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ESTROGENS AND LYMPHOMA GROWTH

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Dedicated to:

The people of Balochistan and my hero great **Imran Khan** (Founder of Shaukat Khanam Memorial Cancer Hospital and Research Centre) source of motivation and inspiration for me.

Most surely ALLAH is Gracious to people, but most people are ungrateful

Quran 2:243

Discovery does not come by design, but through creativity and an open mind

Jan Åke Gustafsson

ABSTRACT

Lymphomas are generally not considered as endocrine-associated cancers. Nevertheless, most lymphoid malignancies show a gender difference in incidence and prognosis, with males being more affected. The molecular mechanism for this gender difference is unknown. Some epidemiological data show a protective function of estrogens against Non-Hodgkin lymphomas (NHL). Recent studies have demonstrated estrogen receptor β (ERβ) to be the major ER expressed in normal and malignant cells of lymphoid lineage.

In **Paper I**, we demonstrated a gender differences in tumor growth by grafting mice with murine T lymphoma cells. We found that male mice developed larger tumors compared to female mice, a difference that was abolished following ovariectomy, suggesting estrogen regulated growth *in vivo*. In addition, we looked into the effects of 17β-estradiol, selective ERα and selective ERβ agonists on lymphoma growth in culture and *in vivo*. Treatment with 17β-estradiol had minor effects on lymphoma growth, whereas the selective ERβ agonists diarylpropionitrile (DPN) and KB9520 showed potent antiproliferative and proapoptic effect. This study for the first time showed *in vivo* that ERβ agonists may be useful in the treatment of lymphomas.

In **Paper II**, we studied ligand-activated ERβ effects on human lymphomas. Treatment with the selective ERβ agonist DPN significantly suppressed lymphoma growth in grafting experiments using Granta-519 Mantle cell lymphoma (MCL) and Raji Burkitt lymphoma (BL) cells in immunocompromised (NOD/SCID gamma) mice in comparison to vehicle treated mice. Importantly, activation of ERβ inhibited vascularization. Furthermore, using a disseminating Raji BL cell line, we showed that ERβ activation reduced dissemination of subcutaneous grafted tumors. We also showed by immunohistochemistry that ERβ is expressed in primary MCL tumors. These results suggest that targeting ERβ with agonists may be valuable in the treatment of some lymphomas, affecting several aspects of the malignant process including proliferation, vascularization and dissemination.

In **Paper III**, we showed that when grafting human DLBCL cells to NOD/SCID gamma mice, tumor growth was faster in males compared to females. We also demonstrated high expression of ERβ1, with small or no ERα expression in DLBCL cells. Furthermore, when treating mice grafted with human DLBCL cells with the selective ERβ agonist diarylpropionitrile (DPN), lymphoma growth was significantly suppressed. Furthermore, ERβ1 expression analysis in primary DLBCL tumors from patients by immunohistochemistry revealed nuclear or cytoplasmic expression of ERβ1 in more than 86% of the cases. No

statistical significant correlation of neither nuclear nor cytoplasmic ERβ1 expression to age, gender, the International Prognostic Index and DLBCL subtype was observed. Nevertheless, high cytoplasmic together with low nuclear expression of ERβ1 was found to be a favorable prognostic factor for overall survival (P=0. 03) in DLBCL patients treated with Rituximab and CHOP.

In conclusion, the studies presented in thesis contribute to an explanation of the clinically observed lower incidence and better prognosis of lymphomas in women than men. This highlights a significant role for estrogens, particularly ERβ signaling, in the pathology of NHL. We also suggest that selective ERβ agonists might be a new and useful therapeutic approach for treatment of ERβ expressing lymphomas.

LIST OF SCIENTIFIC PAPERS

I. K. Yakimchuk, M. Iravani, **M. S. Hasni**, P.Rhönnstad, S.Nilsson, M.Jondal and S.Okret: Effect of ligand-activated estrogen receptor β on lymphoma growth *in vitro and in vivo*. Leukemia (2011) *25*, 1103–1110

II. K. Yakimchuk, **M. S. Hasni**, J. Guan, M.P Chao, B. Sander, S. Okret Inhibition of lymphoma vascularization and dissemination by estrogen receptor β agonists. Blood (2014) *123*, 2054-2061

III. **M. S. Hasni**, M. Berglund, K. Yakimchuk, J. Guan, J. Linderoth, R.M Amini G. Enabled and S. Okret Estrogen receptor β1 in diffuse large B-cell lymphoma growth and as a prognostic marker. (Manuscript).

Publications not included in the thesis

K. Yakimchuk, L. Chen, **M. S. Hasni**, S Okret and M Jondal The selective impact of transgenically expressed glucocorticoid receptor on T cells Autoimmunity (2014) *48*,117-24.

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LIST OF ABBREVIATIONS

ABC	Activated B cell like
AF	Activation function
API	Activator protein1
AR	Androgen receptor
BL	Burkitt lymphoma
BERKO	ERβ knockout mice
cDNA	Complementary DNA
CHOP	Cyclophosphamide, doxorubicin, vincristine, prednisone
CLL	Chronic lymphocytic leukemia
Ct	Cycle threshold
DLBCL	Diffuse large B cell lymphoma
DBN	DNA-binding domain
DPN	Diaryl propionitrile
E1	Estrone
E2	17β-estradiol
E3	Estriol
ERE	Estrogen response element
ERs	Estrogen receptors
ERα	Estrogen receptor alpha
ERβ	Estrogen receptor beta
EBV	Epstein Barr virus
FFPE	Formalin fixed and paraffin embedded
FL	Follicular lymphoma
GCB	Germinal center B cell like
HIV	Human immunodeficiency virus
HRE	Hormone response element
HL	Hodgkin lymphoma

LBD	Ligand-binding domain
MCL	Mantle cell lymphoma
MAPK	Mitogen activated protein kinases
NRs	Nuclear receptors
PPT	Propyl pyrazole triol
PR	Progesterone receptor
PKC	Protein kinase C
NF-κB	Nuclear factor kappa-light chain enhancer of activated B-cells
NHL	Non Hodgkin’s lymphoma
NK	Natural killer
NSG	NOD scid gamma
qPCR	Quantitative polymerase chain reaction
R-CHOP	Rituximab-cyclophosphamide, doxorubicin, vincristine, prednisone
SERM	Selective estrogen receptor modulater
Sp1	Specificty protein 1
TMA	Tissue microarry
WHO	World health organization

1 BACKGROUND

1.1 NUCLEAR RECEPTORS

Nuclear receptors (NRs) are intracellular receptors out of which almost all are ligand-controlled transcription factors that play an important role in many physiological functions such as metabolism [1], immune response [2], reproduction [3] and development [4]. Apart from their functions in normal physiology, NRs have been identified to play an important role in many pathological processes, such as cancer, metabolic and inflammatory diseases [5]. In humans, 48 NRs have been identified [6] (Table 1), and these receptors now represent one of the most important therapeutic drug targets for human diseases, for example diabetes, cancer, heart disease, as well as for lifestyle and behavioral conditions [7, 8].

NRs can be split into 3 major classes: Class I is known as the steroid receptor family, which binds to steroids, for example estrogens. Class II is known as the thyroid /retinoid receptors family, which binds to non-steroids e.g thyroid hormones and the third class is termed the orphan receptor family which may not have a ligand (defined as true orphan NRs) or where the ligand has yet not been identified [9-11]. The steroid hormone receptor family that include estrogen (ER), androgen (AR), progesterone (PR), glucocorticoid (GR) and mineralocorticoid receptors (MR) generally bind as homodimers to inverted hexamer sequences separated by 3 nucleotides present in target genes. NRs are transcription factors that share a common overall structure comprising of 3 main domains, namely the N-terminal domain (NTD), a central DNA binding domain (DBD) and a C-terminal ligand binding domain (LBD). The NTD domain has an active transactivation region that contributes to transcription of target genes. The DBD contains two Zn-finger motifs responsible for DNA binding specificity and the LBD harbors the ligand binding pocket and has a ligand dependent transactivation function [12]. NRs generally have a nuclear localization but are for some NRs localized in the cytoplasm before binding its cognate ligands. This is particularly the case for GR, AR and MR which are localized in the cytoplasm in the absence of ligand but upon ligand binding translocate to the nucleus. In contrast ER and PR are predominantly present in the nucleus also in the unliganded state [13].

NRs bind specific DNA response elements in regulatory regions of target genes (called hormone responsive elements (HREs) and regulate transcription in response to ligand binding by recruiting co-regulatory molecules that subsequently modify the chromatin and contact the basal transcription machinery [14, 15]. Coregulators, which is the universal term for

coactivators and corepressors, are receptor interacting proteins that regulate the transcriptional activity of nuclear receptors on target genes or may modify the chromatin [16]. NR may also regulate transcription by modifying the activity of other transcriptional factors. This leads to either stimulation or repression of transcription. In contrast to other transcriptional factors, the activity of most NRs, are regulated by binding to their corresponding ligands. These are usually small lipophilic molecules that easily penetrate biological membranes. In addition to regulation by ligand, NR activity may be regulated by post-translational modification, such as phosphorylation or acetylation [17]. Increasing evidence also reveals that NRs may act through non-genomic actions by directly modifying signaling mediated by kinases e.g. PKC and MAPK [18]. NR genes are present and expressed in some of the simplest animal organisms, but are missing in fungi, plants and choanoflagellates, the closest known relatives of metazoans [19].

Table 1 Members of the human nuclear receptor adapted and modified from [6].

Name	Abbreviation	Example of Ligands
Androgen receptor	AR	Testosterone, flutamide
Constitutive androstane receptor	CAR	Xenobiotics, phenobarbital
Chicken ovalbumin upstream	COUP-TFI, COUP-TFII	Orphan
DSS-AHC critical region on the chromosome gene 1	DAX-1	Orphan
Estrogen receptor	ERα	Estradiol-17β, tamoxifen, raloxifene
	ERβ	Estradiol-17β, various synthetic compounds
Estrogen receptor-related receptor	ERR α	Orphan
	ERRβ, ERRγ	DES, 4-OH tamoxifen
ErbA2-related gene-2	EAR2	Orphan
Farnesoid X receptor	FXRα	Bile acids, fexaramine
	FXRβ[a]	Lanosterol
Germ cell nuclear factor	GCNF	Orphan
Glucocorticoid receptor	GR	Cortisol, dexamethasone, RU486
Human nuclear factor 4	HNF4α, HNF4γ	Orphan
Liver X receptor	LXRα, LXRβ	Oxysterols, T091317, GW3965
Mineralocorticoid receptor	MR	Aldosterone, spirolactone
Neuron-derived orphan receptor 1	NOR1	Orphan
Nerve growth factor-induced factor B	NGFI-B	Orphan
Nur-related factor 1	NURR1	Orphan
Photoreceptor-specific nuclear receptor	PNR	Orphan
Peroxisome proliferatoractivated receptor	PPARα	Fatty acids, leukotriene B_4, fibrates
	PPARβ	Fatty acids
	PPARγ	Fatty acids, prostaglandin J_2, thiazolidinediones
Pregnane X receptor	PXR	Xenobiotics, 16α-cyanopregnenolone
Progesterone receptor	PR	Progesterone, medroxyprogesterone acetate, RU486
Retinoid X receptor	RXRα, RXRβ, RXRγ	Retinoic acid
Reverse erbA	Rev-erbα	Orphan
	Rev-erbβ	Orphan

Retinoic acid receptor-related orphan receptor	RORα	Cholesterol, cholesteryl sulfate
	RORβ	Retinoic acid
	RORγ	Orphan
Retinoic acid receptor	RARα, RARβ, RARγ	Retinoic acid
Steroidogenic factor-1	SF1	Orphan
Short heterodimeric partner	SHP	Orphan
Testis receptor	TR2, TR4	Orphan
Tailless	TLL	Orphan
Thyroid hormone receptor	TRα, TRβ	Thyroid hormones
Vitamin D receptor	VDR	Vitamin D

1.2 ESTROGENS

Estrogens are steroid hormones synthesized from cholesterol and are present physiologically mainly in the form of 17β-estradiol (E2), estrone (E1), and estriol (E3). Particularly E2 serves as the main female sex hormone and acts locally and systemically on target organs and cells. However, estrogens are present both in men and women and serve important functions in both sexes. In postmenarche to premenopausal women, the ovaries are the main site of estrogen synthesis producing the predominant estrogen, E2, while in postmenopausal women estrogens derive from androgens by aromatization in peripheral tissues of adrenal-derived androgens. In adult males, estrogens are mainly formed from aromatization of gonadal produced testosterone. The most potent and dominant estrogen is E2 with weaker activity of E1 and E3. Estrogens play a key role in controlling development, sexual behavior and reproductive functions, but also have significant roles in the physiology of a wide range of other tissues and organs, including the cardiovascular, nervous, the immune systems, as well as in bone metabolism [20]. In addition to the effects of estrogens on normal cells and normal physiology, estrogens also play an important role in various pathological processes, including cancers, particularly breast, ovary, uterus and prostate cancer [21, 22].

1.3 ESTROGEN RECEPTORS

The biological effects of estrogens such as cell growth, reproduction, development and differentiation are mediated by two specific estrogen receptors, α (ERα) and β (ERβ), which belong to the steroid receptor family and to which E_2 binds with similar affinity [23]. Estrogenic effects can also be mediated by a membrane associated-couple protein, the G protein estrogen receptor 1 (GPER1) [24]. The classical ERα was first cloned in 1986 [25], while the second ER, ERβ, was discovered and cloned in 1996 from rat prostate [26]. ERα and ERβ are transcribed from different genes located on separate chromosomes (6 and 14, respectively) [27, 28] and display discrete expression patterns as well as partially distinct ligand specificities [29]. The three main domains of the NRs as described above can for the ERs be sub-divided into six functional structural domains, A-F [30, 31] (Fig. 1). The A/B-domain contains the activation function 1 (AF-1), which is hormone-independent. The C-domain holds the DBD, the D-domain or hinge region contains nuclear localization signals and interacts with AP-1, and the E/F-domain is the LBD with a ligand-dependent activation function, AF-2 [32, 33]. In mammalians, ERα is expressed in reproductive tissues (uterus, breast, ovaries, testis), kidney, bone, white adipose tissue and liver, while ERβ has been found to be expressed in many tissues, including the central nervous system, the cardiovascular system, the immune system, the urogenital tract, the gastrointestinal tract, the

kidneys and the lungs [34-36]. However, in many cells/tissues both ERα and ERβ are co-expressed. e.g breast, ovaries. The generation of three different knockout models, ERα [37], ERβ [38] and ERα/ERβ double knockout [39] have provided vital information about ER signaling. For example, it was found that ERα is involved in the development of the uterus, mammary gland, bone physiology and male fertility, whereas ERβ is important for neuronal development [40].

Both ERs function as homodimers but may sometimes heterodimerize and possess a high degree homology in the DBD but differs in the N-terminal domain and the LBD [26, 27]. In addition to the full length expression of ERα and ERβ, isoforms derived from alternative splicing are found both in normal and malignant cells [41]. Splice variants of ERα and ERβ have been detected in e.g. normal peripheral leukocytes [42] and many cancers including breast [43, 44], endometrial, ovarian and colorectal cancers [45-47].

With regard to ERβ, several isoforms (ERβ2 to 5) have been reported. These isoforms are generated by alternative splicing in which the terminal exon (exon 8) of the full length wild-type ERβ (ERβ1) has been replaced by unique sequences [48, 49]. The lack of or replacement of the last exon (exon 8) has rendered these isoforms unable to bind ligands and unable to activate transcription of an estrogen response element (ERE)-driven gene [50]. However, they might interfere with ERβ1 function by acting as dominant negative mutants [51]. For example, ERβ2 has been suggested to enhance cell proliferation and invasion [52], and in several cancers it was demonstrated that high expression of ERβ2 is associated to poor prognosis [53-55]. Like most other members of the NR family, ERs are mainly found in the nucleus. However, ERα has also been shown to be present in the cytoplasmic membrane of breast cancer cells, where it upon ligand binding can quickly transmit signals affecting cell proliferation and survival [56]. Membrane expression of ERβ has also been reported [57]. ERβ1 can be found both in the nucleus and cytoplasm of normal and cancerous cells [57, 58]. It has been shown that nuclear ERβ1 expression is linked to better overall survival in breast cancer, but the function cytoplasmic ERβ has not been adequately explored [59].

The classical mode of ER action involves a ligand-induced conformational change in the ER that results in ER binding to estrogen response elements (EREs) present in target genes and subsequent transcriptional activation [60]. However, by transcription factor cross-talk, ligand-activated ERs can also modulate gene expression indirectly through interaction with other transcriptional factors (TFs), such as members of the activating protein-1 (AP-1), nuclear factor kappa B (NF-κB) or specificity protein-1 (Sp1) [41, 61, 62]. In addition,

several reports describe an impact of ER on non- or pregenomic signaling pathways [63-65]. This involves regulation of various protein kinase pathways such as PI3K/AKT and MAPK signaling [66]. These examples demonstrate that the ERs have the ability to regulate cellular responses without binding directly to DNA. Finally, protein kinase pathways may activate ER in a ligand-independent way [67].

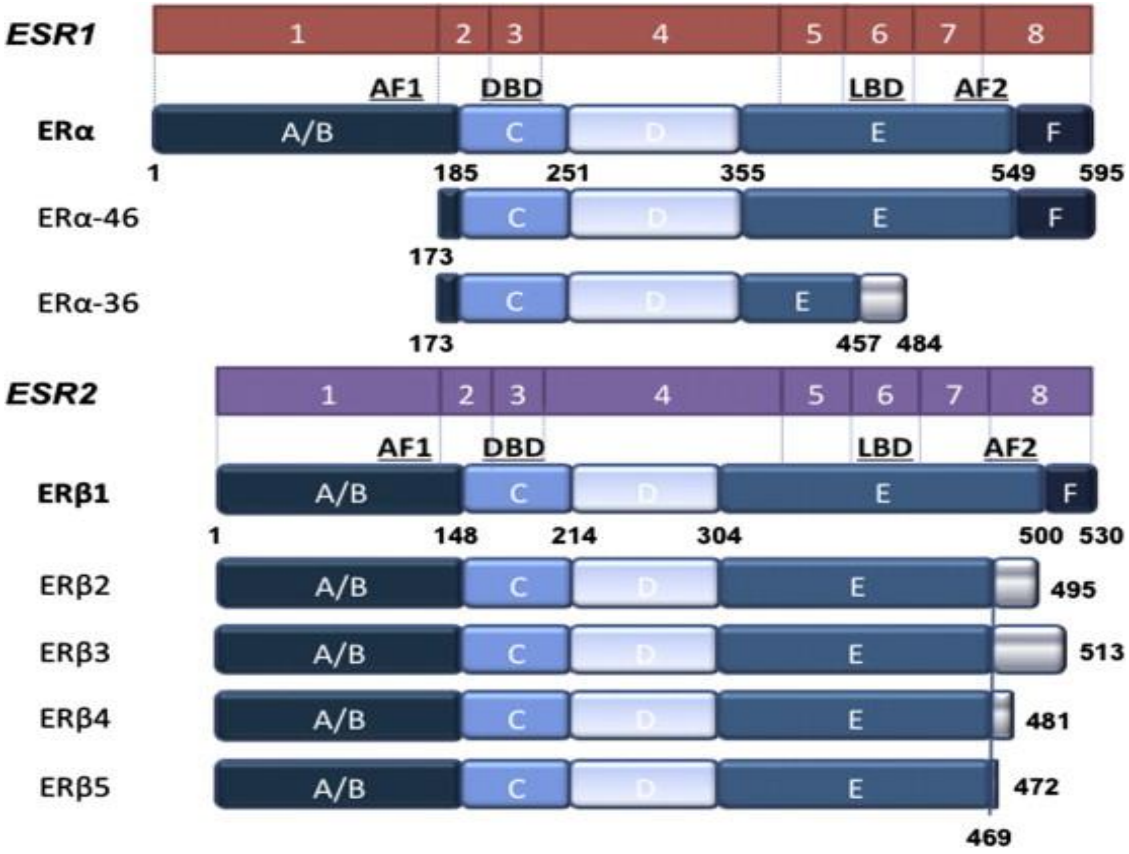

Figure 1: Schematic representation of the functional domain organization of wild-type estrogen receptors and their splice variants [68].

1.4 ESTROGEN RECEPTOR LIGANDS

The word "ligand" comes from the Latin word "ligare" which means "to bind. In biochemistry and pharmacology, a ligand is defined as an allosteric regulator that is able to attach to and shape a biomolecule to serve a biological function, such as a steroid hormone which binds to its corresponding NR. There are different classes of ligand, such as high affinity agonists that require only low concentrations of ligands to bind with maximal occupancy and which elicit a maximal physiological response. Antagonists on the other hand are ligands that bind, but do not provoke a physiological response (e.g., tamoxifen for ER in breast tissue) and compete with agonists for binding. Partial agonists or antagonists are ligands that do not give rise to a maximal activation or inhibition of a response despite full occupancy [69].

E2 is a natural, non/selective ligand for ERα and ERβ and is the most potent natural ER ligand, while the two metabolites, estrone (E1) and estriol (E3), are only very weak agonists. In addition to the natural ligands, there are several synthetic compounds able to function as an agonist or antagonist on the two ERs. For instance, ICI182,780 is considered to be a pure antagonist while other ligands are defined as selective estrogen receptor modulators (SERM) e.g tamoxin and raloxifene that act as an agonist or an antagonist dependent on the tissue [70, 71]. Importantly, the structural differences in the ligand-binding pockets of ERα and ERβ, respectively, have allowed development of subtype selective ligands. One of the most widely used ERα selective agonist, propyl pyrazole triol (PPT), shows a 410-fold selectivity for ERα versus ERβ [72]. Multiple selective ERβ agonists have been synthesized, one of the most well characterized is 2,3-bis (4-hydroxyphenyl)-propionitrile (DPN) which shows higher relative binding affinity and relative potency for ERβ compared to ERα in the range of 70–300-fold [73-75]. To note is that DPN does not bind to GPER1 [76]. Another newly developed ERβ selective agonist is KB9520, which shows a 700-fold selectivity for ERβ versus ERα [77] (Fig 2).

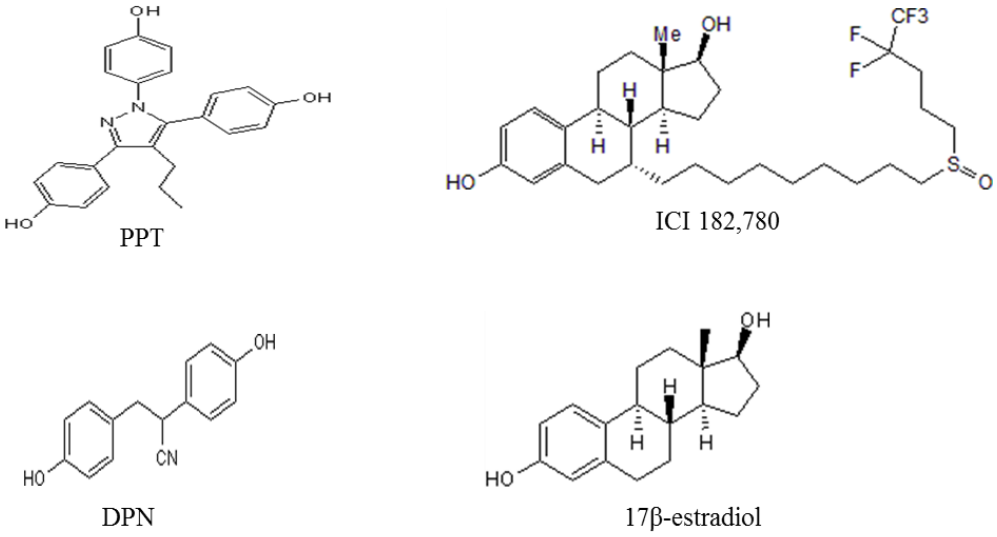

Figure 2: Representation of the structures for some ER ligands.

Also, some Phyto-estrogens (plant-derived) compounds have steroid-like structures and estrogen-like attributes. Examples of these are genistein, dadzein and glycitein, which are the main dietary-derived isoflavones [78]. Many of these compounds show a partial ERβ selectivity. For instance, the isoflavone genistein has a 20-fold greater binding affinity for ERβ compared to ERα [34, 74, 79].

1.5 ESTROGEN RECEPTORS AND CANCERS

Cancer is an abnormal growth of cells which tend to proliferate in an uncontrolled way and often metastasize or disseminate (spread) to other parts of the body via the blood or the lymphatic system (Fig 3). Cancer is a major contributing cause of death worldwide. Despite advances in the early detection methods and improved therapies, cancer still remains a big health challenge. It is a world wide health problem with 12.7 million new cases and 7.6 million deaths reported for 2008. As the world's population continues to grow and live longer, the incidence of cancer is thought to increase to a sum of 22.2 million cases by the year 2030 [80].

Hanahan and Weinberg described different features which cancer cells acquire in order to sustain their growth and to escape different anti-tumor mechanisms [81]. These features, termed "Hallmarks of cancer", include sustaining of proliferative signaling, evading growth suppressors, resisting cell death, enabling replicative immortality, inducing angiogenesis, and activating invasion and metastasis [81]. The primary causes which lead to cancer development are environment and lifestyle factors, including smoking, exposure to radiation or UV light, high alcohol use, inadequate diet or physical activity, obesity, frequent contact with carcinogens (e.g. benzene and asbestos), bacteria or virus infection [82-84]. Chronic inflammation is thought to be a major cause of cancer development [85]. Genetic factors like mutations in oncogene or tumor repressor genes usually accompany cancer development of [86].

In the cells, the overall effect of E2 is determined by a balance between ERα and ERβ as E2 binds to both of these receptors with similar affinity. Many tumor cells express ERs and several studies have shown that ERs impact on cancer development in several tissue types, mainly in endocrine-related reproductive organs [87-90], but also in tissues not generally considered to be endocrine-related like lung cancer [91] and colon cancer [92].

The different expression of the ERs usually account for their effects in cancer tissues. The role of ERs in breast cancer pathogenesis are becoming increasingly elucidated by several clinical and *in vitro* studies. Normally both ERs are found in the mammary gland where ERβ seems to be predominant in normal breast glands [93]. In breast tissue ERα promotes proliferation thereby increasing the risk of tumor development and progression, whereas ERβ seems to suppress proliferation and promote differentiation [94, 95]. In early breast cancers, the expression of ERα usually increases when going from low to high grade tumors [96], but in contrast to ERα, a reduction and loss of ERβ expression was observed when cells

transformed from normal tissue to invasive carcinomas [29, 97-99]. Furthermore, It was shown that administration of ERβ into T47D breast cancer cells inhibits cancer cell proliferation and tumor formation in mouse xenograft models [95, 100]. ERβ also has proapoptotic effects. For example, it has been shown that ERβ induces apoptosis of prostate and ovarian cancer cells, while ERα promotes proliferation and survival [94, 101, 102]. ERβ has also putative anti-proliferative effects on the ovary and endometrium [103, 104]. It was also demonstrated that ovarian cancer shows high expression of ERα when compared to normal ovarian tissue, and loss of ERβ correlated with shorter overall survival in ovarian cancer [105]. ERβ is also predominantly expressed in the normal colon epithelial cells [106], but decreased expression has been observed with the progression of cancer. It has also been shown that there is a reduced intestinal tumorigenesis in mice with the spontaneous development of intestinal adenomas (ApcMin/+) after treating with diarylpropionitrile (DPN), an ERβ specific agonist [107, 108]. An antiproliferative effect of ERβ has also been shown in malignant mesothelioma [109, 110]. Similar to e.g. breast and colon, which express high nuclear ERβ in normal tissue, but lesser levels after tumor transformation, normal pleura expresses high ERβ levels which after tumor progression decrease. It was also indicated that ERβ expression was an independent prognostic factor for better survival [109]. ERs have been found in lung tissue, however the role of ERα or ERβ in lung cancer is less well-defined.

ERβ is the predominant ER in human peripheral blood leukocytes and spleen [111], and therefore generally thought to be the main target receptor for estrogen action in the immune system. In line with an antiproliferative effect of ERβ, the absence of ERβ in ERβ knockout mice resulted in a myeloproliferative disease resembling human chronic myeloid leukemia with lymphoid blast crisis [112]. Monocytes also express mainly ERβ and have been shown to be sensitive to estrogen-induced programmed cell death [113].

ERβ is also expressed in lymphoid cancer cells and studies have indicated an anti-proliferative function of ERβ in these cells. In different human lymphoma cell lines including both B and T cell lymphomas, ERβ is abundant while ERα is not detectable [77, 111]. It has been shown from our lab that ERβ activation *in vitro* and *in vivo* leads to the inhibition of proliferation and increase of apoptosis in T- lymphoma cells [77]. It has also been reported that CLL cells express ERβ [55]. Furthermore, CLL cells also express the ERβ non-ligand binding splice variant ERβ2 [55] and in contrast to the wild-type ERβ1 it has been reported to be a poor prognostic factor for CLL [55], similar to the case for prostate cancer [53]. In

prostate cancer cells, it also has been shown to increase proliferation [52]. It has also been shown in breast cancer that ERβ2 expression correlated to worse overall survival [59].

However, although most studies support antiproliferative and proapoptotic effects of the wild-type ERβ1 leading to inhibition of tumor proliferation and survival [95, 114-117], the role of ERβ1 in tumor progression is not completely consistent as contradictory results in proliferation have been demonstrated. In fact, some studies show a stimulatory effect on tumor growth by activation of ERβ1 [117–119].

Considering that most studies favour anti-proliferative and pro-apoptotic effects of ERβ1 as in the case of e.g breast, prostate and colon cancer, and as demonstrated by us for lymphomas, it has been denoted as a tumor suppressor.

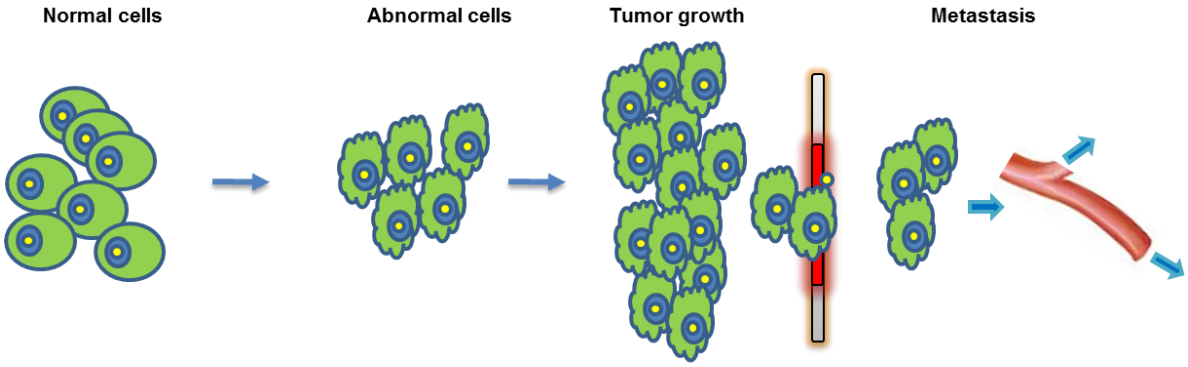

Figure 3: Tumor development stages from normal cells to metastasis.

1.6 THE LYMPHATIC SYSTEM

The human body has two major circulatory systems: the blood and the lymphatic system, respectively. The lymphatic system is essentially a drainage system inside the body which carries excess fluid and metabolic waste products from interstitial spaces into the blood circulatory system. It consists of a network of tissues and organs that include lymphatic vessels, lymph and lymph nodes. Except being important for drainage, the lymphatic system has an important role in the immune system. Furthermore, many cancer cells use the lymphatic system to metastazise/disseminate to other parts of the body [120].

The lymph nodes are part of the group of so called secondary lymphoid organs, which except the lymph nodes include the spleen, Peyer's patches, appendix and tonsils. In contrast to the primary lymphoid organs (the thymus and the bone marrow which are responsible for the production and maturation of lymphocytes), the secondary lymphoid organs are responsible for further maturation of the lymphocytes and initiation of immune responses [121]. Most of the lymphomas arise in the lymph nodes, from B (most common), T or Natual killers (NK) cells. Figure 4 shows a cartoon of the lymphatic system and lymphoid organs.

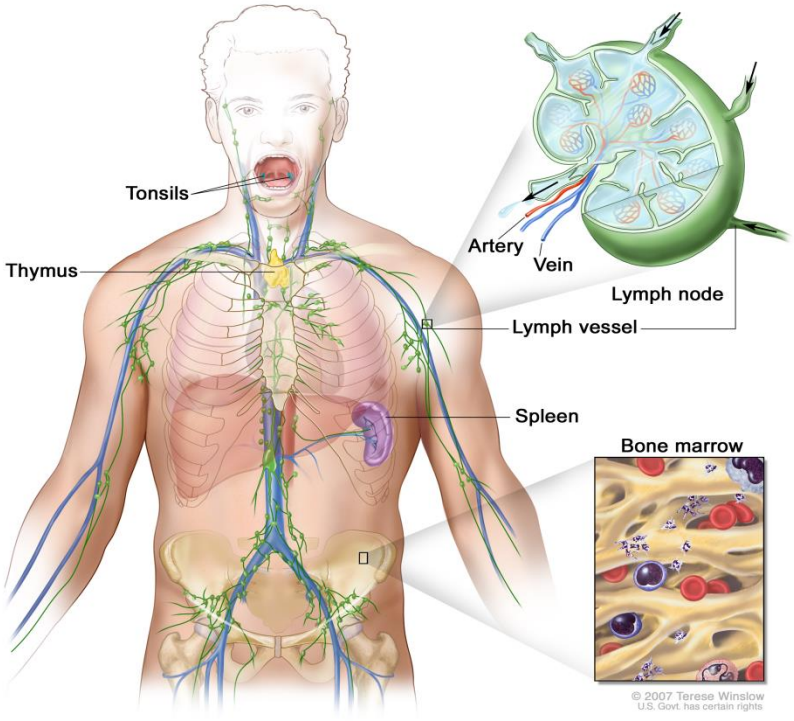

Figure 4: The lymphatic system. Reprinted with the permission by the author. Available at: https://visualsonline.cancer.gov/details.cfm?imageid=7154

The lymphocytes are produced in the bone marrow (B, NK cells) or the thymus (T cells). When the lymphocytes are mature, they are released into the blood stream and migrate to the secondary lymphoid organs. There are three main types of mature lymphocytes:

- B lymphocytes, which produce antibodies that attack germs (bacteria, viruses, etc.)
- T lymphocytes, which have several functions including assisting the B lymphocytes to make antibodies and to regulate immune responses. They may also be cytotoxic, including killing cancer cells as part of a cancer immunosurveilance system.
- Natural killer lymphocytes, which are part of the innate immune system and exert a cytotoxic effect on viral infected cells and on tumors.

1.7 LYMPHOMA

Lymphoma is a heterogeneous group of malignancies derived from the lymphoid system that accounts for roughly 4 % of cancers worldwide [122]. The annual incidence in the Western countries is approximately 20 cases per 100,000 persons [123]. According to the Swedish Cancer Registry, about 2000 to 2200 new lymphoma cases are diagnosed each year in Sweden (http://www.socialstyrelsen.se). In Sweden it is the 8th most common cancer form both in men and woman although the number of males are affected slightly more (see below). Lymphoma incidence increases with age although it may develop at any age. Hodgkin lymphoma (HL) and Burkitt lymphoma (BL) are preferentially seen in young adults or children [124]. Lymphomas derive from lymphocytes. While its primary sites are the main or secondary lymphoid organs (lymph nodes most common), it can affect any site in the body due to infiltration by malignant lymphocytes.

The classical division of lymphomas is into Hodgkin (HL) and non-Hodgkin lymphomas (NHL). About 90% of the lymphomas are NHL. Today, and especially in the clinic, a division based on more clinical lymphoma characteristics is used, classifying lymphomas into aggressive or indolent (non-aggressive) types [125]. Most NHL derive from B or T cells at various stages of differentiation with 95% of all being of B cell origin [126] and the remaining are derived from T cells or NK cells. The most common types of B cell lymphomas are diffuse large B cell lymphoma (DLBCL) 30-40%, follicular lymphoma (FL) 20%, B-cell chronic lymphocytic leukemia (CLL) 7%, mantle cell lymphoma (MCL) 5-10%, and Burkitt lymphoma (BL) 2%. The various B lymphomas arise during different steps of B lymphocyte differentiation (depicted in Fig. 5).

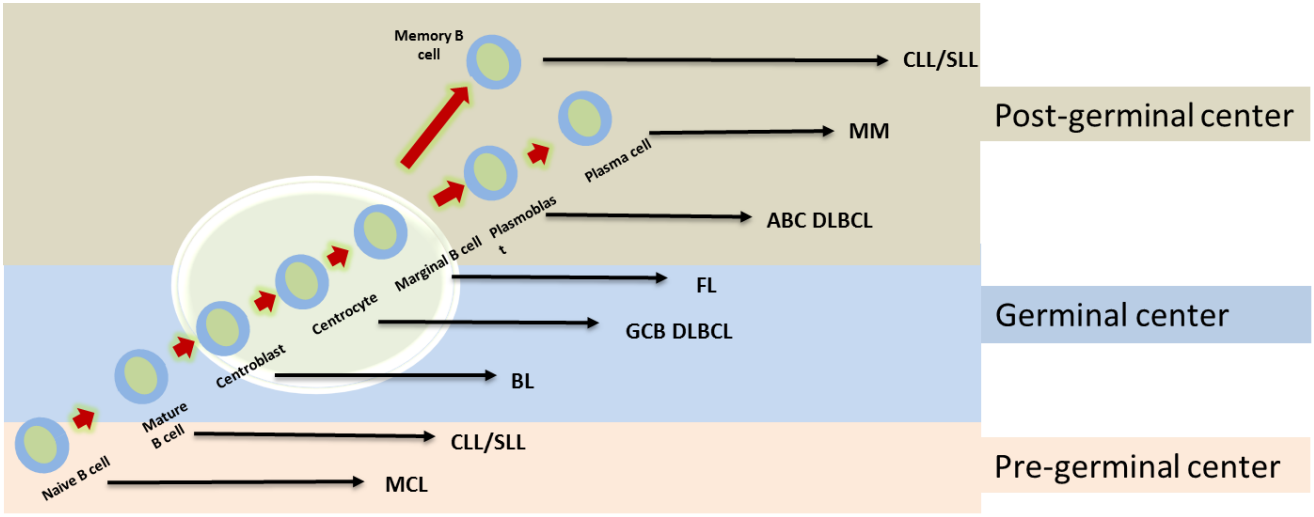

Figure 5: B cell development stages and relation to biology of lymphoma. CLL (chronic lymphocytic leukemia), SLL (Small lymphocytic leukemia), GCB-DLBCL (Germinal center B cell like diffuse large B-cell lymphoma), FL (Follicular lymphoma), MZL (Marginal zone lymphoma), ABC-DLBCL (Activated B cell like diffuse large B-cell lymphoma), MM (Multiple myeloma), BL (Burkit lymphoma) and MCL (Mantle cell lymphoma) Adapted and modified [127].

In most cases, the cause of lymphoma is unknown, although several risk factors for B-cell lymphomas have been described like age, ethnicity, gender, viruses, bacterial infections, life style, impaired immune function, such as immunodeficiency and autoimmune disease. Patients who are treated with immunosuppressive drugs following solid organ or hematopoietic stem cell transplantation are at substantially increased risk (30–50 times) to develop NHL [128, 129], especially during the first year after transplantation [130]. Systemic lupus erythematosus and rheumatoid arthritis has also been associated with B cell lymphoma [131]. Certain lymphomas are related to viral infections, such as Epstein-Barr virus (particularly BL) and hepatitis C virus [132]. Infection with EBV is highly prevalent in the adult population with approximately 90% of individuals in developed countries having evidence of a previous EBV infection by age of 40. Patients infected with HIV show a significantly increased risk of B cell lymphomas [133]. Bacterial infections by e.g. Helicobacter pylori has also been associated to NHL [134]. Interestingly, the incidence of NHL increased for two decades by 3-4% yearly until the mid 90-ties after which the increase has leveled off [135]. The reason for this is unknown.

Several classifications have been described for NHL according to their histologic characteristics, but the most recent system is the fourth edition of the WHO classification of tumors of haemopoietic and lymphoid tissues, published in 2008 (Table 2) [136]

Table 2

Subtypes of non-Hodgkin lymphoma, according to the 2008 WHO classification

Name	Diseases
B-cell lymphomas	
Precursor B cell	Precursor B-cell lymphoblastic leukemia or lymphoma
Mature B cell	Chronic lymphocytic leukemia/small lymphocytic lymphoma; lymphoplasmacytic lymphoma; splenic marginal-zone lymphoma; extranodal marginal-zone B-cell lymphoma of mucosa-associated lymphoid tissue; nodal marginal-zone B-cell lymphoma; follicular lymphoma; mantle-cell lymphoma; diffuse large B-cell lymphoma; Burkitt's lymphoma
B-cell proliferations of uncertain malignant potential	Lymphomatoid granulomatosis; post-transplantation lymphoproliferative disorders (polymorphic)
T-cell and NK-cell lymphomas	
Precursor T cell	Precursor T-cell lymphoblastic leukemia or lymphoma
Extranodal mature T cell and NK cell	Mycosis fungoides; cutaneous anaplastic large-cell lymphoma; extranodal NK-cell or T-cell lymphoma; enteropathy-type lymphoma; hepatosplenic lymphoma; subcutaneous panniculitis-like lymphoma; primary cutaneous CD8-positive lymphoma; primary cutaneous γ/δ T-cell lymphoma; primary cutaneous CD4-positive lymphoma
Nodal mature T cell and NK cell	Peripheral T-cell lymphoma, not otherwise specified; angioimmunoblastic lymphoma; anaplastic large-cell ALK-positive lymphoma; anaplastic large-cell ALK-negative lymphoma; adult T-cell leukemia/lymphoma

Chemotherapy with mustine for NHL malignancy was first used in 1940's and resulted in a dramatic but transient reduction in tumor mass [137]. Since then a number of advances in lymphoma treatment have contributed to improved outcomes for people affected by NHL and HL with decreasing mortality rates observed mainly during the last 5-10 years or so. A significant improvement during the last decade was made possible with the introduction of an anti-CD20 monoclonal antibody, rituximab, which has been utilized widely in the treatment of B cell lymphomas leading to significantly better outcomes, which in part have contributed to the reduced mortality rates. Today overall five-year survival rate in Sweden for all HL subtypes is around 90%, while that for NHL it is around 72% and it has increased during the last 15 years (1996-2011) with about 15% (Stockholm Gotland Cancer region report 2013, www.cancercentrum.se). However and notably, still about one third of patients with NHL don't respond or become therapy resistant [138]. Furthermore, the overall survival rate is very much dependent on lymphoma subtype, age and grading with e.g. MCL and T cell lymphomas having a poor outcome [139]. The need for new therapies utilizing new drug targets, particularly for NHL's with poor prognosis or which become resistant to

present therapies, is therefore still high. In line with this several new drugs targeting especially intracellular signaling pathways important for lymphoma proliferation and survival are under development (together with immunotherapies) or have recently been introduced (still not used as first line therapies). One example of a recently developed drug affecting an intracellular signaling pathway is Ibrutinib, which is a Bruton tyrosine kinase inhibitor preventing B-cell receptor signaling thereby inhibiting B-cell proliferation and survival [140]. However, in a recent study, still 32% of first-line treatment resistant MCL did not respond to the BCR signaling inhibitor Ibrutinib [141], suggesting that a subpopulation of MCL is resistant even to one the most newly developed drugs. Therefore, the need for additional improved therapies, preferentially utilizing new concepts, is still there.

1.8 LYMPHOMA INCIDENCE IN RELATION TO GENDER AND ESTROGENS

Several epidemiological studies demonstrate a gender differences with respect to both the incidence and prognosis of NHL [142-145]. The overall incidence of NHL is 30% lower in women compared to men although the ratio between males and females differ for the different lymphoma subtypes or even with age [146]. NHL with the highest male to female incidence ratio is seen among e.g. MCL and BL with ratios of 3-6:1. In contrast, FL shows no difference in incidence between genders. The most common lymphoma type, DLBCL, show a male to female incidence ratio of 1.2:1 to 1.6:1 [147, 148]. A relative high male to female ratio is also seen among T cell lymphomas where a male/female incidence rate ratio of 1.8 for T-cell peripheral lymphomas was reported [149]. The incidence of CLL in adult males is nearly two times higher than that in women. Interestingly, it has also been reported, and similar to humans, that canine lymphoma is significantly more common in male dogs compared to female dogs [150].

Various additional population based studies have shown that that reproductive factors, particularly estrogens, may explain the gender difference in NHL incidence [151, 152]. Although not fully consistent, epidemiological data from several studies in females show an association between reproductive hormonal factors and oral contraceptives with a reduced risk for NHL with up to 50% [146, 153, 154]. Furthermore, pregnancy has also been reported to reduce the incidence of NHL, supporting an involvement of female sex hormones in NHL development [146, 155]. It has also been proposed that hormone replacement therapy may be associated with a reduced risk of NHL in postmenopausal women [156-158]. Recently in a Swedish population-based study, it was demonstrated that females younger than 52 years of age (average age for menopause) have a better overall survival of the NHL subtype DLBCL compared to men at the same age. However, the gender difference disappeared after menopause [159]. Furthermore, gender also seems to influence response to standard treatment, as some studies suggest that the male gender is associated with a poorer treatment outcome in patients with NHL [160-162]. It line with this it was demonstrated that women show a better response to R-CHOP therapy compared to men [163]. This gender-associated response to therapy is not limited to one single therapy but seen for several drugs [164]. However, some of the gender difference in therapy response may relate to gender specific metabolism of the drugs used [165].

Although there is strong evidence from epidemiological data that reproductive factors and particularly estrogens have a protective effect on lymphoma development as described above, lymphomas are not generally considered as hormone-related cancers. This despite that several lymphoma subtypes have been shown to express ERs [77, 112]. In addition, it has been shown that normal lymphocytes that express ERs were regulated by estrogen [166]. Subsequently, very little is known about the molecular mechanism of sex hormone action on lymphomas and the idea of exploring hormone therapies in lymphoma treatment has not really been recognized.

Malignant lymphoma (excl. Hodgkin's lymphoma)
Number of cases per 100 000 in Sweden

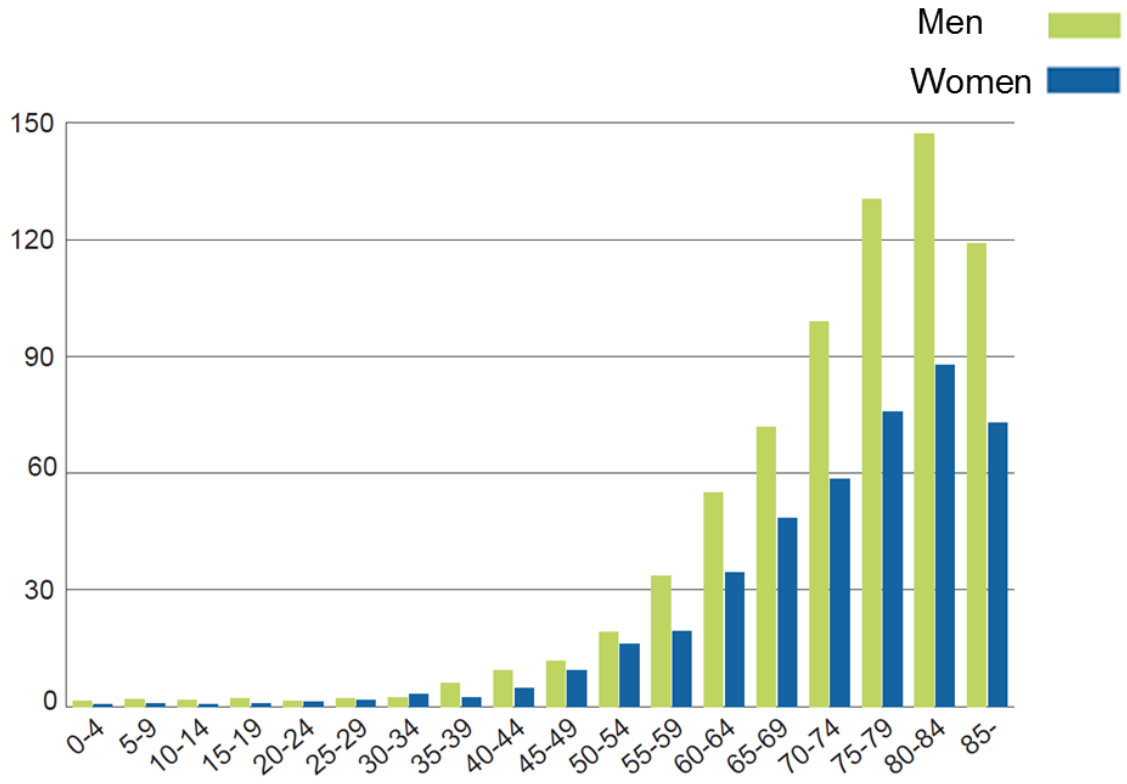

Figure 6. The incidence of malignant lymphoma per year in Sweden defined by age. Source: Socialstyrelsens statistikdatabas; Cancer i siffror, 2013

2 AIM OF THE STUDY

The overall aim of my research project was to investigate the effects of estrogens, particularly selective ERβ agonists, on tumors of lymphoid origin and to study the mechanisms of action of ERβ selective agonists and their usefulness for inhibiting growth of murine and human lymphoma. More specifically the following aspects were studied.

The specific aims were:

- To look into the impact of gender in an experimental lymphoma animal model *in vivo*. To determine ER expression and to investigate the effects of selective ER agonists on murine lymphoma tumor proliferation *in vitro* and *in vivo*.

- Establish *in vivo* models in mice to test ERβ agonists effects on human non-Hodgkin lymphoma (NHL) and their mechanisms.

- To analyze the impact of gender, ERβ1 expression and its correlation to clinical parameters of DLBCL

3 MATERIAL AND METHOD

3.1 CELL LINES AND PATIENTS

In this thesis six different types of lymphoma cell lines were used representing four types of NHL. A murine T lymphoma cell line termed EG7 [167], a Mantle cell lymphoma (MCL) cell line (Granta 519) [168], two Burkitt lymphoma cell lines (Raji and Ramos) [169] and two Diffuse Large B cell lymphoma (DLBCL) cell lines (SU.DHL4 and U2932) [170, 171]. SU.DHL4 is considered to be a germinal center B cell like (GCB) DLBCL while U2932 is an activated B cell type (ABC) DLBCL. All these cell lines were shown by qPCR and/or immunostaining to express relative high amounts of ERβ mRNA and protein, but low or no ERα. Primary MCL material was from Karolinska University Hospital and tissue micro-arrays of primary DLBCL's were from two separate cohorts (Uppsala Akademiska Hospital and Skåne University Hospital, respectively).

3.2 LABORATORY ANIMALS AND TUMOR GRAFTING

It was in mid-1960s when the first *in vivo* models for tumors were developed that were mouse leukemia models [172]. Many inbred, outbred and transgenic strains are used now a days in the labs. Among all animal models, *Mus musculus* is the most extensively used species for transplantation of both mouse and human tumors. These are mammals, small in size and easy to handle. They multiply rapidly and one of the most significant advantages is that the whole genome is sequenced and resembles the human genome to a big extent.

C57Bl/6J mice (8-10 weeks of age) were bought from Charles River (Lille Skensved, Denmark). NOD.Cg-*Prkdcscid Il2rgtm1Wjl*/SzJ commonly known as NOD scid gamma (NSG) mice [173] and ERβ$^{-/-}$ knockout (BERKO) [38] mice were bred at the Department of Biosciences and Nutrition, Karolinska Institutet, Stockholm, Sweden. NSG mice lack B and T cells as well as NK cells. Macrophages number is reduced in NSG mice and they are also deficient in cytokine signalling. They are very reciprocal for tumor cell grafting.

For tumor grafting, 0.5 to 15 x 10^6 lymphoma cells of different lymphoma types were inoculated subcutaneously once daily into the right flank of the mice. Hormone treatment started from the day after injection of lymphoma cells (Paper I) or when lymphomas were first palpaple (Paper II and Paper III). Mice were injected subcutaneously in the left flank with selective agonists of ERs (PPT, DPN, KB9520), non-selective E2 or antagonist (ICI182,780). (See individual paper for details). Tumor growth was assessed daily and tumor volume (TV) was calculated as TV (mm^3) = 0.5 x length (mm) x width^2 (mm^2) using a

caliper. Experimental manipulations of the mice were, according to the ethical approval and local Karolinska Institutet regulations. No signals of pain or physical discomfort among the mice were noted. Figure 7 shows a flow chart for animal experiments.

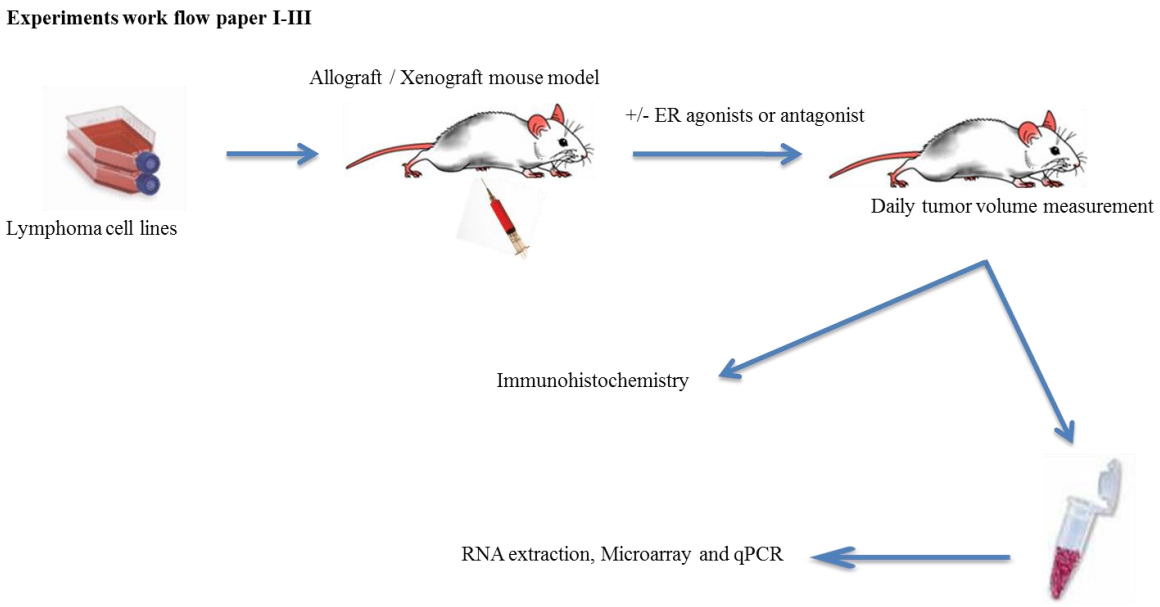

Figure 7: Flowchart for experiments

3.3 IMMUNOHISTOCHEMISTRY

IHC is the most commonly applied immunostaining technique. It is widely used in the basic and clinical research to identify presence and localization of biomarkers and differentially expressed proteins in the different tissues. From animal experiments, tissue samples were fixed in 4% paraformaldehyde overnight, rinsed in 50% ethanol and embedded in paraffin for sectioning. Primary lymphoma specimens were taken at diagnosis for diagnostic purposes, formalin fixed and embedded in paraffin. See individual papers for details regarding conditions used (Paper I-III). In Paper III, normal liver, which lacks ERβ expression as well as omission of the primary antibody served as negative controls for ERβ1 staining. Human tonsils (positively stained germinal centers) were used as positive control. To confirm the ERβ specificity of the 503 antibody and the PPG5/10 antibody used for immunofluorescence/immunohistohemistry, murine hepatoma cells Hepa1c1c7 or human Breast cancer cells MDAM231 were stained for ERβ and showed to be negative, unless the cells were transfected with ERβ-expressing vector, confirming the ERβ specificity of the 503 antibody and the mouse monoclonal anti- human ERβ (PPG5/10) antibody used. To note is that both anti-bodies used to only recognize ERβ1 and not the splice variants. The tissue microarray (TMA) and control slides were evaluated independently by two investigators, out of which one is an experienced hematopathologist.

3.4 QUANTITATIVE POLYMERASE CHAIN REACTION

SYBR Green based quantitative polymerase chain reaction (qPCR) was used to measure gene expression in real time. It is based on detection by the fluorochrome SYBR Green which binds non-specifically to produced PCR products. The SYBR Green based qPCR assay is a sensitive and reliable method to determine the relative level of specific RNA between two samples. To quantify the PCR product, we used the standard 2-ΔΔCt method [174]. The specificity of PCR product was examined by dissociation curves and results were calculated by the 2−ΔΔCt method. The final exponential value 2-ΔΔCt represents the relative fold change between two samples normalized to the Ct values from a housekeeping gene. Three of the most commonly used housekeeping genes GAPDH, 36B4 and 18S, were used in our studies, due to high and stable expression levels in the used cell lines and tumor materials. We obtained the same results with all used housekeeping genes.

4 RESULTS AND DISCUSSION

4.1 PAPER I:

Effect of ligand-activated estrogen receptor β on lymphoma growth *in vitro* and *in vivo*

As mentioned above, several epidemiological studies have shown a gender difference in NHL incidence as well as suggested a protective role for estrogens in NHL development. However, almost no experimental studies, particularly not *in vivo*, have been performed to establish an experimental animal model that resembles this gender difference and to evaluate the mechanism of action of estrogens and involvement of ERs in this response. This is despite that ERβ is expressed in immune cells and numerous studies have identified an anti-proliferative effect of ERβ [36, 110, 175]. In our first experiments, we observed that a murine T cell lymphoma (EG7) grew faster in male mice as compared to female mice, a difference that was abolished following ovarioectomy. This suggested that estrogens may exert an anti-proliferative effect on EG7 lymphoma growth. When analyzing ER expression in the murine T- and in several human B-cell lymphoma cell lines, we detected relatively high ERβ expression with minor or no ERα expression. This is in line with previous studies that showed that normal lymphoid cells, mainly express ERβ [111]. Furthermore and notably, this in contrast to many other cells, e.g. breast, prostate and colon, which seem to drastically down-regulate or loose ERβ expression when transformed into cancer cells [176]. This has been claimed to be due to ERβ promoter methylation occurring during the malignant transformation [177]. In addition, we examined the effects of E2 and selective ERα and ERβ agonists on lymphoma growth in culture and *in vivo*. In culture, selective ERβ agonists exerted a anti-proliferative effects while E2 only showed a weak anti-proliferative effect. A selective ERα agonist, propylpyrazole trisphenol (PPT), did not influence the proliferation of the EG7 T-lymphoma cells. We also investigated whether lymphoma growth may be suppressed *in vivo* by selective ERβ agonist treatment. Mice grafted with the murine lymphoma cells were treated with the selective ERβ agonists DPN or KB9520. Both ERβ selective agonists strongly inhibited lymphoma growth *in vivo* while the ERα selective agonist PPT had no effect and E2 only had a minor effect. Further analysis showed that the reduced tumor size of EG7 lymphoma seen following either DPN or KB9520 treatment was due to reduced proliferation and increased apoptosis. The antiproliferative effect of ERβ activation is in line with previous studies demonstrating that administration of ERβ into breast cancer or colon cancer cells inhibits cancer cell proliferation and tumor formation in mouse xenograft models [95, 178]. Furthermore, it has been shown that ERβ agonist also

induces apoptosis in prostatic epithelial cells of estrogen-deficient aromatase knockout mice [179]. That lymphoma cells seem to maintain high ERβ expression is a positive feature as targeting ERβ may be a successful approach in the development of a new lymphoma therapy.

In summary, to our knowledge this is the first demonstration in an experimental *in vivo* model of the gender difference in lymphoma development and the influence of estrogens on lymphoma growth. We have shown that ligand-activated ERβ by the selective ERβ agonists can suppress tumor growth and induces apoptosis. Furthermore, it gives a possible explanation to the clinically observed lower incidence and better prognosis of lymphomas in women than men. Finally, the results suggest that ERβ agonists may be useful in the treatment of lymphomas, especially considering that lymphoma cells seems to maintain ERβ expression.

4.2 PAPER II:

Inhibition of lymphoma vascularization and dissemination by estrogen receptor β agonists

In the second paper, we extended our studies to see if the anti-proliferative effect following ERβ activation *in vivo* seen on a murine T cell lymphoma (paper I) also is valid for human B cell lymphomas. For this purpose, we studied two lymphomas, Mantle cell lymphoma (MCL) which is one of the NHL types with the poorest prognosis [139], and Burkitt lymphoma (BL). Both MCL and BL are two of the lymphoma subtypes that show the high male to female incidence ratio [143]. NOD/SCID gamma (NSG) mice were grafted with human MCL cells (Granta-519) or Raji BL cells and were treated with the ERβ agonist DPN. ERβ agonist treatment inhibited tumor growth of both the MCL and BL. In a way to investigate the mechanism by which the ERβ agonist inhibited lymphoma growth, we studied genes in Granta-519 MCL whose expression was regulated following selective ERβ agonist (DPN) treatment and previously have been demonstrated to be important for B cell proliferation and survival, namely BAFF and GRB7 [180, 181]. Both these genes were significantly down-regulated following ERβ agonist treatment *in vivo*. Furthermore, as tumor stimulated vascularization plays an vital role in tumor progression and metastasis [182-184], including for the development and progression of hematologic malignancies [185], we examined if this process was affected by the treatment with the selective ERβ agonists. We demonstrated that ERβ agonist treatment reduced vascularization, both with regard to lymphangiogenesis and angiogenesis as determined by reduced expression of markers for these processes. The

reduced vascularization could be explained by a reduced expression of VEGF-C, previously known to be involved in the process of tumor vascularization [186]. Tumor angio- and lymphangiogenesis is a key process enhancing tumor cell dissemination [187]. We also could show by using a disseminating Raji BL cell line [188] that ERβ agonist treatment reduced dissemination *in vivo*.

Growth of cancers *in vivo* require contact between tumor cells and the cells of the tumor microenvironement, such as immune, endothelial and stromal cells, which provide multiple regulatory signals to cancer cells [189]. Although previous studies have shown expression of ERβ in immune and endothelial cells [36, 190], the effect of ERβ in the cells of the microenviroment on lymphoma growth was not clear. We by using mice deficient in ERβ (BERKO mice) [38] could show that ERβ agonist effects on the tumor microenvironment did not influence the tumor response as lymphomas grafted to wild-type and BERKO mice responded equally well to ERβ agonist treatment. Finally we demonstrated nuclear expression of ERβ protein in biopsy sections from MCL patients.

Despite a significant progress in MCL research, the understanding of MCL pathogenesis is not complete, and considering the poor prognosis, new therapeutic approaches are required [191]. In summary, our results suggest that selective ERβ agonist treatment inhibits lymphoma growth, angiogenesis, lymphangiogenesis and dissemination and may therefore be useful in the treatment of at least a selected number of lymphomas, including MCL and BL.

4.3 PAPER III

Estrogen receptor β1 in diffuse large B-cell lymphoma growth and as a prognostic marker

In our third paper, we focused our studies on Diffuse Large B Cell Lymphoma (DLBCL). DLBCL is the most common form of NHL and usually presents as an aggressive form. DLBCL also shows a higher incidence and poorer prognosis in males vs. females [147, 148]. Despite improved treatment strategies of DLBCL, around 1/3 of the patients do not respond or become resistant to standard therapy [138], which highlights a need for alternative therapies. In various studies, the male sex has been associated to a negative impact on overall survival and tumor progression in DLBCL when treated with current therapy [161, 162]. Furthermore, in several studies it was found that age is a negative prognostic factor for overall survival [192, 193], and in a more selected material it was shown that the effect of age was a negative prognostic factor for men at all ages but only for women 60 years or older (Berglund, unpublished data).

DLBCL consists of two main subtypes, namely activated B cell like (ABC)- and germinal center B cell like (GCB)-DLBCL, where the former one is characterized by high NF-κB expression [194] and the latter one for low NF-κB expression. NF-κB signaling has been shown to be important for proliferation and survival and the ABC-DLBCL usually presents as a more aggressive form with poorer prognosis [194, 195]. In this paper, we show that when grafting the ABC-DLBCL cells (U2932) to male and female mice, U2932 DLBCL tumors grew faster in the males, possibly relating to the gender difference seen in the clinical situation for DLBCL incidence and prognosis. Treating tumors derived from the ABC-DLBCL U2932 cells or the GCB-DLBCL SU.DHL4 with the ERβ selective agonist DPN resulted in both cases in inhibition of tumor growth. Notably, this was despite the major difference in NF-κB signaling between the DLBCL subtypes, demonstrating that ERβ agonists are effective in reducing tumor growth independent of the level of NF-κB activity. Considering that ERβ has characteristics of a tumor repressor, we investigated ERβ1 expression by immunohistochemistry in clinical DLBCL samples and correlated nuclear and cytoplasmic ERβ1 expression to clinical data and overall survival. 130 primary DLBCL cases were analyzed. Importantly, very few (14%) of the DLBCLs do not express nuclear or cytoplasmic ERβ1, indicating that most DLBCLs may be responsive to endocrine therapy by ERβ agonists. It is also interesting that most of the DLBCL retain ERβ1 expression as it has previously been suggested that ERβ expression is commonly lost upon malignant transformation, possibly due to promoter methylation of the ERβ promoter [177, 196]. However, no statistical significant correlation of neither nuclear nor cytoplasmic ERβ1 expression to age, gender, International Prognostic Index (IPI) or DLBCL subtype was observed. Nevertheless, there was a tendency that extra-nodal tumor sites express less nuclear ERβ1 compared to nodal sites. Larger cohorts are most likely necessary to fully establish whether a correlation exists or is lacking between ERβ1 expression and one or several of the clinical parameters. In our IHC studies of primary DLBCL, we observed a variation of ERβ1 distribution between nuclear and cytoplasmic localization. The relative role for nuclear vs. cytoplasmic ERβ1 for responsiveness is still unclear. However, we found that DLBCL's with low nuclear and high cytoplasmic ERβ1 expression correlated to a significant better overall survival as compared to DLBCL's that had high nuclear and low cytoplasmic ERβ1 expression (P=0. 03). Considering ERβ to be primarily a nuclear receptor acting (mainly) through genomic mechanisms, these results are surprising. However, ERβ1 expression is not uncommonly found in the cytoplasm in other cancers [197].

5 CONCLUDING REMARKS AND FUTURE PERSPECTIVES

Estrogens have important physiological functions in numerous tissues of the body, but are also involved in the pathogenesis of many diseases. Estrogen effects are mediated by two well know receptors called ERα and ERβ, respectively. It has been shown in several studies that the two ERs influence cancers like prostate, breast and colon but in opposite direction. While signaling via ERα has pro-proliferative properties in cancers, ERβ has anti-proliferative and pro-apoptotic effects. The purpose of this thesis was to increase our understanding of estrogen effects, the role of the two ER's in lymphomas and to identify molecular mechanisms and prognostic factors related to ERβ signaling and expression. Furthermore, this may give an explanation for the gender difference in incidence seen for most lymphomas. Finally, we wanted to explore the possibility of using ERβ agonists as a new treatment option in lymphomas.

To our knowledge, this is the first thorough study where the impact of estrogens, the role of ERα and ERβ, respectively, and their mechanisms of action have been studied more in depth in experimental lymphoma models, particularly *in vivo.* Based on our findings from both *in vivo* and *in vitro* experimental studies, and ERβ the expression analysis in clinical lymphoma material , it was concluded that

- Both murine T cell lymphoma and human B-cell lymphoma express ERβ.
- Treatment of T cell lymphoma cells (EG7) in culture with ERβ selective agonists inhibited cell proliferation.
- Grafted murine lymphoma (EG7) and human lymphoma (DLBCL) tumor growth is estrogen dependent and grew slower in female vs. male mice, a difference that was abolished following ovarioectomy.
- Treatment of mice carrying allografted murine lymphoma (EG7) or xenografted human lymphoma (BL, DLBCL and MCL) by selective ERβ agonists suppressed tumor growth, due to decreased proliferation and increased apoptosis. Treatment with a selective ERα agonist PPT had no effect on lymphoma growth. Selective ERβ agonist treatment also decreased tumor vascularization (blood and lymphatic vessels) and reduced dissemination.

- Primary lymphoma material (MCL and DLBCL) express ERβ1 protein, which suggests that selective ERβ agonists may be useful in the treatment of ERβ expressing lymphomas.
- Analysis of ERβ expression in lymphoma (DLBCL) may be valuable as a prognostic factor for overall survival.

Based on the results we suggest that selective ERβ agonists will be very useful for therapy of ERβ-expressing lymphomas and may be utilized alone or in combination with currently existing therapies, particularly for lymphomas resistant to present drugs. Furthermore, the use of highly selective ERβ agonists will avoid side effects normally associated with the use of un-selective estrogens. The use of selective ERβ agonists in clinical trials for other disease indications has shown that they are safe. The identification of ERβ expression in primary lymphoma biopsies derived from patients and important target genes regulated by ERβ agonists will also provide a tool to predict ERβ agonist sensitivity, thereby identifying patients which will benefit from ERβ agonist treatment, thus contributing to personalized treatment. However, additional studies are required to fully dissect the molecular mechanisms by which ERβ affects lymphoma growth, particularly signaling pathways and additional target genes involved. This involves e.g. the effect of ERβ signalling on dissemination as well as ERβ-mediated regulation of miRNA as dysregulated miRNA expression is frequently occurring in lymphomas [198]. To keep in mind is that most likely there is no general mechanism by which estrogens via ERβ operate in the different NHL's. Furthermore, as we see weaker responses *in vitro* compared to *in vivo* after ERβ agonist administration, further studies on the impact of tumor microenviroment on ERβ agonist responses are worth to explore.

In addition, other cohorts of clinical lymphoma material will have to be studied to further evaluate nuclear and cytoplasmic ERβ expression and its correlation to clinical parameters, especially considering the unexpected results showing a correlation between high cytoplasmic and low nuclear ERβ1 expression in R-CHOP treated DLBCL to good prognosis. The ERβ expression in lymphoma material can also be correlated to the expression of additional markers previously shown to be valuable tools for lymphoma characterization and prognosis. Finally, the role of ERβ splice variants in the process of lymphoma growth and prognosis should not be forgotten.

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