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Elucidating the role of 17β hydroxysteroid dehydrogenase type 14 in normal physiology and in breast cancer

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ABSTRACT

Oestrogens play key roles in the development of the majority of breast tumours, a fact that has been exploited successfully in treating breast cancer with tamoxifen, which is a selective oestrogen receptor modulator. In post-menopausal women, oestrogens are synthesised in peripheral hormone-target tissues from adrenally derived precursors. Important in the peripheral fine-tuning of sex hormone levels are the 17β hydroxysteroid dehydrogenases (17βHSDs). These enzymes catalyse the oxidation/reduction of carbon 17β of androgens and oestrogens. Upon receptor binding, the 17β-hydroxy conformation of androgens and oestrogens (testosterone and oestradiol) triggers a greater biological response than the corresponding keto-conformation of the steroids (androstenedione and oestrone), and the 17βHSD enzymes are therefore important mediators in pre-receptor regulation of sex hormone action.

Breast tumours differ substantially with regards to molecular and/or biochemical signatures and thus clinical courses and response to treatment. Predictive factors, which aim to foretell the response of a patient to a specific therapeutic intervention, are therefore important tools for individualisation of breast cancer therapy. This thesis focuses on 17βHSD14, which is one such proposed marker, aiming to learn more of properties of the enzyme in breast cancer as well as in normal physiology. We found that high 17βHSD14 levels were correlated with clinical outcome in two separate subsets of breast tumour materials from trials evaluating adjuvant tamoxifen therapy. Striving to understand the underlying mechanisms, immunohistochemical 17βHSD14 expression patterns were analysed in a large number of human tissues using an in-house generated and validated antibody. The 17βHSD14 protein was expressed in several classical steroidogenic tissues such as breast, ovary and testis which supports idea of 17βHSD14 being an actor in sex steroid interconversion. Furthermore, using a radio-high pressure liquid chromatography method, cultured cells transiently expressing *HSD17B14* were found to oxidise both oestradiol and testosterone to their less potent metabolites oestrone and androstenedione respectively. The evaluation of a mouse model lacking *Hsd17b14* revealed a phenotype with impaired mammary gland branching and hepatic vacuolisation which could further suggest a role for 17βHSD14 in oestrogen regulation.

Although other mechanisms of the enzyme cannot be ruled out, we suggest that 17βHSD14 relevance in tamoxifen-treated breast cancer is related to oestradiol-lowering properties of the enzyme which potentiate the anti-proliferative effects of tamoxifen. Translating into the

clinical setting, patients with oestrogen receptor positive tumours expressing low levels of oestradiol-oxidising enzymes such as 17βHSD14 would likely receive more clinical benefit from alternative treatments to tamoxifen such as aromatase inhibitors or in the future possibly inhibitors of reductive 17βHSD enzymes.

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SUMMARY IN SWEDISH/SAMMANFATTNING PÅ SVENSKA

Det kvinnliga könshormonet östrogen spelar en nyckelroll i utvecklingen av en stor andel bröstcancer, och detta kan utnyttjas i behandlingssyfte. Tamoxifen är ett läkemedel som kompetetivt binder till östrogenreceptormolekylen och därmed förhindrar östrogenet från att stimulera tumörutveckling. Denna verkningsmekanism har gjort att tamoxifen har blivit en viktig och framgångrik behandlingsmetod mot bröstcancer. Hos post-menopausala kvinnor, vars ovarier slutat producera östrogener, syntetiseras östrogen från prekursormolekyler som utsödras från binjurarna. 17β hydroxysteroid dehydrogenaser (17βHSDs) är en familj enzymer som genom kemisk oxidation eller reduktion kan påverka potensen hos östrogener. Reducerat östrogen (östradiol) genererar ett starkare biologiskt svar vid inbinding till östrogenreceptormolekylen än motsvarande oxiderade östrogen (östron), och 17βHSD-enzymerna utgör därför en viktig mekanism i regleringen och finjustering av hormoners effekt.

De inbördes olikheterna mellan olika tumörer kan vara väldigt stora, och det är därför viktigt att hitta så kallade prediktiva markörer som tidigt kan ge information om vilka patienter som mest gynnas av olika typer av behandling. En sådan prediktiv markör som föreslagits är enzymet 17βHSD14. Syftet med denna avhandling är att studera 17βHSD14s egenskaper och roll i normalfysiologi såväl som i brösttumörer. Vi fann att patienter med tumörer som uttryckte höga nivåer av 17βHSD14 hade förbättrad överlevnad och svarade bättre på tamoxifen. För att försöka förstå de underliggande mekanismerna undersökte vi uttrycksmönster av 17βHSD14 i ett stort antal friska humana vävnader och fann att enzymet uttrycktes i klassiskt sett hormondrivna vävnader såsom bröst, äggstock och testikel vilket stödjer teorin om att proteinet har betydelse för hormonreglering. Vidare utvecklade vi en metod för att kunna undersöka östrogen- och androgenmetabolism i odlade celler i vilka vi överuttryckte genen som kodar för 17βHSD14. Vi fann att dessa celler signifikant oxiderade östradiol till den mindre potenta metaboliten östron. Utvärdering av en mus som genetiskt modifierats för att inte uttrycka musvarianten av 17βHSD14 visade tecken såsom försämrad förgrening av bröstkörtelstrukturer och blåsbildning i levern, vilket även det kan tyda på störd östrogenmetabolism. Även om man inte kan utesluta att 17βHSD14 verkar på andra substrat och verkar inom andra metabola system, så föreslår vi att relevansen av 17βHSD14 i tamoxifen-behandlad bröstcancer är relaterad till effekter som leder till lägre nivåer av potent

östrogen i bröstet och tumören, vilket därmed ökar effekten av tamoxifen. Som prediktiv faktor skulle därför 17βHSD14 nivåer i tumören kunna avgöra huruvida patienter gynnas av en behandling såsom tamoxifen eller om andra behandlingar, till exempel läkemedel som hämmar tidigare steg i östrogenmetabolismen skulle vara med effektiva.

LIST OF ORIGINAL PAPERS

This thesis is based on the following papers, which will be referred to in the text by their Roman numerals (I-IV)

PAPER I

Jansson AK, Gunnarsson C, Cohen M, Sivik T, Stål O

17beta-hydroxysteroid dehydrogenase 14 affects estradiol levels in breast cancer cells and is a prognostic marker in estrogen receptor-positive breast cancer

Cancer research 2006; 66:1147-7

PAPER II

Sivik T, Vikingsson S, Gréen H, Jansson A

Expression patterns of 17β hydroxysteroid dehydrogenase 14 in human tissues

Hormone and Metabolic Research, 2012

PAPER III

Sivik, T, Gunnarsson C, Fornander T, Nordenskjöld B, Skoog L, Stål O, Jansson A

17β-hydroxysteroid dehydrogenase type 14 is a predictive marker for tamoxifen response in oestrogen receptor positive breast cancer

PLoS ONE 2012; 7(7):e40568

PAPER IV

Sivik T, Hakkarainen J, Hilborn E, Fernandez-Martinez H, Zhang F, Poutanen M, Jansson A

Characterisation of *Hsd17b14* knockout mice

Manuscript

ABBREVIATIONS

17bHsd	17beta hydroxysteroid dehydrogenase (mouse)
17βHSD	17beta hydroxysteroid dehydrogenase (human)
ACTH	Adrenocorticotropic hormone
A-diol	Androstenediol ((3β,17β)-Androst-5-ene-3,17-diol)
A-dione	Androstenedione (androst-4-ene-3,17-dione)
ArKO	Aromatase knockout
CYP11A1	Cytochrome P450 cholesterol side chain cleavage enzyme
DHEA	Dehydroepiandrosterone ((3β)-3-Hydroxyandrost-5-en-17-one)
DHT	Dihydrotestosterone ((5α,17β)-17-Hydroxyandrostan-3-one)
E1	Oestrone (3-hydroxyestra-1,3,5(10)-trien-17-one)
E2	Oestradiol (17β)-Estra-1,3,5(10)-triene-3,17-diol)
ER	Oestrogen receptor
FCA	Freunds complete adjuvant
HER2	Human epidermal growth factor receptor 2
HPLC	High pressure liquid chromatography
KLH	Keyhole limpet hemocyanin
LH	Luteinising hormone
MIQE	Minimum Information for Publication of Quantitative Real-Time PCR Experiments
NAD	Nicotinamide adenine dinucleotide
PR	Progesterone receptor
RIN	RNA integrity number
RT RT-PCR	Real-time reverse transcription polymerase chain reaction
SDR	Short chain dehydrogenase reductase
StAR	Steroidogenic acute regulatory protein
T	Testosterone ((17β)-17-Hydroxyandrost-4-en-3-one)
αERKO	Oestrogen receptor alpha knockout
αβERKO	Oestrogen receptor alpha and beta knockout
βERKO	Oestrogen receptor beta knockout

BACKGROUND

Importance

Rather than being a single entity, breast cancer comprises a large number of heterogeneous neoplasms, each with substantially different molecular and/or biochemical signatures and therefore clinical courses and response to treatment. In addition to the numerous differences detected between tumours, cancer cells within one tumour of an individual patient may display remarkable heterogeneity. This multi-level diversity poses a great challenge to modern medicine, and has directed breast cancer care and therapy towards a more individual-based focus. Prognostic and predictive markers are important tools for this desired individualisation of breast cancer therapy. The aim of the research presented in this thesis was to investigate the role of one such proposed marker, 17β hydroxysteroid dehydrogenase type 14 (17βHSD14), which in initial studies analysing tumour tissue, was shown to correlate with clinical outcome in breast cancer. The elucidation of the role of 17βHSD14 in normal physiology enables mechanisms of this enzyme in breast cancer to be better understood. Enhanced knowledge about mechanisms associated with breast cancer development is a prerequisite for improving diagnostic and predictive tools.

Breast cancer

Definition

A breast carcinoma is a malignant tumour emanating from epithelial cells of glandular milk ducts or lobuli of the breast, defined as either non-invasive (carcinoma *in situ*), or invasive, depending on whether or not the transformed epithelial cells forming the duct/lobule have breached through the basal membrane upon which they rest. Invasive breast cancers are cancers which have spread to surrounding connective tissue and have the propensity to metastasise to other parts of the body. Diagnosis of breast cancer is based on so called triple diagnostics including clinical examination, mammographic screening and biopsies [1].

Epidemiology

Breast cancer is the most commonly diagnosed tumour disease among women of all nationalities [2]. In Sweden the disease accounts for 30% of all female cancer diagnoses, with nearly 8000 cases being reported in 2010 [3]. The breast cancer incidence worldwide is steadily increasing. As the disease is most common among women aged 50 years or older, an

ageing population is likely a major cause of this development, however factors such as improved diagnostics are also thought to contribute. There are considerable geographical differences in breast cancer incidence. Disease rates vary from 19.3 per 100,000 women in Eastern Africa to 89.9 per 100,000 women in Western Europe. Yet, as incidence rates grow more in less affluent countries than in western countries, this gap is decreasing [2]. In terms of mortality, breast cancer is among the most frequent causes of cancer death in women; both in developed and developing countries, but survival rates vary greatly. As an example, the age standardised relative 5-year survival ranges from over 80% in Sweden to less than 49% in Algeria [2, 4] (Fig. 1).

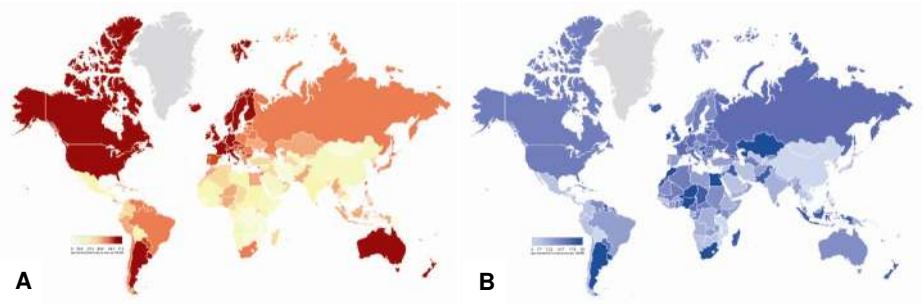

***Figure 1**. Breast cancer incidence (A) and mortality (B) worldwide. Modified from Globocan 2008, International Agency for Research on Cancer [5].*

Treatment

Surgery is the first line treatment of breast cancer. Most commonly, breast-conserving surgery is performed, typically followed by local radiation therapy, and for a large group of breast cancer patients this treatment is curative [6]. Cases where removal of the entire breast (mastectomy) is needed have decreased due to mammographic screening programmes which has led to tumours being discovered at an earlier stage [1]. In order to prevent potential undetected micrometastases from developing into clinical recurrence, most patients receive adjuvant treatment after surgery. Adjuvant treatments include chemotherapy, hormonal treatment such as tamoxifen and aromatase inhibitors (AIs) and more recently targeted treatments such as those directed at human epidermal growth factor receptor 2 (HER2) [7-10].

Prognostic and predictive markers

Prognostic markers aim to foresee the natural course of the disease regardless of treatment, whereas predictive markers intend to foretell the response of a patient to a specific therapeutic intervention. Prognostic and predictive factors fill an important role in breast cancer care as they enable treating clinicians to discriminate between patients who likely will be cured after primary surgery and still suffer from toxic side effects of the, for them unnecessary adjuvant treatment, and patients for whom the side effects can be outweighed by the benefit from treatment. The most established prognostic marker is the TNM (Tumour, Node, Metastasis) - classification which gives information regarding tumour size, lymph node involvement and the presence of distant metastasis. Other prognostic factors used clinically include histological grade, which relates to the presence and appearance of tubules, degree of nuclear atypia and mitotic activity [11]. Predictive markers used clinically include oestrogen receptor (ER) and progesterone receptor (PR), the presence of which are the basis for endocrine therapy. HER2, which is also a prognostic marker, needs to be over-expressed/amplified in order for certain targeted therapy directed at this receptor to be administered, and is thus a predictive factor [1]. In addition to established markers, a large number of prognostic and predictive markers have been proposed, most of which fail to reach sufficient evidence levels to be brought into the clinics. Nevertheless, these proposed markers have provided valuable insight into the biology of breast cancer [12].

Molecular subtypes of breast cancer

Breast cancer gene expression profiling can give additional prognostic and predictive information to the sometimes crude prognostic/predictive tools used today. In the original study, Perou *et al*, [13] analysed cDNA microarrays representing 8,102 human genes from 42 individuals including 36 infiltrating ductal carcinomas, 2 lobular carcinomas, 1 ductal carcinoma *in situ*, 1 fibroadenoma and 3 normal breast samples. Using hierarchical clustering, 3 major molecular breast cancer subgroups of tumours were identified. The grouping has since been confirmed and refined, [14, 15] and presently includes one normal-like group, two different epithelial luminal groups (A and B), one basal like group, and a HER2-enriched group. Both the luminal A and B profiles describe tumours stemming from luminal epithelial cells of breast ducts. Luminal A tumours which constitute most breast cancers tend to be ER and PR positive and have a low expression of the proliferative marker Ki67, whereas luminal B tumours usually are either ER and/or PR positive and have a higher expression of Ki67 and sometimes express *HER2*. The HER2-subtype is ER/PR negative but expresses *HER2*. The

basal-like group of tumours, which is thought to originate from cells of the basal layer of breast ducts, expresses neither ER/PR nor *HER2*. The prognostic power of the this subclassification was shown by performing survival analysis on a group of patients with locally advanced breast cancer uniformly treated in a prospective study which showed that patients belonging to the various subgroups had significantly different outcomes, including a poor prognosis for patients with basal-like tumours, and a significant difference in outcome for patients in the two luminal groups [14].

Oestradiol disposition

Oestrogens play key roles in the development and maintenance not only of sexual and reproductive organs, but also in a large number of extra-reproductive tissues and biological systems such as the immune system, the circulation and the central nervous system [16]. The most potent endogenous oestrogen is 17β-oestradiol (E2). Two metabolites of E2, oestrone (E1) and oestriol bind to the ER with high affinity but are less potent agonists [17]. The connection between the ovaries, which are the primary site for oestradiol synthesis in pre-menopausal women, and the development of breast cancer, was first suggested by Thomas William Nunn in the mid 1800s, several years before the discovery of oestrogens. Surgical removal of the ovaries as a treatment for breast cancer was pioneered by Albert Schinzinger, and later followed George Thomas Beatson who published his results from several oophorectomy cases in the Lancet in 1896 [18]. Factors relating to the life-time exposure to oestrogen such as early menarche and late menopause are linked to breast cancer risk [19]. Furthermore, epidemiological studies provide strong evidence for an influence of plasma oestrogen levels on the risk of breast cancer in postmenopausal women [20].

Principles of estrogen action

The classical mechanism of direct action of oestrogen involves the binding of the hormone to ERs which then translocate to the nucleus and bind as dimers to oestrogen response elements in the regulatory regions of oestrogen responsive genes. The bound receptor will associate with basal transcription factors, co-activators and co-repressors to alter gene expression [21, 22]. In addition to the classical relatively slower genomic signalling mechanism, ERs have also been implicated in rapid non-genomic actions. Rapid ER-signalling is usually initiated by the binding of E2 to ERs located in the cellular plasma membrane which results in the

activation of signal transduction through e.g. calcium flux and kinase cascades [23]. The first ER, ERα, was discovered in the early 1960s by Jensen and Jacobsen [24]. The discovery of an additional ER, the ERβ in 1996 [25, 26], has added to the complexity of ER-signalling. ERα and ERβ are paralogous proteins arisen evolutionary through gene duplication. Although they are largely identical in the DNA-binding domains, ERα and ERβ share only 59% homology in the ligand binding domains, and they have major differences in domains responsible for the binding of co-activators/co-repressors [26, 27]. Thus, the end-result of ligand binding, whether E2 or other natural or synthetic ligands, will significantly differ between the two receptors [28, 29]. ERα is highly expressed in hormone sensitive tissues such as uterus and breast where oestrogen is an important regulator of proliferation and survival, whereas ERβ, which displays a more widespread tissue distribution, in some cases opposes the action of ERα [21, 27, 30-34]

Targeting oestrogen action in breast cancer

The perhaps strongest evidence for the role of oestrogen in breast cancer comes from the successful experiences from treatments with the selective oestrogen receptor modulator tamoxifen and the oestrogen metabolism modulators AIs. Tamoxifen was in 1967 launched as a morning-after contraceptive pill, however it was soon tried as a therapeutic compound for the treatment of breast cancer [35]. Gathered information of clinical trials performed since provide evidence that adjuvant tamoxifen is beneficial for patients with both invasive and *in situ* tumours expressing ERα, with reductions after 5 years of therapy in both breast cancer recurrence and mortality of approximately 50% and 30% respectively [36-38]. The effect of tamoxifen in patients with tumours not expressing ER is minimal, and the drug is in those cases therefore not offered. AIs target the oestrogen conversion from androgens, which is the only pathway for oestrogen supply in women after menopause. Randomised clinical trials comparing 5 years of AI versus tamoxifen, as well as trials where patients have been assigned to two or three years of tamoxifen and then an additional two or three years of tamoxifen or AIs report superior clinical benefit in terms of reduced recurrence in favour of AIs. In terms of over-all survival, there is however no major difference between the two therapies [8]. The different modes of action between tamoxifen and AIs are reflected by significant differences in adverse effects. Whereas tamoxifen use is associated with endometrial cancer and

thromboembolic events, AI use is rather associated with increased risk of bone fracture and joint pain [39].

Endocrine resistance

Despite the efficacy of hormonal treatment in reducing the number of recurrences, about one third of patients will eventually develop a relapse and are considered resistant to the therapy [37]. This resistance can be either developed against the particular treatment drug, or a complete hormonal resistance in which the tumours are no longer reliant on hormones for growth and proliferation. Patients relapsing on e.g. tamoxifen can still potentially benefit from other endocrine treatments such as AIs (or vice versa), or treatments aimed at non-endocrine targets [40].

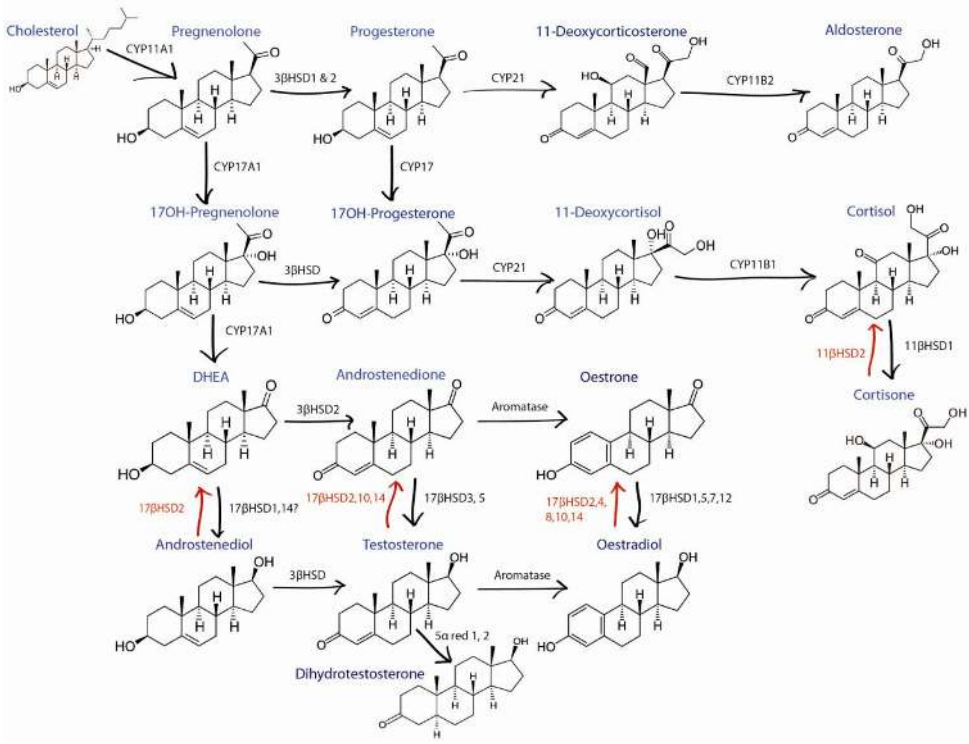

Figure 2. *Steroid biosynthesis from cholesterol. DHEA, dehydroepiandrosterone; 5α red, 5α reductase.*

Pathways of estrogen biosynthesis

Initial steps of steroidogenesis

Cholesterol is the precursor of all bioactive sex hormones. The primary tissues expressing the main enzymes necessary for steroid biosynthesis from cholesterol include the adrenal glands, testes in males and ovaries and placenta in females. Steroid synthesis is initiated by the pituitary hormones adrenocorticotropic hormone (ACTH) in adrenal cells and luteinising hormone (LH) in testicular Leydig and ovarian cells. As a result, cholesterol, which in these cells is stored in the outer mitochondrial membrane, is transferred by the steroidogenic acute regulatory protein (StAR) to the inner mitochondrial membrane. There the cholesterol molecule comes into contact with the cytochrome P450 cholesterol side chain cleavage enzyme (CYP11A1) which catalyses the conversion of cholesterol to pregnenolone [41]. The fate of the pregnenolone molecule (summarised in Fig. 2) is dependent on tissue specific expression of downstream steroid-converting enzymes. In the zona reticularis of the adrenals, as well as in the gonads, the enzyme CYP17A1 catalyses the conversion of pregnenolone to dehydroepiandrosterone (DHEA) in a two-step reaction with 17-hydropxypregnenolone as an intermediary metabolite. DHEA, which is the general precursor of all androgens and estrogens, is converted to androstenedione (A-dione) by 3β hydroxysteroid dehydrogenase type 2 (3βHSD2). 3βHSD2 is also responsible for the conversion of pregnenolone to progesterone in the corpus luteum of the ovaries. [42]. In premenopausal women, A-dione produced in ovarian follicular theca cells is converted into E1 by aromatase present in the granulosa cells. These cells also express 17βHSD1 which converts E1 into the potent oestrogen metabolite E2 [43, 44].

Intracrinology

In the conventional concept of endocrinology, biologically active sex hormones are synthesised by endocrine organs such as the adrenal glands or the gonads. The hormones are then released into the circulatory system where they eventually diffuse through plasma membranes of cells throughout the body and bind wherever their designated receptors are being expressed. The concept of *intracrinology* deals with the enzymatic systems responsible for synthesis of bioactive sex hormones in tissues that have not classically been considered to be hormone producing, and where hormone synthesis occurs without significant release into the circulatory system [45]. The intracrine steroid biosynthesis (illustrated in Fig. 3) is not *de novo* steroid synthesis, but starts with adrenally derived DHEA which diffuses into target tissues where expression of relevant enzymes converts the DHEA to bioactive sex steroids

such as E2 and dihydrostestosterone (DHT). In post-menopausal women, for whom the ovaries have ceased to produce oestrogen, oestrogens are synthesised in peripheral hormone-target tissues from adrenally derived precursors [46]. The intracrine machinery involves enzymes such as aromatase, which converts A-dione and testosterone (T) to E1 and E2 respectively, oestrogen sulfatases and sulfotransferases, which through their action regulate tissular concentrations of inactive versus active oestrogens, and 17βHSD enzymes, which regulate the pool of more and less active androgenic and oestrogenic metabolites.

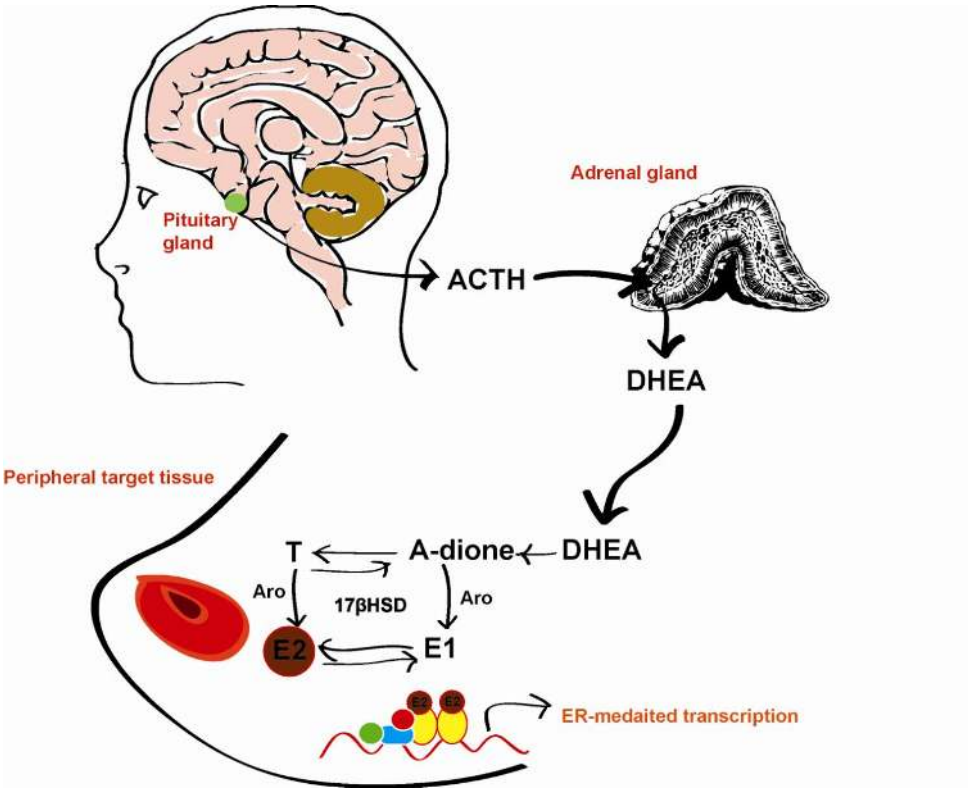

Figure 3. *Schematic representation of intracrine sources of oestrogens. ACTH, adrenocorticotropic hormone; DHEA, dehydroepiandrosterone, A-dione, androstenedione; T, testosterone; E1, oestrone; E2, oestradiol; ER, oestrogen receptor; Aro, aromatase; 17βHSD, 17β hydroxysteroid dehydrogenase.*

17β Hydroxysteroid dehydrogenases

The short-chain dehydrogenase/reductase family

Using the reduced or oxidised forms of nicotinamide adenine dinucleotide (NAD) as hydrogen donors or acceptors, 17βHSD enzymes catalyse the stereospecific oxidation/reduction at carbon 17β of androgens and oestrogens [47]. Upon receptor binding, the 17β-hydroxy conformation of androgens and oestrogens triggers a greater biological response than the corresponding keto-conformation of the steroids [17]. The 17βHSD enzymes are thus important mediators in pre-receptor regulation of sex hormone action. Selective expression in peripheral tissue of enzymes that catalyse this on-off switch is essential for the regulation of hormonal homeostasis. To date, 15 members of the 17βHSD enzyme family have been described (Table 1). All but 17βHSD type 5, which is an aldo-keto reductase, belong to the short chain dehydrogenase reductase (SDR)-family [48, 49]. The SDR-family is a large category of enzymes which show only 20-30% sequence identity. Common to all SDRs is the presence of three structural elements; the Rossman fold, which is composed of central parallel beta sheets flanked by three to four alpha helices creating a co-factor binding site, an active site structure composed of three or four specific amino acids residues, and structures associated with substrate binding. The substrate binding motifs vary considerably between individual SDR enzymes, which explains the large variability in substrate specificity of the SDRs in general, and the 17βHSDs in particular [50]. While the major substrates of 17βHSD enzymes are sex steroids [51-56], a few are believed to be dedicated primarily to other substrates, such as fatty acids [57, 58], cholesterols [59] bile acids [60] or retinoids [61].

Purified 17βHSDs can work essentially as both reducing and oxidising agents depending on cofactor concentrations and pH. However, *in vivo* they tend to be unidirectional, and according to experimental data the 17βHSD enzymes are grouped as either oxidative or reductive enzymes based on the preferred co-factor utilisation under biological conditions. From this classification, human 17βHSD types 2, 4, 6, 8, 10, 11 and 14 are considered *in-vivo* oxidative enzymes catalysing the NAD+-dependent inactivation of oestrogens/androgens whereas types 1, 3, 5, 7, 12 and 15 catalyse the NADH-dependent reduction, and hence activation, of oestrogens and androgens [48, 49].

Table 1. *17β hydroxysteroid dehydrogenases and their primary functions*

Type	gene	Ref	
1	*HSD17B1*	E1, DHEA reduction,	[62, 63]
2	*HSD17B2*	E2, T, A-diol, 20α progesterone oxidation	[54, 64]
3	*HSD17B3*	A-dione reduction	[52]
4	*HSD17B4*	Fatty acid β-oxidation, E2 oxidation	[65, 66]
5	*AKR1C3*	A-dione, E1, progesterone and prostaglandin reduction	[67-69]
6	*HSD17B6*	Androgen oxidation	[70]
7	*HSD17B7*	Cholesterol biosynthesis, E1 reduction	[71, 72]
8	*HSD17B8*	E, T and DHT oxidation	[73]
9	*Hsd17b9*	Retinol and E2 oxidation (mouse)	[74]
10	*HSD17B10*	E2, T and progesterone oxidation, fatty acid and bile acid metabolism	[60]
11	*HSD17B11*	3α androstanediol to androsterone oxidation	[51, 75]
12	*HSD17B12*	Fatty acid elongation, E1 reduction	[58, 76]
13	*HSD17B13*	Not demonstrated	
14	*HSD17B14*	E2 and T oxidation	[56][paper II]
15	*HSD17B15*	Androgen biosynthesis	[49]

E1, oestrone; E2, oestradiol; DHEA, dehydroepiandrosterone; A-dione, androstenedione, A-diol, androstenediol; DHT, dihydrotestosterone

Evolution of 17βHSD enzymes

Hormonal signalling is, from an evolutionary point of view, a relavtively recent event. The earliest evidence for ER and AR are in sharks, and most likely, 17βHSD-activity arose at a similar time, from several ancestral enzymes that did not have 17βHSD-activity [77]. Both gene duplication with subsequent divergence and convergent evolution has been important in 17βHSD-activities. 17βHSD2 and 17βHSD3 are two examples of gene duplication. Whereas 17βHSD2 arose from an ancestral vertebrate retinoid oxido-reductase, 17βHSD3 is believed to stem from an ancestral dehydrogenase in invertebrates. In both cases, divergent evolution created 17βHSD-activity. For the larger part of the 17βHSD family, selective pressures led to convergent evolution of the 17βHSD activity from different ancestral oxido-reductases. 17βHSD4 and 10 are two examples of convergent evolution from ancestors that metabolised fatty acids [77].

17βHSD enzymes in breast cancer

In malignant breast tissue, the tissue/plasma ratio of E2 is elevated compared with benign breast tissue [78, 79]. As the main androgenic substrate in the circulation is A-dione which is aromatised to E1 either in e.g. peripheral fat tissue or in the breast or tumour itself, this pinpoints the importance of reductive 17βHSD enzymes for the generation of potent E2 in breast cancer. Indeed, several 17βHSDs have been implicated in breast cancer. Tumoural expression of 17βHSD1, which catalyses the reduction of E1 to E2, and also the reduction of DHEA to androstenediol (A-diol), has been associated with poor clinical outcome in breast cancer [80-82]. 17βHSD2, which was the second 17βHSD enzyme to be described and cloned, efficiently oxidises E2 to E1 and thereby balances the action of 17βHSD1. When low or absent, 17βHSD2 is associated with poor clinical outcome in ER positive breast cancer [80, 81, 83]. The ratio of 17βHSD1/17βHSD2 is an even stronger predictor, both of disease outcome and tamoxifen benefit, in breast cancer [81, 84]. 17βHSD5, which has been studied primarily in prostate cancer, reduces not only E1 to E2 but also A-dione to T as well as progesterone and prostaglandins. The 17βHSD5 enzyme is expressed in both benign and malignant breast tissue [85, 86], and tumour expression of this enzyme has in one study been associated with poor clinical outcome in breast cancer [87]. In a recent study, intratumour E2 levels were found to be negatively correlated with 17βHSD2 but positively correlated with 17βHSD7, an enzyme implicated in E1 to E2 reduction [88]. Another 17βHSD enzyme with possible implications in breast cancer is 17βHSD12. Whereas immunoreactivity in breast tumours for this enzyme has been found to predict of poor prognosis [89], it is unclear whether or not the suggested E1 reductional properties are responsible for this as conflicting data regarding catalytical properties of 17βHSD12 have been published [76, 90].

17βHSD enzymes as therapeutic targets for breast cancer

Their involvement in endocrine cancer makes the 17βHSD's interesting as targets for therapeutic intervention. Since 17βHSDs catalyse the final steps of steroid hormone biosynthesis, selective inhibition should result in fewer side effects compared with inhibition of preceding steps such the aromatisation of androgens. However, as many of the 17βHSDs in addition to catalysing the final steps of biosynthesis also perform other enzymatic reactions, which would make them unsuitable as drug targets, relatively few of them have entered pre-clinical testing. The main enzyme being evaluated in breast cancer is 17βHSD1. In addition to a demonstrated role in the disease, this enzyme has a relatively strict substrate specificity and

expression profile. Indeed, 17βHSD1-inhibitors have shown promising results in reducing tumour load in mouse xenograft models [90, 91].

17β Hydroxysteroid dehydrogenase type 14

The gene encoding 17βHSD14, first called retSDR3, was cloned from a retinal epithelium cDNA-library in 2000. Based on sequence analysis, the protein was determined to be an SDR [92]. Enzymatic properties of retSDR3 were assessed by screening the recombinant protein expressed in insect cells against steroid and retinoid substrates; however, no enzymatic activity was detected for the enzyme [92]. Some years later, retSDR3 was re-evaluated by Lukacik and colleagues [56]. The crystal structure of the enzyme was solved (Fig. 4), which supported the enzyme being an SDR. Functional studies showed that the enzyme converted NAD+ to NADH in the presence of E2, T and A-diol. Oxidative 17βHSD- activity was also shown *in vivo* for E2 in cells transfected with the *HSD17B14* cDNA. With structural and functional studies revealing features of the protein equivalent to those of 17βHSDs, the retSDR3 was renamed 17βHSD14 [56].

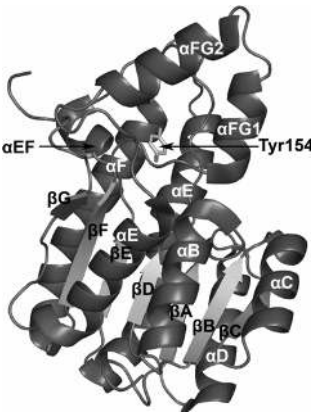

Figure 4. *Secondary structure of 17βHSD14, adapted from Lukacik et al. [56]*

Lessons from mouse models

17βHSD1/2 knockout and transgenic models

A number of mouse models of 17βHSD proteins have been generated, several of them establishing or revealing non-classical functions of the enzymes. Transgenic models of both *HSD17B1* and *HSD17B2* display unexpected phenotypes with regards to hormonal profiles.

As an example, despite the fact that the efficacy of the A-dione to T conversion catalysed by 17βHSD1 is only a fraction of that of E1 to E2 conversion [62], female mice over-expressing *HSD17B1* present with masculinisation caused by elevated T levels [93, 94]. Male mice over-expressing *HSD17B2* present with a phenotype suggestive of impaired retinoid metabolism, which can be rescued by the addition of a retinoic acid receptor agonist [95]. Female mice over-expressing *HSD17B2* have unaltered E2 levels but a delayed puberty, abnormal oestrous cycling and elevated progesterone, prolactin and LH likely being the cause of the mammary gland hyperplasia seen in these animals [96]. Mice deficient in *Hsd17b2* are embryonically lethal due to placental defects that cannot be rescued by anti-oestrogens, suggesting that at least in mice, *Hsd17b2* operates in steroid-independent pathways [97].

Other 17βHSD mouse models

The 17βHSD4 enzyme, also called multifunctional protein-2, catalyses E2 oxidation but is believed to play its primary role in fatty acid metabolism. Male *Hsd17b4* knockout (KO) mice accumulate fatty acids in Sertoli cells of the testis which could explain their infertility [98, 99]. While female *Hsd17b4* KO mice are fertile both female and male mice show CNS lesions and motor deficits with growth retardation and death before 6 months [99, 100]. Rodent as well as human *HSD17B7* catalyse E1 to E2 reduction [71], but the enzyme has also been found to be involved in cholesterol biosynthesis [72]. The relevance of cholesterol biosynthesis for 17βHSD7 is evident from a mouse model lacking *Hsd17b7* [101]. This model, which is embryonical lethal, accumulates early cholesterol intermediates, whereas late intermediates such as cholesterol are reduced. A mouse model has also shed light on the biological role of 17βHSD12. Stem cells of homozygous *Hsd17b12* KO mice, which die *in uteru* and display severe developmental defects, show significantly lower aracidonic acid concentrations compared with wild type cells, suggesting that fatty acid synthesis is a primary role of the 17βHSD12 [102].

ER and aromatase knockout models

Both male and female ERα and ERβ knockout mice (αERKO and βERKO respectively) develop normally and are grossly identical to their wild type littermates; however these animals have more or less severe abnormalities in reproductive tissues. Female αERKO display hypoplastic uteri and hyperemic ovaries which render them infertile whereas βERKO female mice are subfertile due to reduced ovarian function [103]. αERKO males have markedly reduced or no fertility due to abnormalities in testis development [103-106]. Young

male βERKO mice have no reproductive pathological phenotype and are fertile; however older βERKO mice develop bladder and prostate hyperplasia [106]. Double ER knockout models (αβERKO) of both sexes survive to adulthood and exhibit no marked outer abnormalities, however both male and female αβERKO are infertile [107]. Specifically, in adult female αβERKO ovaries, follicular structures rather resemble seminiferous tubules of the testes, suggesting a sex-reversal in the ovaries of these female mice [108]. Aromatase deficient (ArKO) female mice display abnormal development of reproductive organs, yet different from that seen in the αERKO. Whereas the αERKO females display hyperemic ovaries with few granulose cells, the ArKO females have hyperplastic ovaries with numerous granulosa cells [109]. Both female and male ArKO mice have specific phenotypes in bone, (e.g. excessive long bone growth and osteopaenia), and adipose tissue (e.g. increased serum lipids and intra-abdominal fat) [110, 111].

HYPOTHESIS AND AIMS OF THE THESIS

General aim:

The overall aim of this thesis work was to study the biological role and relevance of the 17βHSD14 enzyme. By evaluating expression and function of this enzyme in normal physiology, hypotheses can be generated on its role in breast cancer.

Specific aims:

- To investigate expression levels and clinical relevance of 17βHSD14 protein and *HSD17B14* mRNA expression levels in primary breast tumours
- To assess expression levels and patterns of the 17βHSD14 protein in normal human tissues
- To investigate oxidative/reductive enzymatic activities of 17βHSD14
- To study the phenotype of the *Hsd17b14* KO mouse

MATERIAL AND METHODOLOGICAL CONSIDERATIONS

Study populations

For the 17βHSD14 project we analysed two subsets of breast tumour material, each derived from randomised clinical trials evaluating tamoxifen benefit. The trial protocols were approved by the Research Ethics Committee of the Karolinska Institute. Retrospective tumour analysis was approved by the Research Ethics Committee of the Karolinska Institute (dnr 97-451, with amendments) and the Research Ethics Committee of Linköping University (dnr 02-314).

Paper I

For paper I, tumour material from post-menopausal patients enrolled in a clinical trial evaluating the optimal duration of adjuvant tamoxifen [112] was analysed. Patients were 75 years or less and recruited from five Swedish health care regions. A total of 3887 patients were recruited during the years 1983 to 1991. Study participants entering into the study were allocated to a daily dose of tamoxifen (20 or 40 mg). Patients who were alive and recurrence-free after two years (91%) were randomised to an additional three years of tamoxifen or no further systemic endocrine treatment, enabling comparison of adjuvant tamoxifen treatment for 2 or 5 years. Paper I covers analysis of tumours from patients in the South-East health care region. At the time of trial inclusion, these women had stage II to IIIa breast cancer (tumours larger than two cm and no lymph node involvement or patients with tumours equal to or larger than two cm with positive regional lymph nodes), rendering them “high risk” patients. Patients had modified radical mastectomy or breast-conserving surgery in combination with axillary lymph node dissection. Radiotherapy to the breast and chest wall was offered to all patients with breast-conserving surgery and positive lymph nodes. For paper I, data on tumoural expression of *HSD17B14, HSD17B5* and *HSD17B12* was obtained from 131 patients. The median follow-up period was 13.5 years.

Paper III

Tumour material analysed in paper III was derived from a randomised tamoxifen trial conducted in Stockholm 1976-1990 which comprised 1780 breast cancer patients [113]. At the time of diagnosis, all patients were postmenopausal and had lymph node-negative primary breast cancer with tumours of ≤ 30 mm, and they were thus classified as “low-risk” patients. Patients were treated with either breast-conserving surgery with post-operative radiotherapy

or modified radical mastectomy. After surgery the patients were randomised to tamoxifen treatment (40 mg daily) or no endocrine treatment. ER status was not an indication for allocation to tamoxifen as the predictive benefit of this hormone receptor was considered uncertain at the time. After two years of tamoxifen treatment, disease free patients were offered to participate in a trial comparing tamoxifen for an additional three years or no further therapy. Protein expression of 17βHSD14 was analysed in tumours from 912 patients. The median follow-up time was 17 years.

The second tumour material is unique in the sense that it enables true prognostic values of biomarkers to be determined since half of the study population received no endocrine treatment. Although not likely to be accepted today, the presence of an untreated control-group was possible at the time since the effect of the drug was uncertain. In 1990, it was concluded from a meta-analysis that tamoxifen therapy was beneficial, and continued patient inclusion in the Stockholm trial was thus cancelled. In both studies, information about relapse was supplied by the responsible clinician to the trial centre. Among other deceased patients, follow-up data was collected from regional population registers and the Swedish Cause of Death Registry.

The generation and validation of an in-house polyclonal anti-17βHSD14 antibody

Antibodies are among the most commonly used research tools. For the production of polyclonal antibodies, a mammal, commonly a rabbit or a goat is inoculated with the antigen of interest. Antibody-producing B-cells of the host animal will recognise different epitopes present on the antigen, and thus several clones of antibodies will be generated against the specified antigen. In comparison, a monoclonal antibody is the result of antibody-production from a single B-cell clone and is produced by fusing that B-cell with a myeloma cell, creating an antibody-producing hybridoma. The fact that polyclonal antibodies are capable of recognising multiple epitopes on any one antigen makes them more robust and less sensitive than monoclonal antibodies to changes in the antigen due to e.g. denaturation. However, while the polyclonality may be an advantage compared with monoclonal antibodies in terms of sensitivity, it can also be a disadvantage as it may lower specificity due to the increased risk of individual clones cross-reacting with non-specific antigens, causing background noise.

Although this is not necessarily the case, it highlights the need to validate any antibodies prior to using them for research or clinical applications.

The polyclonal anti-17βHSD14 antibody used in project II and III was generated in association with AgriSera (Vännäs, Sweden) with permission from the Swedish animal welfare authority (dnr A112-06). A peptide corresponding to amino acid 255 to 269 of human 17βHSD14 was inoculated into a breed of New Zeeland White Rabbits/French Lop. Since the peptide in itself is not expected to evoke an efficient enough antibody-response, it was coupled to a keyhole limpet hemocyanin (KLH) carrier. The addition of KLH, which is a copper-containing protein, will create a larger molecule more likely to activate the immune system of the host animal. In order to increase the immune response and the antibody titers, the antigen was co-administered with an adjuvant. For the production of the anti-17βHSD14 antibody, the peptide was emulsified in Freunds Adjuvant, which is a water-in-oil emulsion that will enable a slow release of the peptide within the animal. With the initial injections, Freunds Complete Adjuvant (FCA), which also contains heat inactivated *Mycobacterium tuberculosis,* was used. FCA efficiently stimulates the antibody immunity against e.g. denatured proteins or peptides. As most adjuvants are toxic to the animals, their use, and especially the use of FCA, is strictly regulated by animal welfare laws. In compliance with current legislation, the three subsequent booster administrations where therefore administered with Freunds *incomplete* adjuvant which lacks bacteria component and therefore is less toxic to the animals. After the final bleeding and sacrifice of the animals, the anti-17βHSD14 antibody was affinity-purified on a column containing a peptide-coated gel matrix (Ultralink; Thermo Fischer Scientific, Waltham, MA).

Validation

The anti-17βHSD14 antibody was validated for immunohistochemistry using a peptide-neutralisation assay. Briefly, the antibody was incubated for two hours at room temperature with the peptide used for rabbit immunisation, in a molar ratio of 1:100 after which the antibody/peptide solution was added to tissue slides. Signals from tissue specimens incubated with the antibody/peptide solution were absent, confirming that the antibody binds to the peptide (Fig. 6). As a positive control of the antibody specificity, the 17βHSD14 protein was over-expressed in cultured cells using a vector containing the *HSD17B14* cDNA insert (see paragraph "Cell culture *transient transfection of HSD17B14"*). The cells were thereafter lysed and separated using gel electrophoresis. The proteins in the gel were transferred to a

membrane after which the membrane was incubated with the anti-17βHSD14 antibody. Up-regulated expression of *HSD17B14* increased the binding to the protein band corresponding to 17βHSD14 and no other protein bands were visible. In cells transfected with a vector lacking the HSD17B14-insert no increase in the 17βHSD14 corresponding band was noted (Fig. 5C).

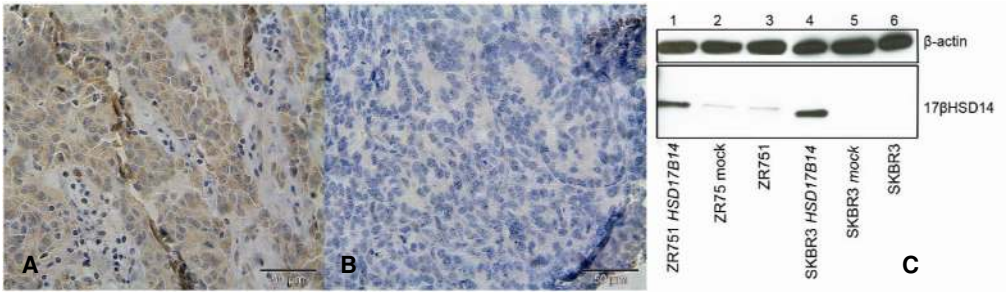

Figure 6. *Validation of 17βHSD14 antibody. 17βHSD14-immunohistochemical staining of (A) tumour tissue, and (B) corresponding tumour tissue from the same individual with antibody pre-incubated with a 17βHSD14 peptide. (C) Immunoblot analysis of lysates from ZR75-1 and SKBR3 breast cancer cells. Lower bands represent 17βHSD14 at an estimated size of 28 kDa. In lane 1 and 4 cells transiently over-expressing HSD17B14, lane 2 and 5 mock-transfected cells and lane 3 and 6 untreated cells. B-actin serves as a control for equal loading.*

Assessing 17βHSD14 protein expression with immunohistochemistry

Immunohistochemistry, which is the detection of antigens in tissue sections using antibodies, is a relatively simple and inexpensive technique which offers the advantage that the actual morphology and subcellular expression sites of antigens can be assessed.

For the assessment of 17βHSD14 in normal tissue (paper II) and tumour tissue (paper III), formalin-fixed and paraffin-embedded tissue in the tissue micro array (TMA)-format, was cut using a microtome and mounted on glass slides. The samples were de-paraffinised and hydrated in descending concentrations of ethanol. Antigen-retrieval, which restores the reactivity of antigens which have been crosslinked during formalin fixation, was performed by boiling the samples in a decloaking buffer (Biocare Medical, Concord, CA). Prior to antibody incubation, samples were immersed in a high protein-buffer that blocks the reactive sites to which the primary or secondary antibodies may otherwise bind. The optimal antibody concentration was empirically determined. After washing away unbound primary antibody, a

secondary antibody carrying a horse radish-peroxidase (HRP) enzyme was added. The slides were then immersed in a solution containing hydrogen peroxide and 3,3'-diaminobenzidine tetrachloride, which is the substrate for the HRP-enzyme. The resultant brown insoluble end-product will only develop where the immune reaction has occurred and is thus an indication of where the target antigen is localised in the tissue. The specimens were counterstained with hematoxylin, dehydrated in increasing ethanol concentrations and finally mounted. Representative images of 17βHSD14-immunostaining are shown in Fig. 7.

Although the reliability of immunohistochemistry analysis is directly related to the specificity of the antibody and the quality of the tissue to be analysed, the ultimate evaluation of the immunohistochemical staining is based on the subjective view of the investigator which can be seen as a disadvantage of the immunohistochemistry method. By involving multiple investigators blinded to the material data this subjectivity can be somewhat overcome.

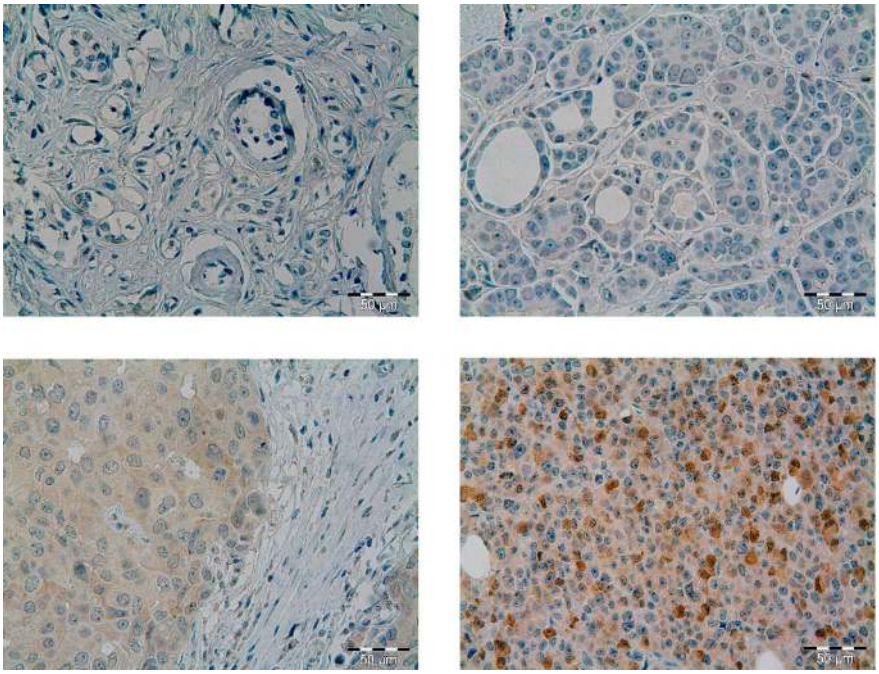

***Figure 7**. Representative images of 17βHSD14 immunostaining in breast tumours. (A) Negative, (B) weak, (C) intermediate, (D) strong staining.*

Analysing gene expression in breast tumours and mouse tissue using real-time reverse transcription polymerase chain reaction

Real-time reverse transcription polymerase chain reaction (RT RT-PCR) is a highly sensitive method that enables gene expression to be quantified. In the TaqMan approach, which was utilised in our studies, the detection of PCR products is dependent on the addition of a gene specific probe which carries a reporter fluorochrome at the 5' end and a quencher at the 3' end. When the probe is intact, the proximity of the reporter and the quencher dyes allows the quencher to suppress the fluorescence signal of the reporter dye through fluorescence-resonance energy transfer. As the Taq DNA polymerase extends the strand from the 3' primer, the exonuclease activity of the enzyme will cleave the probe, releasing the reporter and allowing the fluorescence to be detected. The process is repeated each cycle with the speed of accumulation of emitted light being related to the amount of starting material [114].

The quality of the original template is the most important determinant of the biological relevance of conclusions drawn from the PCR results. Although the DNA molecule is stable and not easily degraded, RNA, which serves as the template in our analyses, is very fragile and prone to degradation. As the oldest tumour samples analysed in paper I have been stored for more than 20 years, RNA quality needed to be considered prior to PCR-analysis. We analysed RNA integrity using an Agilent 2100 system. The Agilent apparatus performs electrophoretic separation of the added RNA-cointaining sample (mainly comprised by ribosomal RNA), and generates an RNA integrity number (RIN) which gives an indication of RNA integrity. RIN-values range from 10 (intact) to 1 (totally degraded) [115]. The gradual degradation of RNA is reflected by a continuous shift towards shorter fragment sizes. Only samples with high-enough RIN-values were used for subsequent RT-PCR analysis.

In paper I, the expression levels of *HSD17B5, HSD17B12,* and *HSD17B14*-genes were analysed using Taqman Gene expression assays (Applied Biosystems, Warrington, UK). For normalisation, standard curves for all analysed genes were run on each plate, using 8-fold serially diluted cDNA derived from normal breast tissue from women aged 45-81. Obtained data from *ACTB* was used to standardise sample variation in the amount of input cDNA. The use of only one reference gene which was common practice at the time when paper I was conceived and written, may represent a flaw to that study. According to the Minimum

Information for Publication of Quantitative Real-Time PCR Experiments (MIQE) guidelines from 2009 [116], at least two different validated endogenous reference genes should be used. This relates to the fact that also genes used as reference are likely to be regulated and that regulation can differ between individuals, bringing a confounding factor into the analysis. *ACTB* was chosen after testing several control genes for their variability between breast cancer samples. Moreover, as *ACTB* found to correlate significantly with the total amount of input mRNA in the sample, the use of this gene as reference was considered justified.

In paper IV, the expression of *Hsd17b14* and *Hsd17b2* were analysed in mouse tissue. Gene expression of the respective genes was calculated by subtracting the average Ct value of the two different reference genes *Cxxc1* and *Srp1*. The selection of the two reference genes was based on careful review of the literature covering mouse reference genes and theoretical testing of respective primers. The Ct value of the positive control was subtracted from the resultant value generating the ΔΔCt value. Relative *Hsd17b14* and *Hsd17b2* expression was based on the calculation $2^{-\Delta\Delta Ct}$.

Cell culture

Adherent cells were cultured in media supplemented with bovine serum and incubated at 37°C in 5% CO_2. For analysis involving detection or stimulation with hormones, a medium lacking phenol red was used as this supplement, which is used as a pH-indicator, has oestrogenic properties [117]. Furthermore, charcoal treatment of the serum which removes the hormone component, is essential for oestrogenic stimulation to be controlled.

Transient transfection of *HSD17B14*

In papers I-III, cultured cells were transfected with a plasmid containing *HSD17B14* cDNA. An in-house plasmid was used in paper I, whereas a commercial plasmid (Origene, Rockville, MD) was used in paper II and III. The most common approaches to transfection of mammalian cells include the use of liposome-based reagents and electroporation [118]. In transient liposome-based transfection, which was the approach used in our studies, the plasmid encoding the gene for up-regulation is delivered to the cells bound in liposomes which will fuse with the lipid bilayer of the target cells. The genetic material becomes released within the cells where it can be transcribed using the enzymatic machinery of the host cells. Both electroporation and liposome-based transfection approaches are more or less toxic to the cells. The transfection reagent is a stressor on its own, but also the plasmid, being

recognised by the cells as foreign nucleic acid, can lead to off-target transcriptional effects. Perhaps not surprisingly, it has been shown that transient transfection leads to transcriptional responses corresponding to the intrinsic cellular response to a viral infection [119]. Such off-target effects can mask or complicate the interpretation of events that really are biologically related to the gene of interest. The potential for misinterpretation of results when transfecting cells transiently can be reduced by incorporating suitable controls, e.g. plasmids lacking the gene-insert, to experiments. Another way of circumventing the problems relating to the acute immune response to foreign nucleic acids is to transfect cells stably, i.e. allow for the gene of interest to be fully integrated into the genome of the target cells.

Analysing sex hormone 17βHSD-conversion with radio-HPLC

Several methods describing efforts to analyse 17βHSD-activity have been described. Commonly, a thin-layer chromatography approach is applied [47, 68, 76, 90], however, also high pressure liquid chromatography (HPLC)-based methods have been used [56, 62, 120]. In paper I, we demonstrated E2 lowering in breast cancer cells transiently transfected with *HSD17B14* using an immunoassay kit. However, as the end-product was not shown, it was not possible to determine whether 17β-oxidation performed by 17βHSD14 was causing the reduction in E2. By integrating labelled hormones into the assay, this problem can be circumvented as it enables the metabolism of the added steroid to be traced. In paper II, basal sex-hormone 17βHSD-activities of *HSD17B14*-transfected/mock-transfected cells were assessed using an optimised and validated radio-HPLC method described in Sivik *et al*. [121]. For separation of the tritiated steroids, we developed a HPLC system that allowed for E1, E2, T and A-dione to de detected simultaneously with good peak separation between E1/E2 and A-dione/T respectively, making the method suitable for assessment of 17βHSD-activity, but also for analysis of aromatase activity. The validation of the method was based on samples containing the four steroid metabolites in varying ratios, and showed robustness in terms of e.g. intra-and inter-day variation, linearity and accuracy. A typical chromatogram with retention times for E2, T, E1 and A is shown in Fig. 8.

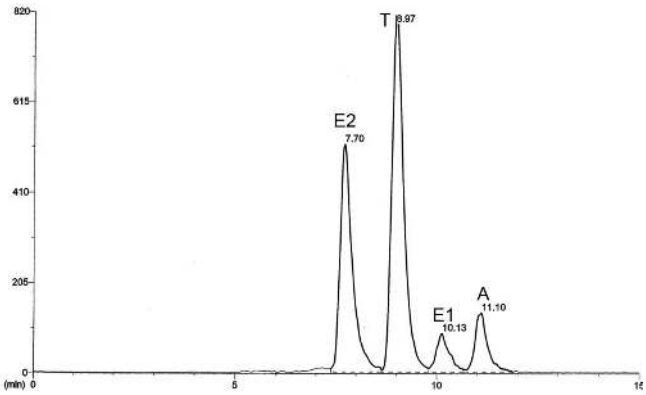

Figure 8. *Typical chromatogram with retention times for E2, T, E1 and A. E2, oestradiol; T, testosterone; E1, oestrone; A, androstenedione.*

Knock-out technology

In 2007, the Nobel Prize for physiology was awarded to Capecchi, Evans and Smithies for their discoveries of principles for introducing specific gene modifications in mice with the use of embryonic stem cells [122]. Transgenic and knockout mouse technology has revolutionised science as it allows for the determination of the role of specific genes *in vivo.* In its most basic form, a global knockout mouse, in which the gene of interested is deleted in all tissues, is created through targeted gene disruption in embryonic stem cells collected from a mouse blastocyst. The altered cells are introduced into a blastocyst of a different genetic background. The blastocyst is then implanted into the uterus of a pseudo-pregnant female mouse. The resultant mouse will be a chimera, in which some cells have developed from the altered stem cells lacking the gene of interest, whereas the rest of the cells are stemming from the host blastocyst cells. If the targeted cells were from a strain with a different fur colour than the host blastocyst, the resultant offspring will have mixed fur colour. The chimeric offspring are backcrossed to a mouse of the same genetic background as the host blastocyst. Offspring with a fur colour matching the genetic background of the altered cell is the progeny of a chimera in which the gonads have developed from the recombinant cells. In these animals, all cells carry one copy of the altered gene. By interbreeding mice heterozygous for the gene, one in four offspring will be homozygous for the deleted gene, assuming Mendelian ratios.

The Hsd17b14KO mouse

In paper IV, we studied genotypical and phenotypical alterations in a mouse lacking the equivalent of the human *HSD17B14* gene. Heterozygous breeding pairs generated by targeting exon one to four of *Hsd17b14* in embryonic stem cells of mouse strain 129/SvEvBrd bred to C57BL/6J were purchased from Lexicon Genetics, Inc. (The Woodlands, TX, USA). Homozygous animals lacking *Hsd17b14* and WT animals were obtained through heterozygous breeding. The animals were housed at the Central Animal Laboratory unit of University of Turku, Finland. The housing and analysis of the mice was approved by the ethical committee for experimental animals, University of Turku, complying with international guidelines on the care and use of laboratory animals.

Potential pitfalls concerning KO-models in endocrine research

Knockout mice provide powerful tools to delineate the role of various genes in physiology, however, whereas some KO-models faithfully mimic human phenotypes, it is sometimes not possible to directly extrapolate findings made in mouse models to the human as important species specific differences exist. As an example, unlike humans, mice adrenals do not produce androgens as these do not express the Cyp17a1 enzyme which catalyses the conversion of pregnenolone to DHEA [123]. Another major difference is substrate preferences of analogous mice and human enzymes. One such example is 17βHSD1. In humans, this enzyme is expressed not only in the ovaries, but also in tissues such as placenta and breast and endometrium, whereas in mice it is only expressed in the ovaries [124]. In terms of enzymatic activity, the mouse *Hsd17b1* catalyses not only E1 to E2, but also to a substantial degree A-dione to T whereas the human *HSD17B1* catalyses primarily the E1 to E2 reaction [125, 126].

Statistical analysis

Local recurrence-free survival time was defined as the time from diagnosis to the first event of cancer recurrence in the breast, ipsilateral chest wall or regional lymph nodes. Recurrence-free survival time was defined as the time from diagnosis to the first event of a local recurrence or distant recurrence. Overall breast cancer survival was the time elapsed from diagnosis to death due to breast cancer. Relationships between grouped variables such as 17βHSD14 expression and factors such as ER, PR, tumour size were analysed using χ^2 test. Survival curves were produced according to the lifetable method described by Kaplan and

Meier. Analyses of recurrence rates were performed with Cox proportional hazard regression. Multivariate Cox hazard regression was used to evaluate whether e.g. tumour expression of *HSD17B14* was predictive or prognostic independent of other tumour characteristics such as tumour size and lymph node involvement. Tests for interaction between 17βHSD14 and tamoxifen were performed by including the covariate, treatment variable and interaction variable in the multivariate model. Survival analyses were performed using the statistical package STATISTICA 9.0 (StatSoft Scandinavia AB, Uppsala, Sweden). Two-sided p-values of < 0.05 were considered to be statistically significant.

RESULTS AND DISCUSSION

The role of 17βHSD14 in breast cancer

In paper I, we investigated the relevance of tumour expression of 17βHSD-genes for predicting recurrence-free survival and breast cancer specific survival among breast cancer patients. mRNA levels of *HSD17B5*, *HSD17B12* and *HSD17B14* were assessed using semi-quantitative RT-PCR. Expression levels of *HSD17B5*, which has been previously associated with breast cancer prognosis [87], did not fall out as significantly associated with survival when comparing three expression groups. However, when comparing outcome among patients with low/intermediate versus high tumoural *HSD17B5*-expression, high expression was found to predict late relapse in patients with ER positive tumours (Fig. 9A-B). No association between prognosis and *HSD17B12* was found (Fig 9 C). Tumour expression levels of this gene were similar across patients and also comparable to levels detected in reference tissue from healthy post-menopausal women, indicating that expression of this enzyme is not altered during tumourigenesis in these cancers, and is thus plausibly not significantly involved in predicting the clinical course among tamoxifen treated breast cancer patients. High tumoural expression levels of *HSD17B14* were significantly associated with both reduced rates of breast cancer recurrence and improved over-all breast cancer survival (Fig. 9D). The results were significant in a multivariate analysis adjusting for nodal status, tumour size and treatment (tamoxifen 2 or 5 years). As all patients in the 2006 study had received tamoxifen it was not possible to determine whether *HSD17B14* was a prognostic factor in the true sense or not. This discrimination was possible however in paper III, since half of the patients included in that study received no adjuvant treatment. In the group of systemically untreated patients in that study, no relevance of 17βHSD14 was found. In both studies however, tamoxifen-treated patients with high tumoural expression of *HSD17B14* had significantly better recurrence-free survival than those with tumours expressing little or no *HSD17B14*. Moreover, patients with high tumoural expression of 17βHSD14 had a significant benefit from tamoxifen in terms of reducing the likelihood for local relapse (paper III), whereas for patients with absent or low tumoural expression levels of the enzyme, tamoxifen did not reduce the number of local recurrences (Fig. 10). It thus seems likely that rather than being a prognostic factor, the relevance of 17βHSD14 is highlighted in the tamoxifen background.

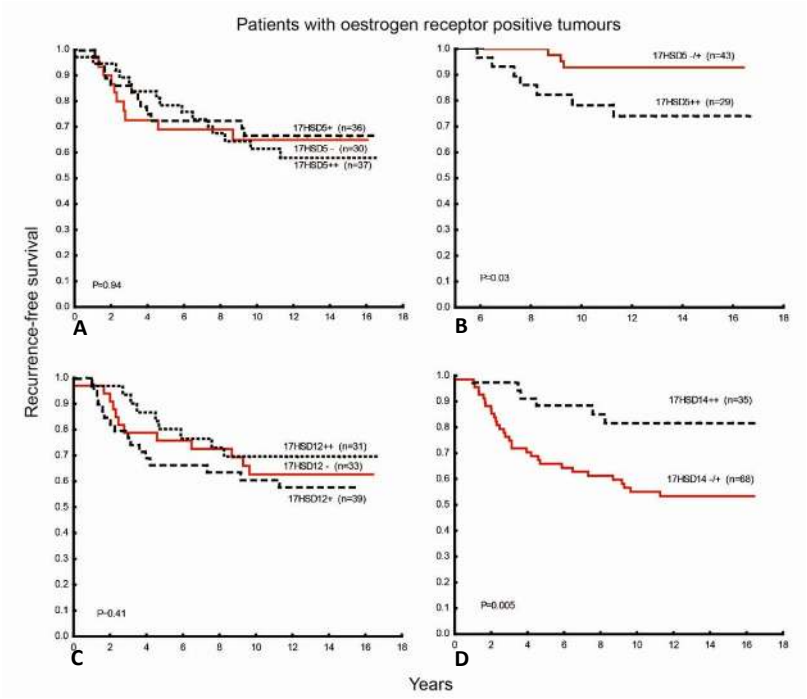

Figure 9. *Kaplan-Meier curves showing recurrence free survival in patients with ER-positive tumours with (A) low (-), intermediate (+) and high (++) expression levels of HSD17B5; (B) low/intermediate versus high HSD17B5 expression five years after diagnosis; (C) low, intermediate and high HSD17B12 expression and (D) low/intermediate versus high HSD17B14-expression.*

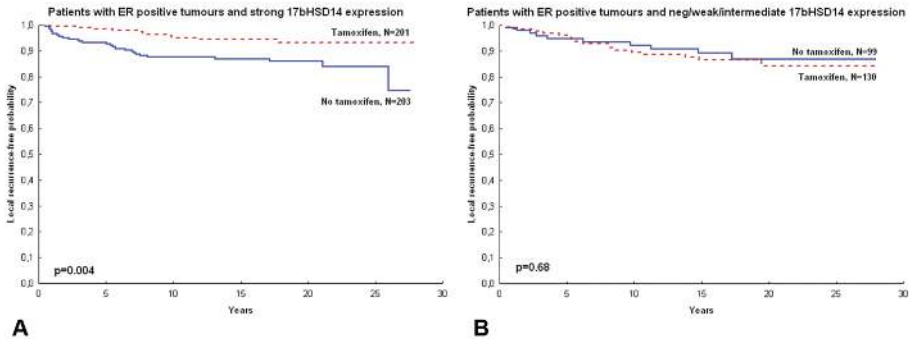

Figure 10. *Kaplan-Meier survival curves of local recurrence-free probability stratified by tamoxifen treatment. (A) Patients with ER+ tumours and strong 17βHSD14 expression. (B) Patients with ER positive tumours and negative/low or intermediate 17βHSD14 expression. ER, oestrogen receptor.*

One of several proposed mechanisms for tamoxifen resistance is illustrated by a situation in which stimulatory effects by excessive E2 levels cannot be counteracted by normal doses of tamoxifen. In a model described by Iino *et al*, it was shown that although tamoxifen inhibited the growth of MCF-7 tumours in athymic mice, the tamoxifen inhibitory effect on tumour growth could be partially reversed by increased E2 levels [127]. Indeed, a recent meta analysis by the Early Breast Cancer Trialists' Collaborative Group reported a greater reduction in breast cancer recurrence in trials of higher daily tamoxifen doses when comparing 20, 30, and 40 mg per day [36]. A mechanism for 17βHSD14 action in breast tumours could thus be E2 to E1 conversion which reduces the levels of E2 competing with tamoxifen for ER binding. This effect could consequently potentiate the effect of the tamoxifen.

A discrepancy between the role of *HSD17B14* expression in paper I and 17βHSD14 expression in paper III was relevance of the gene/protein for different breast cancer end-points. Whereas protein levels of 17βHSD14 significantly predicted tamoxifen benefit as shown in paper III, only when considering local recurrence as end-point, tumour expression of *HSD17B14* analysed in paper I predicted both recurrence-free survival (including distant metastasis) and over-all breast cancer survival. Rather than being an indication of analysis error, this inconsistency could stem from the fact that the two studies are based on analysis of tumours from relatively different patient cohorts. In paper III, tumours from “low risk” patients with smaller tumours and no lymph node spread were analysed. In the 2006 study (paper I) however, tumours from women with stage II to IIIb breast cancer were analysed. It is possible that the driving forces behind tumour development and growth differed between the two tumour groups, and that the role of 17βHSD14 changes given the different tumour environments or different stages of tumourigenesis.

Tumour expression of 17βHSD14 was strongly correlated with expression levels of 17βHSD1 and 17βHSD2, two highly potent enzymes catalysing the reduction of oestrone and oxidation of oestradiol respectively, for which expression levels have been previously assessed in the same tumour material [84]. That study found that high tumoural expression of 17βHSD2 as compared with 17βHSD1 was significantly beneficial for tamoxifen response when considering distant recurrence and breast cancer survival as endpoint, whereas it was not significant when considering local recurrence as endpoint [9]. Interestingly, in paper III, the situation was quite the opposite, with 17βHSD14 having an influence on local recurrence but no impact in predicting distant recurrence or breast cancer-specific survival. The mechanism

behind this divergence is unknown, as one would expect these enzymes to all influence the same pathways, namely steroid conversion. This raises the possibility that although these 17βHSD enzymes are possibly co-regulated, the role of 17βHSD14 is separated from that of 17βHSD1 and 17βHSD2, suggesting that these enzymes operate in different metabolic pathways.

17βHSD-activity of 17βHSD14

In paper II, conversional activities in cultured cells over-expressing *HSD17B14* were analysed using an in-house developed method for radio-detection of HPLC-separated tritiated sex steroids. No reduction was seen when using keto-substrates such as E1, A-dione or DHEA, exceeding that of mock-transfected cells, in media from *HSD17B14*-transfected cells; however cells transfected with *HSD17B14* oxidised E2 to E1 and T to A-dione significantly more than mock-transfected cells. Net efficiencies were approximately 15% for the E2 to E1 reaction and 8% for the T to A-dione reaction within 72 hours (Fig. 11).

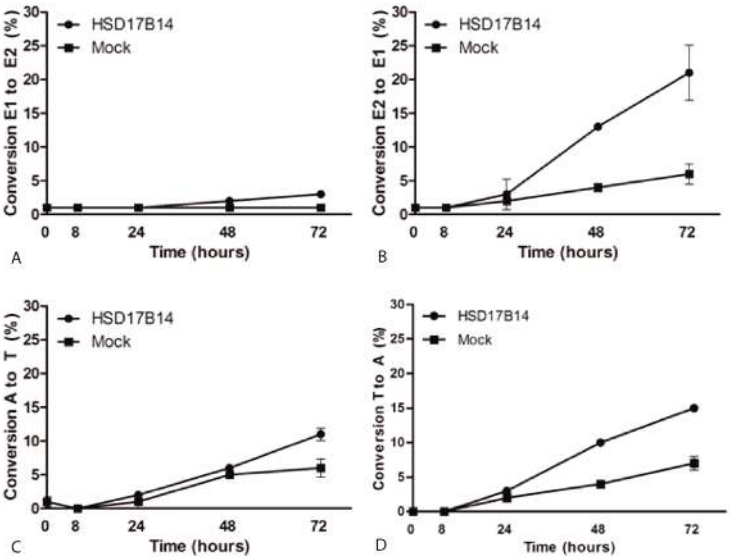

Figure 11. *17βHSD-activity in HSD17B14 transfected cells. (A-B) Reductive (E2 to E1 and T to A) activity, (C-D) oxidative (E1 to E2 and A to T) activity in HEK293 cells transfected with HSD17B14. E1, oestrone; E2, oestradiol; A, androstenedione; T, testosterone.*

In comparison, when transfected with a plasmid encoding 17βHSD2, the same cell line oxidised 100% E2 to E1 within 24 hours. The relatively low catalytic efficiency of 17βHSD14 in oxidising E2 could be related to the fact that the E2 molecule, as shown in docking-experiments, sits rather loosely within the unusually broad active site cleft of the 17βHSD14 enzyme [56]. A low catalytic efficiency however, does not rule out physiological relevance of 17βHSD14 in oxidising E2 and T. As an example, 11βHSD1, which catalyses cortisone to cortisol conversion, has a catalytical activity resembling that of 17βHSD14, and yet has a fully established relevance *in vivo* which is highlighted by its role in metabolic diseases [128]. Nevertheless, the low catalytic efficiency of 17βHSD14 in oxidising substrates such as E2 and T opens up for the possibility that biological relevance of the enzyme is, if not altogether, at least partly within other metabolic pathways. Addressing this question, Lukacik *et al.* screened the recombinant 17βHSD14 enzyme against a library of more than 200 compounds, including steroids and fatty acids, but failed to demonstrate catalytic activity for the enzyme with substrates other than E2, T and A-diol [56]. Interestingly, in a recent publication, DHEA was put forward as a putative substrate for 17βHSD14 [129]. The authors demonstrated that cells transfected with *HSD17B14* reduced DHEA to the weak estrogenic androgen A-diol, which in turn was shown to be a potent activator of ERβ in cells of the nervous system. Further, *HSD17B14*-expression was shown to be negatively regulated by pro-inflammatory LPS and IL-1β, whereas the pro-inflammatory cytokine IL-10 induced the transcription of *HSD17B14,* making the enzyme a possible on/off switch for immune responses. Aiming to repeat this finding however, we failed to detect reduction exceeding that of mock-transfected cells within 72 hours using our HPLC-based system when adding DHEA as a substrate to transiently *HSD17B14* over-expressing MCF10A-cells. In order to test whether this result was cell line specific, we used the very cell line used in Saijo *et al.* 2011, and repeated the experiment. No A-diol was detected, however, a metabolite eluting at a time point matching that of A-dione was formed in media from *HSD17B14* transfected cells stimulated with DHEA. The suggestion that 17βHSD14 would potently act as a reductive enzyme as shown by Saijo *et al.* is intriguing as neither we, nor Lukacik *et al.* could detect reductive activity when adding ketosteroids such as E1 or A-dione as substrates to purified enzyme [56] or cells over-expressing *HSD17B14*. Although it is known that 17βHSD-enzymes catalyse reversible reactions *in vitro*, in intact cells these enzymes cause equilibrium, favouring either the 17β-hydroxy conformation or the 17-keto conformation of the sex steroid, and the 17βHSD-enzymes are thus classified as either reductive or oxidative [130].

Whilst an interesting proposal which may explain some of the discrepancies, e.g. between the roles of 17βHSD1, 17βHSD2 and 17βHSD14, whether or not 17βHSD14 is relevant for DHEA to A-diol reduction remains to be clarified.

The role of 17βHSD14 in human normal physiology

In paper II, we sought to learn more on the biological role of 17βHSD14 by investigating expression patterns of the enzyme in healthy human tissues using the same antibody which in paper III was used for the assessment of tumoural 17βHSD14 expression.

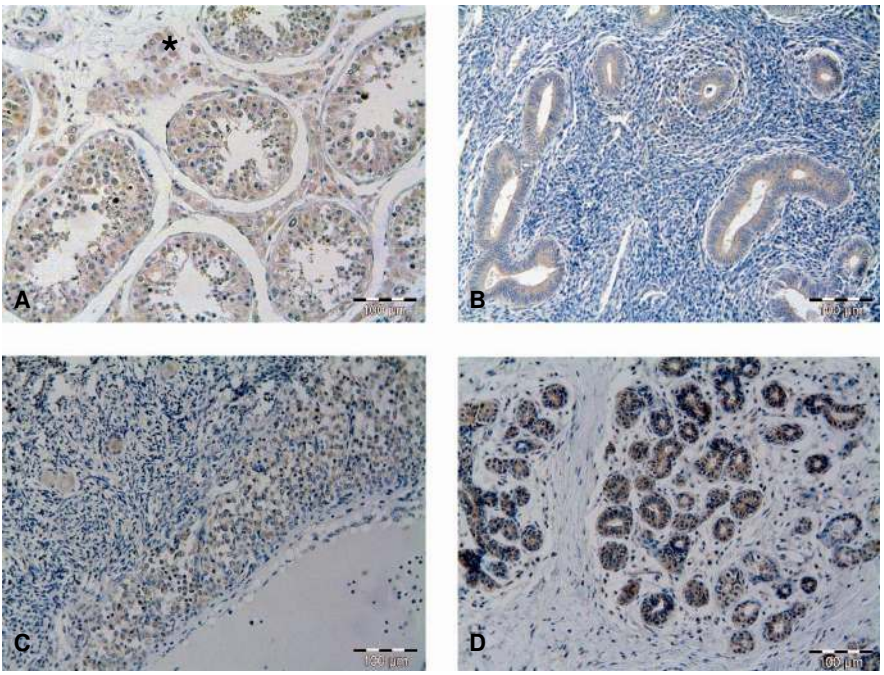

Figure 14. *17βHSD14 immunolocalisation in reproductive tissue. (A) Testis seminiferous epithelium (B) pre-menopausal endometrial glands, (C) ovary with dominant follicle, (D) mammary epithelium. Asterisk indicates Leydig cells.*

Reproductive tissue

17βHSD14 was expressed in both male and female reproductive tissues such as testis, uterus, ovary and breast (Fig. 14). In the testis, 17HSD14 was expressed in both Leydig cells, where 17βHSD14 may participate in the fine tuning of bioactive sex hormone levels which is crucial

for the homeostasis in the testis. 17βHSD14 was highly expressed in breast epithelial cells. It has been suggested that the predominant enzymatic direction in normal breast is oxidative, favouring E2 to E1 conversion, whereas tumour progression is associated with a shift towards a reductive environment, and these changes have been shown to concur with changes in local protein expression of steroidogenic enzymes from a dominance of oxidative enzymes such as 17βHSD2, to increased expression of reducing enzymes, e.g. 17βHSD1 [131-133].

Gastrointestinal tissue

The 17βHSD14 protein was expressed throughout the gastrointestinal tract with strong expression in the stomach (Fig. 15A). Colon and rectal samples displayed prominent immunoreactivity of enterocytes and goblet cells of the mucosal crypts in the outer absorptive border facing the lumen, whereas positivity towards the deeper situated glands gradually diminished (Fig. 15B). The same pattern, although not as marked, was seen in samples from duodenum. The expression patterns in gut resembled those seen previously for oxidative 17βHSD-enzymes such as 17βHSD2 and 17βHSD4 [134, 135]. It has been hypothesised that oxidative enzymes play a role in the gut in protecting gastrointestinal tissues from exposure to excess reduced steroidal agents such as ingested or bacterially derived E2.

Kidney

17βHSD14 immunoreactivity in the kidney was intense and specifically prominent in epithelial cells of proximal and distal tubules whereas renal corpuscles were negative (Fig. 15C). Although the kidney harbours several steroidogenic enzymes [75, 136, 137], the exact significance of steroid metabolism in renal cells has not been extensively studied. Observed gender differences in e.g. blood pressure response to salt stimuli and the tendency to form urinary stones suggests involvement of sex steroids in the regulation of tubular reabsorption [136]. Specific immunopositivity for 17βHSD14 and other 17βHSD enzymes such as 17βHSD5 in sites for selective ion and water transport in the kidney could therefore possibly suggest a role for 17βHSD enzymes in normal reabsorption physiology.

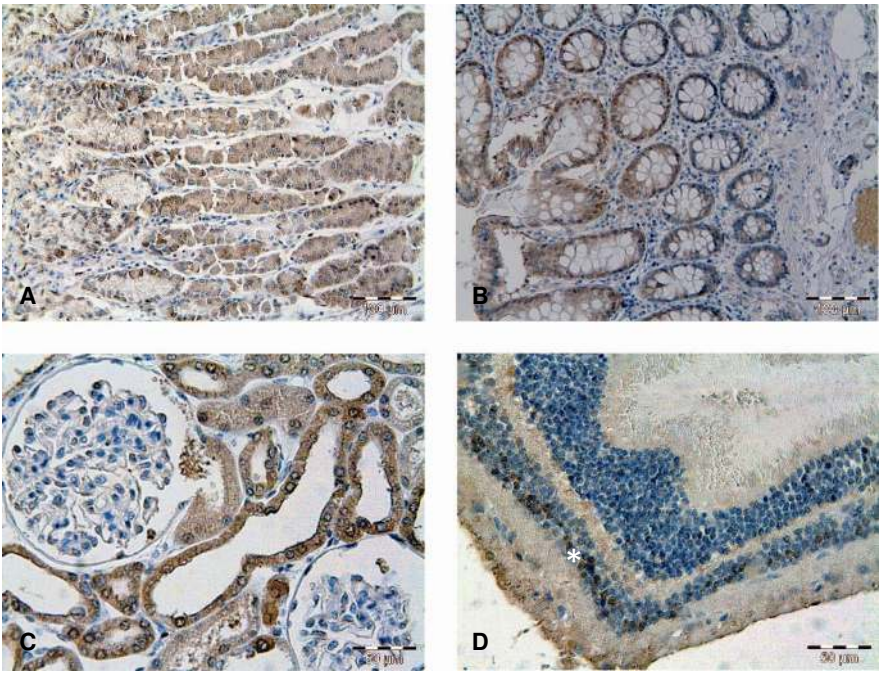

Figure 15. *Immunolocalisation of 17βHSD14 in human tissues. (A) Gastric glands, (B) colonic glands, (C) cortical kidney, (D) retina. The asterisk indicates amacrine cells of the inner nuclear layer of the retina.*

Retina

The *HSD17B14* gene product was first cloned from a retinal cDNA-library and was also found to be highly expressed therein [92]. We found 17βHSD14 immunoreactivity in cytoplasmic projections of the plexiform layers of the retina, whereas nuclear layers were negative, with an exception for the inner nuclear layer, where staining was prominent in specific nuclei, most likely belonging to amacrine cells (Fig. 15D). Interestingly, the inner nuclear layer of the retinal epithelium has been shown to harbour the highest density of steroidogenic enzymes in rat [138]. The presence of 17βHSD14 in retina could be an indication for a role in steroid metabolism, but it could also be related to a possible role in retinoid metabolism. 17βHSD enzymes share homology with retinol dehydrogenase enzymes, and some 17βHSD enzymes have been shown to metabolise retinoids [61, 70, 77]. As recently shown by Haller *et al.*, the exchange of a single amino acid in the 17βHSD1 enzyme makes it efficiently reduce all-*trans*-retinal to its alcohol retinol [139].

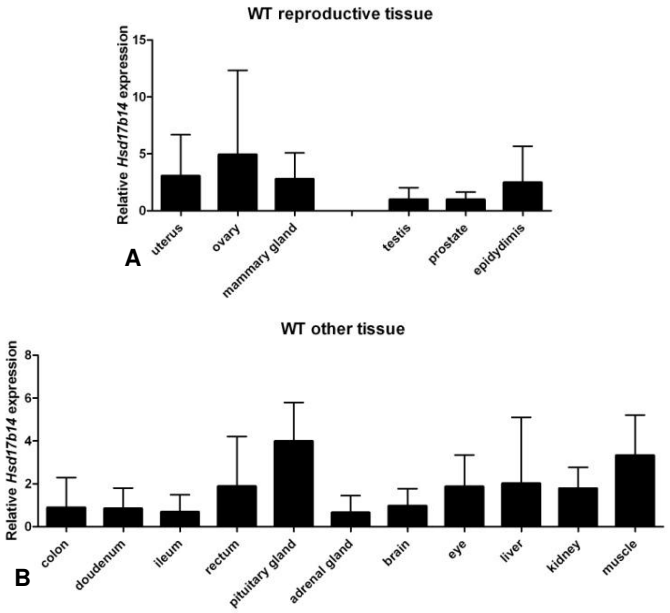

Figure 16. *RT-PCR analysis of Hsd17b14 expression in reproductive tissue (A) and in other tissue (B) in wild type (WT) mice. Hsd17b14 expression was normalised using the average of Cxxc1 and Srp1 expression using testis as a calibrator (n=3-12).*

The Hsd17b14KO Mouse

Mice heterozygous for the targeted deletion of *Hsd17b14* were viable and fertile, and heterozygous mating generated mutant progeny at expected Mendelian ratios. Homozygous *Hsd17b14* were compared with the WT counterpart and were found to be morphologically similar on gross examination. Regretfully, neither the in-house produced anti-17βHSD14 antibody, nor a commercial anti-17βHSD14 antibody was considered specific enough for the detection of mouse-17bHsd14, and subcellular expression patterns of the mouse protein could therefore not be analysed. However *Hsd17b14*-expression was analysed in a wide range of tissues using RT RT-PCR. Furthermore, histological appearance of tissue was compared in H&E stained tissue slides from Hsd17b14KO and WT mice.

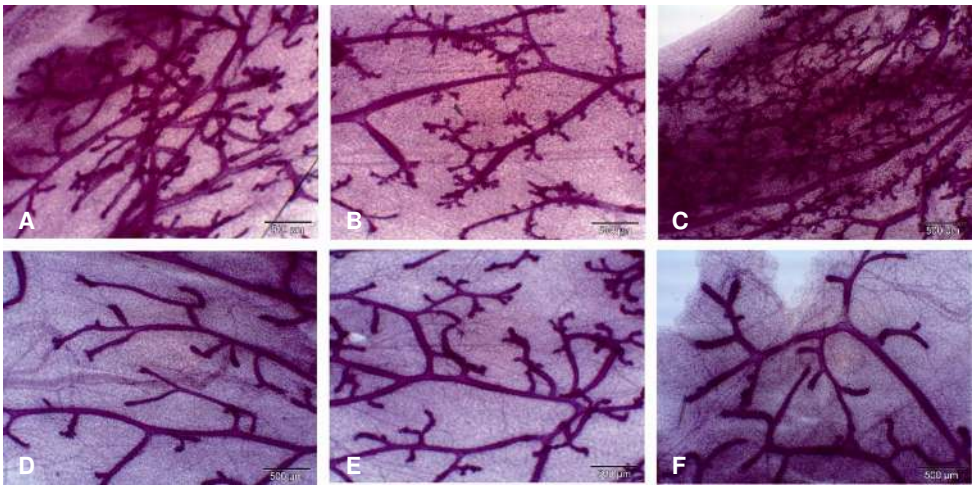

***Figure 17**. Mammary gland whole-mount. (A-C) normal branching in three individual wild type mice (D-F) impaired secondary branching in three Hsd17b14KO mice.*

Reproductive tissue

Epidydimis had the highest *Hsd17b14* expression in WT male mice. Although testis weight varied considerably more for Hsd17b14KO animals than their WT counterparts, testis histology in three to four month old KO males appeared normal. Prostate histology in KO males was identical to that of WT males. Among the WT female reproductive tissues analysed, ovary had the highest *Hsd17b14* expression; however, the gene was also expressed in breast and uterus. The major discrepancy between KO and WT-mice was seen in whole-mount preparation of breast tissue, which showed only primary or in a few cases incipient secondary branching in KO-animals, whereas the expected secondary branching was present in most WT specimens (Fig. 17). Until puberty, mammary gland development in mice is hormone-independent, as demonstrated by pre-pubertal αERKO mice which are identical to pre-pubertal WT littermates. At puberty, hormone-dependent mammary gland development occurs and results in ductal elongation and side branching. As demonstrated by grafting mammary fat pads from αERKO and PRKO mice into WT mice, both ERα and PR signaling is required for ductal elongation as well as branching of mammary glands [140]. The impaired mammary gland branching seen in the Hsd17b14KO females contrasts with the mammary gland hyperplasia seen in *HSD17B2*-overxpressing mice [96], which could be explained by 20α-progesterone oxidation properties of the 17βHSD2-enzyme, which in turn would elevate the levels of progesterone. Whether 17βHSD14, like 17βHSD2, catalyses 20α-progesterone

oxidation is unknown, however, the impaired branching of mammary glands as seen in Hsd17b14KO mice could be an indication that the enzyme affects sex-hormone signaling at some level.

Non-reproductive tissue

Among non-reproductive tissue analysed, pituitary gland as well as muscle, rectum and liver expressed the highest levels of *Hsd17b14*. While pituitary tissue was not examined histologically, examined neural structures of Hsd17b14KO animals including cerebral cortex and retina, which in human has a high 17βHSD14 expression, were normal. Furthermore, no aberrant phenotype was present in gastrointestinal tissues of Hsd17b14KO animals of either sex. In liver however, histological examination revealed increased vacuolisation in hepatocytes of KO animals compared with WT littermates (Fig. 18). The vacuolisation was most prominent in female KO mice. Hepatocellular vacuolisation as a result of triglyceride accumulation has been described in male ArKO mice, which also have a general obese phenotype [111]. The obese phenotype as well as the fatty liver can in these mice be completely reversed by the addition of E2 [141]. Although altered oestrogen signalling could be causing the liver phenotype in the Hsd17b14KO, it is also possible that the vacuolisation is a direct result of altered lipid metabolism. Several 17βHSD enzymes, including 17βHSD11 which was cloned alongside 17βHSD14 in retina [92], have been implicated in lipid metabolism. Agonists of peroxisome proliferator-activated receptor-α, which is a major regulator of lipid metabolism in the liver, induce a rapid increase in 17βHSD11 in the endoplasmic reticulum and lipid droplets of mouse liver [142, 143]. Furthermore, the 17βHSD11 protein has been identified as one of three major enzymes in lipid droplets of hepatocytes [144]. Although initial screens do not support lipids being substrates of 17βHSD14, lipid-metabolising functions of the mouse 17βHSD14 protein cannot be ruled out.

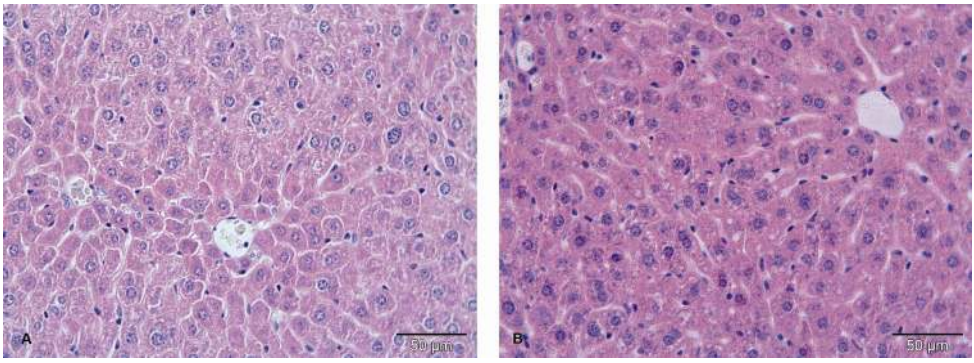

Figure 18. *Mouse liver tissue. (A) Increased vacoulisation in female Hsd17b14KO individual and (B) normal liver parenchyma in female WT littermate.*

CONCLUDING REMARKS

We have shown that high tumoural expression levels of *HSD17B14* significantly associates with beneficial clinical outcome in terms of both breast cancer recurrence and over-all breast cancer survival among tamoxifen treated patients. Furthermore, tumoural expression levels of the 17βHSD14 protein can predict the outcome of adjuvant tamoxifen treatment in terms of local recurrence-free survival in patients with lymph node-negative ER-positive breast cancer.

Several findings support a role for 17βHSD14 being a 17βHSD-enzyme with implications in hormone regulation which could explain our findings in the tumour materials. Among these, cells transiently transfected with *HSD17B14* convert E2 to E1 and T to A-dione. Further, 17βHSD14 is expressed in several classical endocrine tissues such as breast and testis. Finally, the Hsd17b14KO mouse displays aberrances implicating disturbed hormonal homeostasis such as impaired mammary gland branching. Still, the E2 to E1 oxidation efficiency of the enzyme is significantly lower than that of e.g. 17βHSD2, and while a low catalytic efficiency need not necessarily be an indication that E2 oxidation is not the prime physiological role of the enzyme, it raises the question whether 17βHSD14 additionally operates in other metabolic pathways.

The naming of the 17βHSD enzymes refers to their capacity to catalyse the oxidation or reduction of 17-hydroxy or 17-keto groups of oestrogens and androgens. A problem concerning the classification of 17βHSD enzymes is that it primarily refers to conclusions drawn from *in vitro* analysis using recombinant proteins. Information about the role of 17βHSD enzymes in human biology is scarce, with one of the few examples being 17βHSD3, which when mutated in boys causes pseudohermaphroditism [52]. In addition, knockout and transgenic mouse models have revealed functions of the 17βHSD enzymes in higher biological systems.

Of the 17βHSDs described to date at least 6 different enzymes are reductive and another 6 oxidative *in vivo*. One may question why such a large number of enzymes performing the same task are needed. An answer could be that the importance of the fine-tuning of hormonal levels, (which constitutes an economical on-off switch for regulating steroid hormone action since it conserves a complex molecule that requires a large number of enzymatic steps for its

synthesis), has led to an evolutionary redundancy which enables the organism to endure changes in its environment or genetic makeup. Another possibility is that a re-evaluation of the SDR-enzymes, taking substrate specificity, enzymatic kinetics and possibly findings from mouse models into consideration, would reduce the number of enzymes belonging to the 17βHSD-family. Addressing this question, a recent nomenclature initiative undertaken to re-classify the SDR enzymes may help to bring clarity on the biological function of the 17βHSD enzymes [145].

With significant findings in two independent tumour materials, 17βHSD14 emerges as a candidate predictive factor for tamoxifen response in breast cancer. Translating into the clinical setting, assuming that oestrogen metabolism is the primary focus of 17βHSD14, patients with tumours expressing low levels of E2 oxidising enzymes such as 17βHSD14 and 17βHSD2, would likely receive more clinical benefit from alternative treatments to tamoxifen such as AIs or in the future possibly inhibitors of reducing 17βHSD enzymes.

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