosovo and Croatian cohorts

The distribution of the patients in each substage is presented in Table 8.

Table 8. Breast cancer substage distribution of Kosovo and Croatian cohorts

	I	IIA	IIB	IIIA	IIIC	
Kosovo	18 (18%)	37 (37%)	17 (17%)	18 (18%)	10 (10%)	P=0.013
Croatia	58 (51.3%)	34 (30.1%)	11 (9.7%)	8 (7.1%)	2 (1.8%)	

There were no patients in substage IIIB.

6. DISCUSSION

In this study, a significantly higher IL-7 serum concentration in EIBC patients compared to healthy control cases, ($P<0.001$), has been noticed. On the other hand, no correlation has been noticed between IL-7 serum concentrations and tumor size, metastasis in lymph nodes or tumor passes from well to poorly differentiation. The higher IL-7 serum concentration in BC patients can be explained by the production of different cytokines including IL-7 from cancer cells. In a study done by Cheavy et al. -2007, it was established that several cytokines were produced from breast cancer tissue (154). Therefore, these cytokines have been overexpressed when compared to normal breast tissue (155). However, the discontinuing increase of IL-7 serum concentration during the tumor growth and disease progression is controversial. It was also shown in another study that most of cytokines that were overexpressed in the BC tissue have not shown correlation with patient age, tumor size, histological type, nor lymph node status (155).

Another reason for the increase of IL-7 serum concentration in the start of tumor growth can be the counteraction of immune system to the tumor, producing various cytokines, among them IL-7. Interleukins are cytokines or immunomodulatory proteins that are activated in response to changes that occur in organs and tissues (156). Most of them modulate growth and differentiation of different immune cells and activation of several mechanisms during immune response (157). Interleukins are closely involved in cellular defence against various pathological agents as well as the physiological response to infection. They have also been shown to participate in many processes of tumor growth and development (158). It is already known that the immune system plays a key role in the mechanism of cancer development through various processes involved under the concept of immunosurveillance and immunoediting (159, 160, 161). Most of the interleukins are secreted by the cells of the immune system, such as $CD4^+$ T helper lymphocytes, monocytes, macrophages, and endothelial cells. $CD4^+$ T helper lymphocytes have as their main function the regulation of the immune response and the modulation of the function of $CD8^+$ cytotoxic T lymphocytes, B cells, natural killer cells, macrophages and dentritic cells (162, 163). New findings in tumor immunogenetics have shown the role of the tumor microenvironment in tumor progression and its close association with the interleukin-related

immune response and immune regulation (164, 165, 166). Many families of interleukins have impact in cells of the immune system, which participate in tumor progression, co-constructing the immunogenic phenotype of the tumor during its development. The interaction of tumor cells and immune cells in the tumor microenvironment can be divided into three phases of immunoassay: elimination, equilibrium, and escape (167). In the first stage or “elimination” the cells of the immune system attack and destroy the tumor cells. Tumor cells that survive this attack move on the second stage or “equilibrium”, which is characterized by molecular editing (mutations, genetic rearrangement). During this stage, tumor cells and immune cells “coexist” in the tumor microenvironment. At this stage, although the immune system is not able to eliminate all the cancer cells, it does not allow them to multiply and metastasize. In the third stage of this process, the tumor already build from immunological aspect begins to grow, creating an immunosuppressive tumor microenvironment, suitable for the further development of carcinogenesis. Various cells of the immune system participate in these processes, including the ones of innate and adaptive immune system, as well as different interleukins at the different stages. For example, IL-2, IL-10, IL-21, IL-27 participate in the process of immunosurveilance; IL-10, IL-19, IL-23, IL-27 participate in the process of immunosuppression; IL-10, IL-18, IL-22, IL33 participate in the process of immunotolerance (168, 169, 170).

Based on these data, it can be said that the immune system counteracts the growth and the development of the tumor at its beginning through various substances, cells, mechanisms, and different ways.

However, as the tumor progresses in growth, metastasis in lymph nodes or passes from well to poorly differentiation, the immune parameters fail and are replaced by other inflammatory cytokines, which support the tumor growth and progression (171). It is known that the immune system has a key role in a mechanism of preventing the occurrence of cancer, but the immunity fails to control the tumor growth and progression, due to a strong defense mechanisms developed by the tumor (172, 173, 174, 175). In the tumor microenvironment, the immune cells that are tolerant toward tumors such as exhausted1 cytotoxic T lymphocytes, macrophages, and T helper cell type 2, can be found, as well as more MDCS and T regulatory cells

(Treg), responsible for inhibition of effector immune responses (176, 177). Cancer patients commonly have lower T cell counts and consequently immunosuppressive status (178). The low level of the T lymphocytes in the patients with malignant diseases may be one reason for the increase in circulating IL-7 concentrations, based on the fact that IL-7 is responsible for the development, growth, and maturation of T lymphocytes (179). Administration of IL-7 has been shown to be effective in improving and rebuilding the immune system, mainly through homeostatic expansion of peripheral T cells. The application of IL-7 in addition to increasing the number of T cells, also increases the diversity of T cells to recognize different antigens (180). IL-7 has also been shown to increase thymopoiesis to increase the number of naive T cells.

The increase of the IL-7 serum concentrations in the BC patients in our study does not correspond with the Iranian study, in which no elevation of IL-7 serum concentration in the BC patients has been noticed. Moreover, their results show the higher concentration of IL-7 in the control group (107). Furthermore, in the Iranian study, the higher concentration of IL-7 has been seen in the serum of well-differentiated BC patients compared to patients with poor differentiation, while in our study there has been no difference in the IL-7 serum concentration between patients with poor and well-differentiated tumors. The previous study has shown that the impact of IL-7δ5 variant through the activation of Akt has a critical role in cell proliferation, apoptosis, angiogenesis and metastasis (181), but in our study there no correlation of IL-7 serum concentration and lymph node metastasis, LVI and PNI, and neither with tumor size has been noticed. Interestingly, a lower value of IL-7 has been observed in the serum of the patients with ILC compared to patients with invasive ductal carcinoma (IDC) and other histological subtypes, and the difference has been significant (P=0.043).

ILC is the second most common histological subtype in terms of frequency of presentation, immediately after IDC. There are some differences between ILC and IDC both in histological terms and in clinical findings, such as thin line growth, difficulty in detecting the disease, high degree of recurrence; ILC usually presents in older ages, and it is detected in advanced stages (182). Another difference between these two histological subtypes is that ILC in about 90% of cases lacks E-catherine protein overexpression (CDH1). E-catherine is a calcium-

dependent transmembrane protein that enables adhesion between cells, and in its absence, there is a characteristic non-cohesive increase (183). On the other hand, the estrogen receptor is expressed in about 90% of cases in ILC, so many times more pronounced than in IDC, while Her2/neu overexpression is in only about 5% of cases, much lower than in IDC. According to the study done by Davoli et al. - 2017, an overexpression of CD274 (PD-L1), PDCD1 (PD-1) and CTLA4 has been observed in ILC (184). In the study done by Plitzko B et al. - 2018, it has been proven that ILC is characterized by a lower rate of cellular metabolism compared to IDC, confirming it by basal oxygen consumption rate and basal extracellular acidification rate as indicators of oxidative phosphorylation (185). Many genetic profile studies have been conducted to see the difference between ILC and IDC. Zhao H et al. - 2004 and Buerger H et al. - 2000, have found 11 genes that can make the difference between these two subtypes, but in the meantime, many other studies, which have detected functional sets of genes, responsible for the etiological differences between ILC and IDC, have been performed (186, 187). Neoadjuvant chemotherapy treatment is now standard for locally advanced breast cancer, although there has been no increase in disease-free survival and overall survival. However, this method affects down staging, and increases the chance of breast-conserving surgery, while also enabling chemosensitivity to the tumor. In previous studies, it has been observed that patients with IDC have benefited greatly from neoadjuvant chemotherapy, while the effect in patients with ILC was very small (188, 189, 190).

Given the already established differences in terms of clinicopathology, genomic profile, as well as different responses to therapy, it can be assumed that also the immune response to these two histological subtypes may be different, specifically the production of different cytokines, in this case IL-7.

After reviewing the data by the Expert panel at the 13 St Gallen International Breast Cancer Conference (2013), some data regarding the most specific treatment at the local and regional level in BC were approved. They recognize five molecular surrogate subtypes of invasive BC according to the expression of formerly mentioned molecular markers, namely Luminal A-like, Luminal B/Her-2 negative-like, Luminal B/Her-2 positive-like, Her-2 positive type and Triple negative. In this study, patients have been divided into these five groups, respectively into five

surrogate subtypes according to this classification, and no difference of IL-7 serum concentrations between them is shown.

No differentiation is seen in the IL-7 serum concentration in the patients based on the overexpression or not of molecular markers such as ER, PR, Her-2/neu. No differentiation has been found in IL-7 serum concentrations between patients regarding the age.

The menopause is not a factor that causes BC, but there are several risk factors associated with it. The longer exposure to estrogen increases the chance of developing BC. In previous study there were seen several biological and clinical-pathological differences between premenopausal and postmenopausal BC patients. In premenopausal one, it was more pronounced heterogeneity and aggressiveness of the disease, the higher rate of the involvement of the lymph nodes and consequently higher stage of the disease (64). Molecular markers such as: ER, PR, Her2/neu, which also serve as prognostic factors, are distributed differently in premenopausal and postmenopausal BC patients with tendency of overexpression of the triple negative cases in premenopausal one (65). Also, the rate of the cell's poor differentiation is higher in premenopausal patients. Although, based on these data BC tends to be more aggressive and with worse prognosis in premenopausal women, however no difference has been observed in the IL-7 serum concentrations between premenopausal and postmenopausal patients.

In addition, there has been no significant difference of IL-7 serum concentrations between patients coming from Croatia and Kosovo. Given the fact that in this study a large difference in the stages of the disease at the time of diagnosis between patients coming from Croatia and Kosovo has been found, i.e., patients from Kosovo were in more advanced stages, respectively the tumor size was larger and the axillary lymph involvement was more pronounced, there was no difference in IL-7 concentrations between these two cohorts. This confirms once again that the concentration of IL-7 in the serum of BC patients has no correlation with tumor size and its progression.

Tumor markers have an important and ever-increasing role in all the aspects of malignant disease care. Their effective application in clinical practice requires basic knowledge of pathophysiology as well as identification and testing techniques. The detection of a tumor marker

can be done in tissue, or in various body fluids, including serum, pleural fluid, or ascites. Clinical use may be done for screening or early detection, completion of diagnosis, determination of prognosis, monitoring of the effectiveness of therapy, follow-up of disease and recurrence. As tumor markers, there can be different substances: surface antigens, different cytoplasmic proteins, hormones, enzymes, different oncogenes, and their products. The definition of tumor marker may be as follows: the presence of a particular substance produced by the tumor itself or by the body in response to the tumor, which can be used to distinguish between the tumor and the normal tissue, or to determine the presence of the tumor based on blood measurements or other body fluids. There has been constant research on tumor markers. Although many have been detected, only a few of them have managed to get used. An ideal tumor marker should have high specificity for a given tumor, with high sensitivity to exclude false positive results, technically to be easily accessible as well as to be cheap to realize. Such a tumor marker so far does not exist.

In this study, ROC curve analysis has been applied to analyse the diagnostic accuracy of measurements and optimal cut-point values for IL-7 concentration between verified EIBC and control group. IL-7 concentration 99 shows 88.9% sensitivity and 90.9% specificity for carcinoma, AUC was 0.942 (CI 95% 0.819-0.991) at a cut-off value of 41.8pg/ml, and IL-7 concentration 14 shows 73.7% sensitivity and 83.3% specificity for carcinoma, AUC was 0.854 (CI 95% 0.803-0.896) at a cut-off value of 13.4pg/ml, which indicates that it has very high specificity and sensitivity. IL-7 can be measured in patient's serum, and the technical sampling procedure is not different from other biochemical analyses, while now it cannot be said that it is a low-cost analysis.

Although nowadays the concept of screening is very attractive, for the purpose of early detection of malignant diseases and their effective treatment, the use of tumor markers for this purpose as routine is still impossible, because there is still no test 100% specific, which would distinguish between malignant tumor and benign changes. According to current data, tumor markers cannot be considered yet as a diagnostic tool due to insufficient specificity and sensitivity. They are now used in medical practice as laboratory tests to support the diagnosis as well as in monitoring patients during treatment with therapy and follow-up after the end of

therapy. However, when combined with other imaging methods, they help to distinguish between benign and malignant changes.

IL-7 has been seen also as a target for the treatment of BC as an immunotherapy. The purpose of using IL-7 as an immunotherapy has been to increase the efficiency of tumor regression. Some preclinical evidence have shown that the application of IL-7 to tumor-bearing hosts has increased survival in some tumor types. In clinical trials conducted in 2006, 2008 and 2010, in some patients with different types of cancer such as sarcomas or malignant melanoma IL-7 was prescribed in different doses subcutaneously, and in these patients, an increase in levels of CD4+ and CD8+ T cells was observed. However, no satisfactory antitumor activity was observed (191, 192, 193). The most common side effects that have been reported after rhIL-7 injection were decrease in temperature, temporary increase in hepatic enzymes, while at the site of application, erythema and hardening of the skin (194). IL-7 in the human body has had a short half-life of only 6-10 hours, but its effect has continued even after its values dropped to normal concentrations. According to the study of Lynch DH et al. - 1991, in gliomas of experimental mice after administration of dual immunotherapy with interferon- γ and IL-7, an increase in survival was confirmed through the growth and proliferation of T cells (195). In another study, it was observed that the co-administration of IL-7 and IL-2 was more effective than their separate application (196). In the study of Touwn et al, recombinant IL-7 was applied to patients with ALL, respectively in ALL cell lines and it was observed that IL-7 has a key role in the regulation and proliferation of ALL cells (197). Administration of IL-7 has also been shown to be effective in renal cell carcinoma (197). The application of IL-7 can enhance the function of immune cells in vivo, increasing the long-term response of tumor antigen-specific CTLs in quantity and quality (198).

High-income countries are characterized by a decrease in BC mortality rates, whereas BC incidence and mortality rates are increasing in developing countries (199). BC is most frequently diagnosed among women aged 55-64 years in developed countries; the median age at diagnosis is 62 in the USA (200). In this study, median age of the patients is 56 years. This finding can be explained by the young age of the population. The median age of the Kosovo population was 30.2 years in 2020, and that of the Croatian population was 44.3 in 2020 (201, 202).

As one of the main points of cancer control, a cancer registry is designed to collect, store and process data from cancer patients. The collection of accurate data and their statistical evaluation enables sound decisions to be made in the development of national health policies. There are approximately 200 population-based registers worldwide. Through the World Health Organization (WHO), the International Research Agency (IARC) publishes global health statistics covering all countries in the world. GLOBOCAN publishes incidence and mortality analyses on cancer every 3-5 years for use by the scientific community. The SEER (The Surveillance, Epidemiology, and End Results) program is one of the most popular registers and is affiliated with the National Cancer Institute (NCI). SEER data have been collected since 1973 and serve as an official source of data in the US on cancer incidence and survival (203). On the other hand, the European Commission has supported the European Network of Cancer Registries (ENCR), which was established in 1990 (204).

The BC screening program is an evaluation of symptom-free and otherwise healthy females for the early detection of BC. Mammography is the most common BC screening modality worldwide. The major merits of BC screening programs are early diagnosis, prevention of risk factors and timely treatment to reduce morbidity. These programs also reduce the overall mortality rate by 20%. The major disadvantages are overdiagnosis, high cost, ionizing radiation, false-positive biopsy, false-negative results, and their subsequent consequences (205). The recognition of clinicopathological features makes it feasible to undertake the necessary preventive and treatment measures. In addition, at the time of diagnosis, BC patients in developing countries and underdeveloped countries appear in more advanced stages than those in developed countries. This gap between lower and higher-income countries remains unclear. Nevertheless, this gap is attributed to racial and genetic variations, exposure to the external environment and differences in access to screening and early diagnosis methods (206).

Croatia and Kosovo have no major differences in relation to geographical coverage or lifestyle. Based on this study's findings, BC patients from Kosovo University Hospital had a significantly larger median tumor size than Croatian patients (P=0.013). Only 38/113 or 33.6% of patients from the Croatian University Hospital underwent axillary lymph node dissection, whereas 47% of

patients from Kosovo University Hospital underwent axillary lymph node dissection. This difference is attributed to the application of sentinel lymph node biopsy (SLNB), a widely adopted routine method for axillary involvement assessment in University Hospital Center Zagreb (UHCZ) that is equal to axillary dissection under certain clinical conditions (no evident clinical axillary involvement in preoperative work-up), whereas SLNB is not performed in Kosovo as routine. Axillary lymph node involvement was significantly increased in Kosovo compared with Croatian patients (47% vs 33.6%, P=0.047) based on the clinical stage of disease presentation before surgery of the patients in both countries, regardless of the method for the axillary lymph node involvement assessment. The numbers of total (P=0.014) and metastatic lymph nodes (P=0.011) were also significantly increased in Kosovo patients. The number of total lymph nodes obtained could be explained by different methods of axillary involvement assessment (axillary dissection vs SLNB). The higher number of metastatic lymph nodes in the Kosovo cohort could be explained by the more advanced stage of the disease at the time of diagnosis (larger tumors and more patients with positive axillary lymph nodes). Therefore, BC patients from Kosovo University Hospital at the time of diagnosis had a significantly more advanced stage than patients from Croatian University Hospital, (P=0.013).

There are several reasons to explain such a large difference in the stages of the disease at the time of diagnosis, between BC patients from two neighboring countries. Until recent years, the largest concentration of the population in Kosovo was in rural areas, a situation accompanied by lack of information regarding malignant diseases in general and BC in particular. In many settlements, access to health facilities for routine check-ups has been difficult. Confidentiality of the disease, so even when patients have noticed changes in the breasts or axilla, have not been reported to the doctor due to fear, stigma, low awareness of malignant diseases, low socio-economic position. Major shortcomings have also been noted in the health system, ranging from lack of qualified health personnel, lack of diagnostic tools and technology, poor geographical distribution of health services, delayed referrals, lack of good coordination between levels of the health system. The treatment of cancer patients at the Clinic of Oncology has started in 2012. Until this time, cancer patients have been partially treated at other clinics at UCCK in Prishtina, and a significant part of them have been treated outside of Kosovo. In these conditions, the

information of the population regarding malignant diseases has been very small. With starting of the provision of the health services in the Clinic of Oncology in Prishtina, the gradual increase of information of the population regarding the issue has begun.

This finding also can be explained by the lack of application of screening and early detection methods in Kosovo. While this program was implemented in 2006 in Croatia, Kosovo implemented its first mobile mammography program in 2014. Based on the data of this study it is seen that the difference is still very large and that much work is still needed to be done in this regard.

The exclusion of patients treated with neoadjuvant chemotherapy, endocrine therapy or targeted therapy prior to surgery is one of the reasons that patients with stage III disease are less represented, and this category is more populated in the Croatian cohort due to the larger number of patients treated with neoadjuvant therapy compared to patients in Kosovo. Regarding surgical treatment, a more aggressive approach has been observed in patients from Kosovo, especially expressed axillary dissection, due to the lack of the application of SLNB, diagnosis of the disease in more advanced stages and the lack of the regular application of neoadjuvant therapy.

On the other hand, no significant differences in access and treatment with adjuvant systemic therapy, such as chemotherapy, endocrine therapy and target therapy, or radiotherapy, was noted in the BC patients between two university hospitals in Croatia and Kosovo.

The rate of LVI it seems to be significantly increased in Kosovo BC patients (P=0.012). LVI is characterized by the presence of malignant cells within the definite endothelial-lined space of blood or lymph vessels (207). The presence of LVI is indicative of a higher risk for axillary lymph node involvement and distant metastasis (208). Although the exact mechanism of LVI is not yet known, it is considered a prognostic factor in patients operated for BC and undergoing chemotherapy. Even in the meeting of St. Gallen-2005, LVI has been approved as a prognostic factor for patients with negative lymph nodes. LVI it was seen also to have directly related to tumor size, histological grade, loco-regional lymph node involvement (208), and this is corresponding with our findings. The Kosovo patients have the median tumor size significantly larger than Croatian patients (P=0.013). Additionally, axillary lymph node involvement was

significantly increased in Kosovo compared to Croatian patients (47% vs 38%, P=0.047), as well as the number of total and metastatic lymph nodes was also significantly increased in Kosovo patients (P=0.014). Although based on the data so far, it is seen that LVI as a prognostic factor when it is expressed, indicates a worse prognosis for the patient, still there is no definitive data that proves why in some patients it is more pronounced than in others.

The rate of the expression of PR it was significantly increased in Croatian patients in comparison with Kosovo patients, 39.9% respectively 16% (P=0.014). Based on the data from previous study, PR is more pronounced in Luminal A-like BC (209), and this also is corresponding with our data. In Kosovo cohort there are 34% of patients in Luminal A-like, while in Croatian cohort there are 38.1%. The growth of BC cells that have PR, depends on the hormone Progesterone. Progesterone together with Estrogen play an important role in many functions in women, such as sexual development, pregnancy, childbirth, and menopause.

Human epidermal growth factor (Her2) is overexpressed in approximately 20-30% of breast cancer. The patients with Her2 overexpressed are associated with higher recurrence rate, more aggressive disease, and generally shorter life expectancy. Her2 is a part of the epidermal growth factor (EGF), which includes 3 other receptors such as: Her1, Her3, and Her4. Trastuzumab as a monoclonal antibody which has the Her2 receptor as its target for action, has been in use since 1998. Trastuzumab has been shown to be more effective when combined with chemotherapy.

Differences were noted between the two cohorts regarding Her-2/neu amplification status (24% in the Kosovo cohort and 14.2% in the Croatian cohort), but this tendency did not reach statistical significance (P=0.073). The reason for this in a certain percentage may be the examinations performed in different laboratories in Croatia and Kosovo. Her2 overexpression as a biochemical marker for BC prognosis is also associated with sentinel lymph node metastasis (209, 210).

6.1. Study limitations

This study has some limitations. First, although the criteria for inclusion of the participants in this study were clear and meticulously respected, it is still impossible to conclude absolutely that the patients and participants of the control group, did not have any other malignant or any unconfirmed inflammatory disease, at the same time. Second, except to age and menopausal status, other clinical data on patients are lacking. The lack of data on parity, body mass index, hormone replacement therapy, alcohol and tobacco use, diet, and physical activity, makes it impossible to have a clear picture of the risk factors that affect the occurrence of BC, and at the same time makes it impossible to assess the eventual correlation between IL-7 and these risk factors.

7. CONCLUSION

Based on our results, the following conclusions were drawn:

1. Significantly higher IL-7 serum concentration was assessed in the EIBC patients compared to healthy control cases.

2. There has been no correlation between IL-7 serum concentration and histopathological characteristics of the tumor, with neither age nor menopausal status of the patients.

3. Patients with invasive lobular carcinoma (ILC) seem to have lower IL-7 serum concentration compared to other histological subtypes, and the difference was significant.

4. There is no differentiation in the IL-7 serum concentration between patients coming from Croatia and Kosovo.

5. There have been no significant differences of IL-7 serum concentration among different molecular surrogate subtypes.

6. The median tumor size at the time of diagnosis was significantly larger in the patients from Kosovo than in those from Croatia.

7. The number of metastatic lymph nodes was also significantly increased in Kosovo patients compared to Croatian patients.

8. BC patients from Kosovo at the time of diagnosis had a significantly more advanced stage than the ones from Croatia.

8. SAŽETAK

Uloga serumske razine Interleukina-7 kao biološkog biljega u raku dojke

Uvod. Cilj ove studije bio je istražiti postoji li razlika u koncentraciji interleukina-7 u serumu bolesnica s ranim invazivnim karcinomom dojke (RIRD) u usporedbi sa zdravim ženama. Analiziran je odnos koncentracija IL-7 u serumu i patohistoloških karakteristika tumora.

Metode. Ova presječna i analitička studija uključila je 213 uzastopnih bolesnica s RIRD (113 iz Hrvatske i 100 s Kosova) i 62 zdrave sudionice u kontrolnoj skupini (30 iz Hrvatske i 32 s Kosova). Uzorci krvi uzeti su zdravim ženama i ženama kojima je biopsijom potvrđen rak dojke (RD) prije kirurške intervencije i drugih modaliteta onkološkog liječenja. Također, nakon operativnog zahvata učinjena je patohistološka analiza uzoraka tkiva te imunohistokemija. Prije operacije zabilježena je dob i menopauzalni status bolesnica.

Razlike u distribuciji numeričkih varijabli analizirane su Mann-Whitneyjevim U testom i Kruskal-Wallis ANOVA testom. Povezanost između numeričkih varijabli analizirana je kao Spearmanov koeficijent korelacije ranga. Dijagnostička točnost i optimalna granična vrijednost koncentracija IL-7 između dvije skupine dobivene su na temelju vrijednosti površine ispod ROC krivulje. Kategoričke varijable uspoređene su pomoću hi-kvadrat testa i prikazane su kao učestalosti i postoci. Analize su provedene statističkim softverom SPSS-22.0.

Rezultati. Koncentracija IL-7 u serumu bolesnica s RIRD značajno je viša u usporedbi s kontrolnom skupinom žena (P<0,001). U skupini bolesnica s RIRD nije bilo korelacije između koncentracija IL-7 u serumu i veličine tumora, dobi te vrijednosti Ki-67 (%). Čini se da bolesnice s invazivnim lobularnim karcinomom (ILK) imaju nižu koncentracija IL-7 u serumu u usporedbi s drugim histološkim podtipovima, a razlika je statistički značajna (P=0,043). Nije bilo značajnih razlika u serumskoj koncentracija IL-7 između različitih stadija bolesti, molekularnih surogatnih podtipova i histoloških stupnjeva raka dojke. Srednja veličina tumora u trenu postavljanja dijagnoze bila je

značajno veća u ispitanica s Kosova nego u onih iz Hrvatske (P=0,013). Broj ukupnih i metastatskih limfnih čvorova također je bio značajno veći kod bolesnica s Kosova. (P=0,023).

Stopa LVI bila je značajno veća u ispitanica s Kosova (P<0,012), dok je ekspresija PR bila veća u hrvatskih ispitanica, a razlika je statistički značajna (P=0,014).

Zaključak. Obzirom na značajno povišene koncentracija IL-7 u serumu bolesnica s RIRD u usporedbi sa zdravom kontrolnom skupinom, IL-7 može se razmotriti kao potencijalni dijagnostički marker za RD. Izostanak korelacije vrijednosti IL-7 s veličinom tumora, metastazama u limfnim čvorovima te svim ostalim histopatološkim karakteristikama tumora dovodi u pitanje njegovu upotrebu kao prognostičkog pokazatelja. Bolesnice s RD s Kosova imale su veći stadij bolesti u trenu dijagnoze nego bolesnice iz Hrvatske. Obzirom na geografsku blizinu dviju zemalja i sličan načina života u obje zemlje, može se pretpostaviti da je ovakav rezultat odraz nedostatka mamografskih pregleda, niskog obrazovnog statusa i nedovoljne svijesti o RD na Kosovu.

Ključne riječi: Rak dojke, interleukini, interleukin-7, Kosovo, Hrvatska, biomarker.

9. ABSTRACT

The role of Interleukin-7 serum levels as biological marker in breast cancer

Faton Sermaxhaj, 2023.

Background: The aim of this study has been to explore whether there is any difference of Interleukin-7 serum concentration in early invasive breast cancer (EIBC) patients in comparison with healthy controls. In addition, the correlation between the IL-7 serum concentration and histopathological characteristics of the tumor has been evaluated.

Methods: This cross-sectional and analytical study has included 213 consecutive patients with EIBC and 62 healthy participants as the control group. Blood samples have been taken from patients confirmed with breast cancer, prior to surgical intervention. IL-7 serum concentration has been measured, using the "Sandwich" ELISA Immunoenzyme test. In addition, after the surgical intervention, histopathological specimen examinations and immunohistochemistry have been performed and analyzed. The differences in the distribution of the numerical variables have been analyzed with the Mann-Whitney U test and Kruskal-Wallis ANOVA test. Correlations have been tested with Pearson coefficients. P-value <0.05 has been accepted as statistically significant.

Results: IL-7 serum concentration in the EIBC patients has been significantly higher as compared to control cases, (P<0.001). There has been no correlation between IL-7 serum concentration and histopathological characteristics of the tumor, with neither age nor menopausal status of the patients.

Conclusions: The use of IL-7 as a potential diagnostic indicator for BC, as well as in the follow-up of the patients after the treatment, can be assumed. The lack of correlation with histopathological characteristics of the tumor, questions its use as a prognostic indicator.

Keywords: Breast cancer, interleukins, interleukin-7, Kosovo, Croatia, biomarker.

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11. CURRICULUM VITAE

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