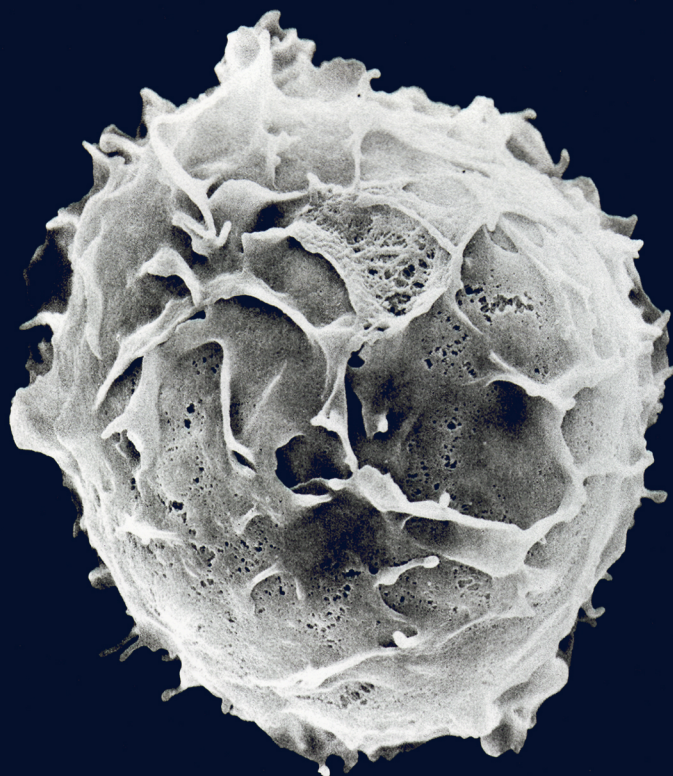


Thesis for doctoral degree (Ph.D.)  
2013

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# Endocrine Signaling and Molecular Aberrations in Primary Hyperparathyroidism



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# ENDOCRINE SIGNALING AND MOLECULAR ABERRATIONS IN PRIMARY HYPERPARATHYROIDISM

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*“Our freedom to doubt was born out of a struggle against authority in the early days of science. It was a very deep and strong struggle: permit us to question — to doubt — to not be sure. I think that it is important that we do not forget this struggle and thus perhaps lose what we have gained. “*

- Richard Feynman

# Thesis defense

Rolf Lufts auditorium  
Karolinska University Hospital Solna L1:00

Friday October 18<sup>th</sup> 2013  
at 09.00

# ABSTRACT

Primary hyperparathyroidism is a common endocrine disorder, characterized by an inappropriate increase in serum parathyroid hormone (PTH) levels. It is most often caused by a single benign parathyroid tumor. The elevated PTH levels cause an increase in serum calcium levels, which in turn may present diffuse neuromuscular symptoms, as well as increased risk of cardiovascular complications. Malignant parathyroid tumors are rare. They have a poor prognosis and constitute a diagnostic challenge for the pathologist.

Studies on hereditary syndromes with parathyroid tumor manifestations have identified a number of genes involved in parathyroid tumorigenesis. The etiology of the common sporadic parathyroid adenoma, however, is yet to be explained. Since postmenopausal women constitute the most frequently affected group of patients, the involvement of female endocrine hormones has been suggested. The aim of this thesis was to elucidate the molecular pathophysiology of this disease, mapping molecular aberrations and endocrine signaling within these tumors.

By a limited screening of the Wnt signaling cascade we identified a number of aberrantly expressed proteins. Changes included the proteins glycogen synthase kinase 3- $\beta$ , and Adenomatous Polyposis Coli. Loss of the latter was distinguishingly restricted to malignant parathyroid tumors, thus being a candidate diagnostic marker of parathyroid malignancy. (Paper I)

The S37A mutation in the *CTNNB1* gene, encoding  $\beta$ -catenin was suggested to be a significant event in the development of sporadic parathyroid tumors. We evaluated this hypothesis by mutational analysis of 98 parathyroid tumors. Finding no S37A *CTNNB1* mutations, we suggest that this genetic variant has a limited significance in development of primary hyperparathyroidism. (Paper II)

Several indications suggested the involvement of prolactin signaling in parathyroid physiology and tumor development. By tumor protein and ribonucleic acid analysis we could identify an overall high receptor expression as compared to other tissues. We showed that physiological levels of prolactin were able to affect PTH secretion and alter gene expression in parathyroid tumor cells. As compared to normal parathyroid tissue, the levels and distribution of the receptor was altered in parathyroid adenomas. In all, the findings support a possible link between prolactin signaling and parathyroid tumors. (Paper III)

We also evaluated the expression of estrogen receptor isoforms in parathyroid tumors. Previous data suggested that the parathyroid glands are targets of estrogen signaling, but that they lacked estrogen receptor (ER) expression. We re-evaluated the ER expression, including recently identified isoforms. Our results suggest that parathyroid tissue lack ER $\alpha$ , but express ER  $\beta$ 1 and  $\beta$ cx isoforms. Parathyroid tumors showed decreased ER  $\beta$ 1 expression, with an inverse correlation to tumor weight. Treatment of primary parathyroid cultures with an ER  $\beta$ 1-specific ligand showed changes in transcriptional activity significantly analogous with nuclear ER transcriptional activity and apoptosis of tumor cells. Thus, this gene expression profiling suggests tumor suppressive properties of ER  $\beta$ 1 in the parathyroid glands. (Paper IV)

Much work remains in elucidating the molecular changes that characterize parathyroid tumors. Our data suggests that female hormone receptors, either during the course of life or the menopausal changes, may play a role in the development and presentation of primary hyperparathyroidism.

# LIST OF PUBLICATIONS

The thesis is based on the following papers, which will be referred to throughout the text by their Roman numerals.

- I Juhlin CC, **Haglund F**, Villablanca A, Forsberg L, Sandelin K, Bränström R, Larsson C, Höög A.  
Loss of expression for the Wnt pathway components adenomatous polyposis coli and glycogen synthase kinase 3- $\beta$  in parathyroid carcinomas.  
*International Journal of Oncology*, 2009, vol 34, pages 481-492
- II **Haglund F**, Andreasson A, Nilsson I-L, Höög A, Larsson C, Juhlin CC.  
Lack of S37A CTNNB1/ $\beta$ -catenin mutations in a Swedish cohort of 98 parathyroid adenomas.  
*Clinical Endocrinology*, 2010, vol 73, pages 552-553
- III **Haglund F\***, Lu M\*, Vukojević V, Nilsson I-L, Andreasson A, Džabić M, Bränström R, Höög A, Juhlin CC, Larsson C.  
Prolactin receptor in primary hyperparathyroidism - expression, functionality and clinical correlations.  
*PLoS One*, 2012, vol 7, e36448
- IV **Haglund F**, Ma R, Huss M, Sulaiman L, Lu M, Nilsson I-L, Höög A, Juhlin CC, Hartman J, Larsson C.  
Evidence of a functional estrogen receptor in parathyroid adenomas.  
*Journal of Clinical Endocrinology and Metabolism*, 2012, vol 97, pages 4631-4639

\* Both authors contributed equally



# LIST OF RELATED PUBLICATIONS

1. Juhlin CC, Villablanca A, Sandelin K, **Haglund F**, Nordenström J, Forsberg L, Bränström R, Obara T, Arnold A, Larsson C, Höög A.  
Parafibromin immunoreactivity: its use as an additional diagnostic marker for parathyroid tumor classification.  
*Endocrine Related Cancer*, 2007, vol 14, pages 501-512.
2. Juhlin CC, Kiss NB, Villablanca A, **Haglund F**, Nordenström J, Höög A, Larsson C.  
Frequent promoter hypermethylation of the APC and RASSF1A tumour suppressors in parathyroid tumours.  
*PLoS One*. 2010, vol 5, e9472.
3. Juhlin CC, Nilsson I-L, Johansson K, **Haglund F**, Villablanca A, Höög A, Larsson C.  
Parafibromin and APC as screening markers for malignant potential in atypical parathyroid adenomas.  
*Endocrine Pathology*, 2010, vol 21, pages 166-177.
4. Juhlin CC, **Haglund F**, Obara T, Arnold A, Larsson C, Höög A.  
Absence of nucleolar parafibromin immunoreactivity in subsets of parathyroid malignant tumours.  
*Virchows Archiv*, 2011, vol 459, pages 47-53.
5. Andreasson A, Sulaiman L, do Vale S, Martins J M, Ferreira F, Miltenberger-Miltenyi G, Batista L, **Haglund F**, Björck E, Nilsson I-L, Höög A, Larsson C, Juhlin CC.  
Molecular characterization of parathyroid tumors from two patients with hereditary colorectal cancer syndromes.  
*Familial Cancer*, 2012, vol 11, pages 355-362.
6. Sulaiman L, Nilsson I-L, Juhlin C C, **Haglund F**, Höög A, Larsson C, Hashemi J.  
Genetic characterization of large parathyroid adenomas.  
*Endocrine Related Cancer*, 2012, vol 19, pages 389-407.
7. Sulaiman L\*, **Haglund F\***, Hashemi J, Obara T, Nordenström J, Larsson C, Juhlin CC.  
Genome-wide and locus specific alterations in *CDC73/HRPT2*-mutated parathyroid tumors.  
*PLoS One*, 2012, vol 7, e46325

\* Both authors contributed equally

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# LIST OF ABBREVIATIONS

4-OHT	4-Hydroxytamoxifen
5'-UTR	5' - untranslated region
7TM	7 transmembrane (receptors, G-protein coupled)
AKT	Protein kinase B
APC	Adenomatous polyposis coli
ATP	Adenosine triphosphate
Bcl-2	B-cell lymphoma 2
Ca <sup>2+</sup>	Calcium ion
cAMP	Cyclic adenosine monophosphate
CaSR	Calcium sensing receptor
<i>CASR</i>	Calcium sensing receptor gene
<i>CCND1</i>	Cyclin D1 gene
<i>CDC73</i>	Cell division cycle 73 gene ( <i>HRPT2</i> )
Cdkn1b/p27kip1	Cyclin dependent kinase inhibitor 1B
cDNA	Complementary deoxyribonucleic acid
CNA	Copy number alteration
CT	Computed tomography
DAG	Diacyl-glycerol
ddNTP	Dideoxynucleotide triphosphate
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleotide triphosphate
DPN	Diarylpropionitrile
E1	Estrone
E2	Estradiol
ER	Estrogen receptor
ERE	Estrogen responsive element
ERK	Extracellular signal-regulated protein kinase
<i>ESR1</i>	Estrogen receptor gene 1
<i>ESR2</i>	Estrogen receptor gene 2
FFPE	Formalin fixated, paraffin embedded
FGF23	Fibroblast growth factor 23
FPKM	Fragments per kilobase of exon per million fragments mapped

FSH	Follicle-stimulating hormone
<i>Gcm2</i>	Glia cells missing 2 gene
GP <sub>ER</sub> /GPR30	G protein-coupled estrogen receptor 1
GSK3- $\beta$	Glycogen synthase kinase 3- $\beta$
G $\alpha$	GTP-binding protein alpha
H3K4	Histone H3 at lysine 4
H3K9	Histone H3 at lysine 9
Hh	Hedgehog (signaling pathway)
HPR	Horseradish peroxidase
HPT	Hyperparathyroidism
<i>HRPT2</i>	Hyperparathyroidism 2 gene (CDC73)
<i>HRPT3</i>	Hyperparathyroidism 3 gene locus
IF	Immunofluorescence
IGF-I	Insulin-like growth factor I
IHC	Immunohistochemistry
IP3	Inositol triphosphate
IP3K	Inositol triphosphate kinase
JAK-STAT	Janus kinase - Signal Transducer and Activator of Transcription
Ki67	Antigen Kiel clone 67
LH	Luteinizing hormone
LOH	Loss of heterozygosity
LRP	Low density lipoprotein receptor-related protein
MAPK	Mitogen-activated protein kinase
Mdm-2	Mouse double minute 2 homolog
MEN 1	Multiple endocrine neoplasia type 1
MEN 2A	Multiple endocrine neoplasia type 2A
MEN 4	Multiple endocrine neoplasia type 4
MHC Class II	Major histocompatibility complex
MRI	Magnetic resonance imaging
Paf1	RNA polymerase II associated factor complex
PCR	Polymerase chain reaction
PHPT	Primary hyperparathyroidism
PI3K	Phosphoinositide 3-kinase
PLA2	Phospholipase A2

PLC	Phospholipase C
PLD	Phospholipase D
PRLr	Prolactin receptor
PTH	Parathyroid hormone
PTH1R / PTH2R	Parathyroid hormone 1 / 2 receptor
PTHrP	Parathyroid hormone related protein
<i>PVALB</i>	Parvalbumin gene
qRT-PCR	Quantitative real-time polymerase chain reaction
Rb1	Retinoblastoma protein
<i>RET</i>	Rearranged during transfection gene
RNA	Ribonucleic acid
rRNA	Ribosomal ribonucleic acid
RT-PCR	Reverse transcription polymerase chain reaction
SERM	Selective estrogen receptor modulator
SHPT	Secondary hyperparathyroidism
SOCS	Suppressor of cytokine signaling
SUV39H1	Suppressor of variegation 3-9 homolog 1
TCF/LEF	T-cell factor / Lymphoid enhancer factor
THPT	Tertiary hyperparathyroidism
TIP39	Tuberoinfundibular peptide of 39 residues
TrCP	Transducin repeat containing E3 ubiquitin protein ligase
VDR	Vitamin D receptor
WHO	World Health Organization
Wnt	Wingless/integration 1 (signaling pathway)

# INTRODUCTION

“... ehuruväl sannolikheten att här anträffa något förut obeaktadt föreföll så ringa...”<sup>1</sup>

- Ivar Viktor Sandström

The parathyroid glands constitute an endocrine tissue whose main function is the regulation of circulating calcium in the body. While these glands are essential to human life and commonly affected by tumorous growth, their role in physiology and disease have only gained a limited proportion of our attention. Until date, the parathyroid glands are the last major organ to be identified in man. The discovery has been accredited to Ivar Viktor Sandström (Figure 1) who at the time was a medical student at Uppsala University. It was in 1877, during his work in the anatomy department, that he noted the presence of an unknown structure in close proximity to the thyroid capsule in dog. After identifying these structures in man, he named them the *parathyroid glands*. (Sandstrom, 1880) (Figure 2) Since his studies were limited to the diseased, he had no possibility of knowing their function. He did however correctly predict the presence of tumors in these glands.



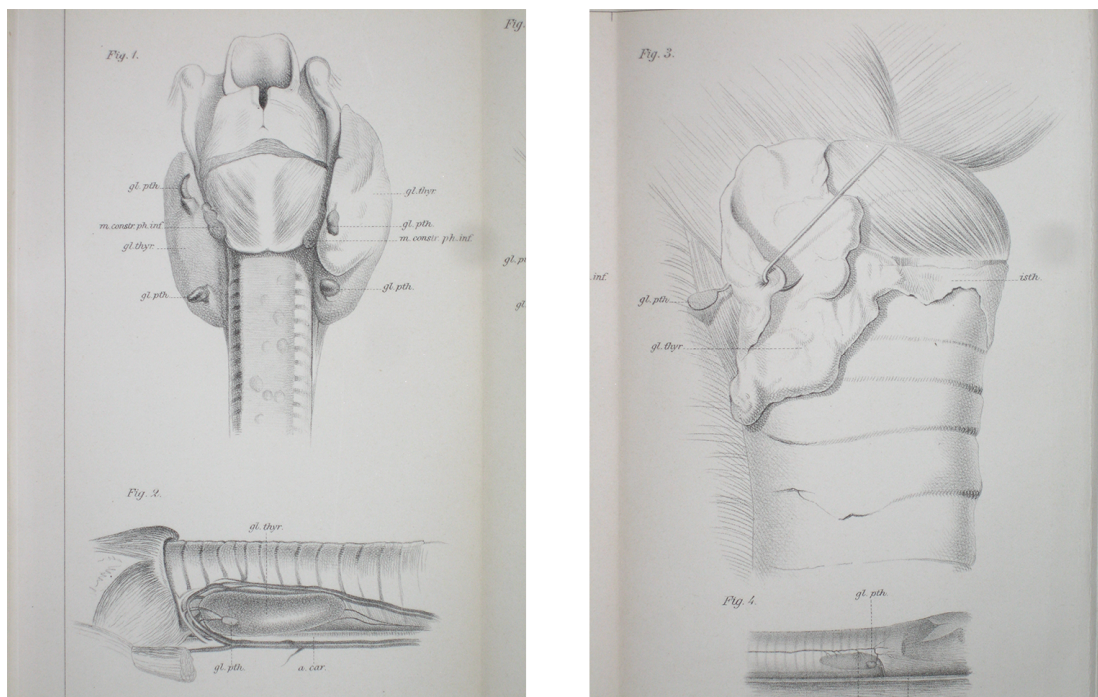
**Figure 1.** Ivar Viktor Sandström, 1852-1889.

Photographer: Heinrich Osti. Digital image: Uppsala University Library.

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<sup>1</sup> eng. "... the possibility of finding something not previously described seemed so small..."

During the late 19<sup>th</sup> century, the surgeons Kocher and Billroth began to perform thyroid surgery. While Billroth used a faster operating technique, Kocher used a more cautious approach - unknowingly sparing the adjacent parathyroid tissue. Many of Billroth's patients died in severe postoperative seizures. Today we know that incidental parathyroidectomy with subsequent loss of parathyroid function was the cause of these seizures. However, at the time these complications were attributed to complete loss of the thyroid gland, and it was not until the work of the experimental pathologist Erdheim that these seizures were coupled to the loss of parathyroid tissue. Erdheim also made the observation that parathyroid function affected the calcium metabolism, and hypothesized that parathyroid gland enlargement could be due to disease of the bone. Several years later Schlagenhauer suggested that parathyroid tumors could cause bone disease, subsequently leading to the first parathyroid adenectomy in 1925, performed by Mandl. Research efforts during the following decades have unraveled several pieces in the puzzle of parathyroid physiology and disease. The recent development of molecular techniques has extended this to the molecular characterization of the parathyroid glands on the subcellular level. Even so, many fundamental questions remain unanswered about these glands.



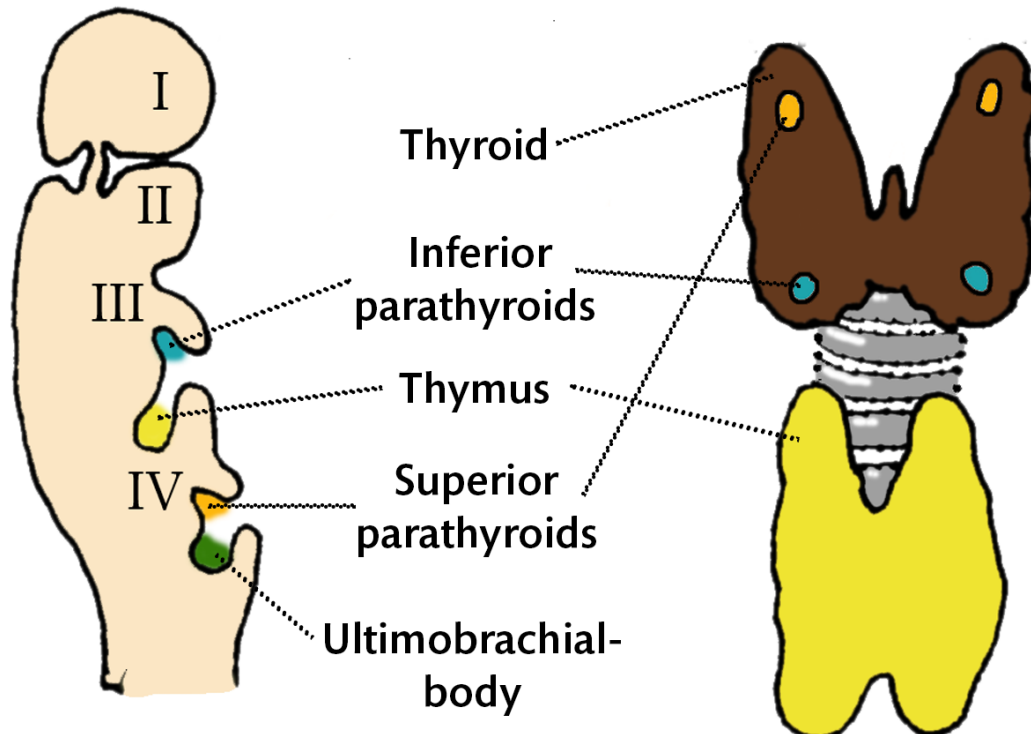
**Figure 2.** Illustrations of the parathyroid glands in Sandströms' thesis. Illustration: E. Nisbeth, *Om en ny körtel hos människan*, 1880. Digital image: Hagströmer Library, Stockholm.



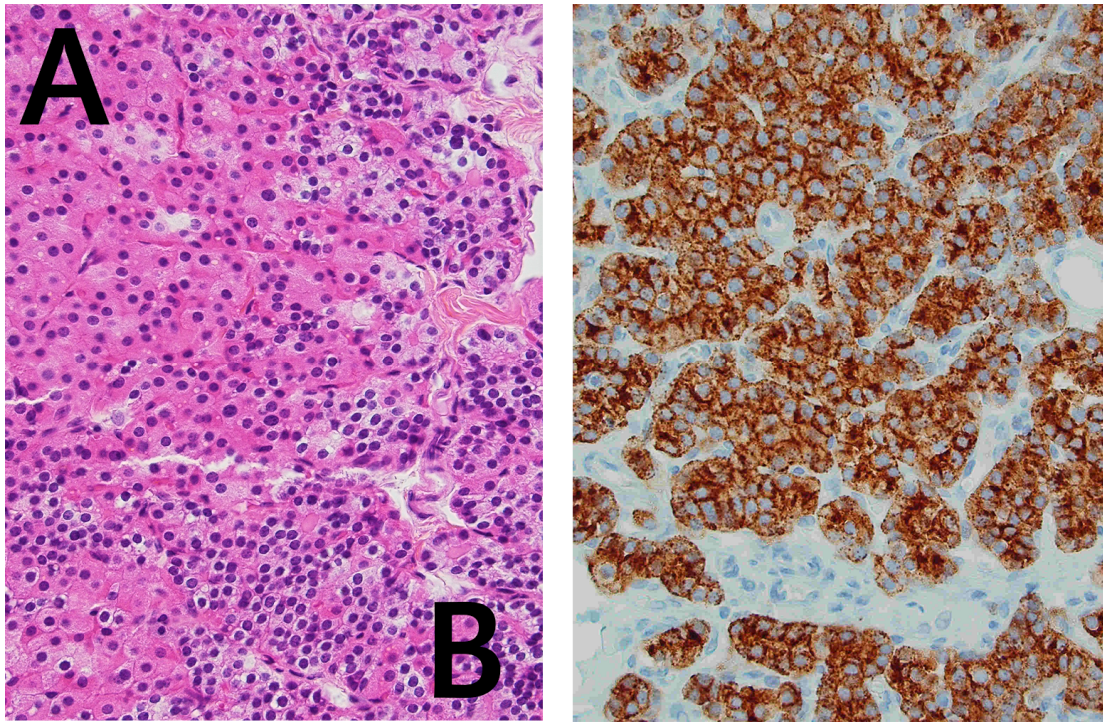
# THE PARATHYROID GLANDS

## Anatomy and Embryology

Most commonly there are four parathyroid glands – two superior and two inferior. However, additional glands may occasionally be present. The arterial blood flow is generally supplied by the *arteriae thyroidea inferior*. In some variants branches from superior thyroid arteries supply the parathyroid glands. During embryogenesis the parathyroid glands develop from the dorsal endoderm of the third and fourth pharyngeal pouches. The superior parathyroid glands in the fourth pharyngeal pouches migrate together with the parafollicular C-cells of the thyroid. This pair of glands frequently presents themselves on the dorsal side of the upper thyroid lobes, inferior to the cricoid cartilage. In the third pharyngeal pouches the inferior glands migrate down, crossing the fourth pouches, together with the thymus precursor. This pair of glands is commonly located proximal to the inferior pole of each thyroid lobe, but may end up in the carotid sheath or the superior mediastinum within the thymus as a result of alternate migration. (Figure 3)



**Figure 3.** Migration of the third and fourth gill pouches and their respective tissues. *Left:* Human embryo, gill arches are represented by their Roman numeral (I-IV). The ultimobranchial body migrates with the superior parathyroid glands, forming C-cells of the thyroid gland. *Right:* Anterior view of a human adolescent neck. Note that the parathyroid glands are located behind the thyroid gland.



**Figure 4.** Microscopic appearance of parathyroid adenoma cells. *Left:* Hematoxylin & eosin staining showing areas with oxyphilic cells (A) and chief cells (B). *Right:* Immunohistochemical detection of parathyroid hormone (PTH) in a chief cell adenoma.

## Histology

Macroscopically the glands color from brown to dark yellow and the normal size is equivalent to that of a rice grain, usually weighing less than 75 mg. (Dufour and Wilkerson, 1983, Akerstrom et al., 1984, DeLellis, 1993) The glands are composed of parenchyma and stroma. The majority of parenchyma cells are chief cells. These are PTH producing, compact cells with a pale cytoplasm and central dense round nuclei. The other main parenchymal cell type is the oxyphil or oncocytic cell, which is larger than the chief cell with eosinophilic cytoplasm and rich in mitochondria. (Figure 4) Oxyphilic cells first appear at puberty, and while they produce PTH the nature of these cells is considered equivocal. (Tanaka et al., 1996) A current hypothesis proposes that oxyphilic cells are aged chief cells, based on the fact that parathyroid tissue from older patients has a higher amount of oncocytic cells. In addition, glycogen-rich cells referred to as clear chief cells may be present. The stroma is a framework of supportive cells consisting of fibroblasts-, adipose-, and immune cells. Furthermore, each gland is encapsulated by a thin layer of connective tissue.

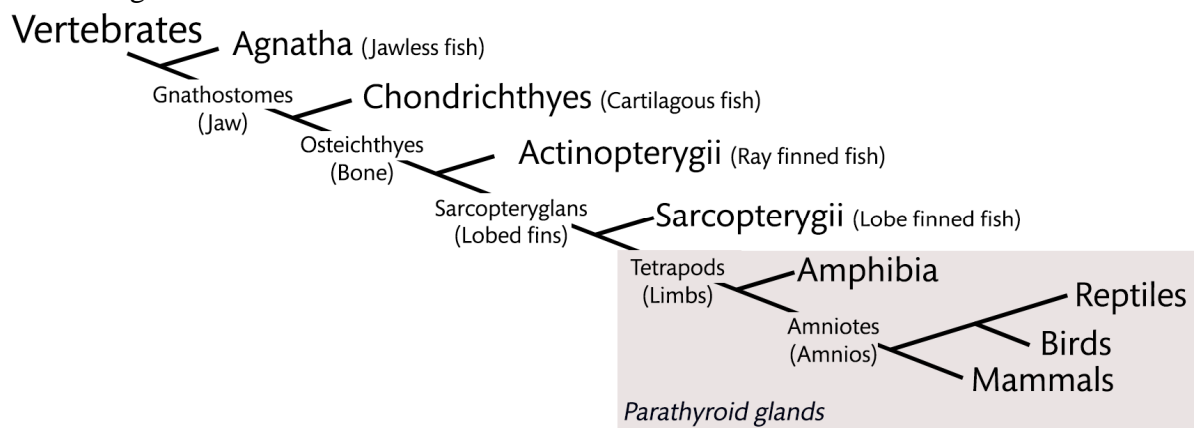
## A Darwinian perspective

“The chicken is only an egg’s way for making another egg.”

– Richard Dawkins

The calcium metabolism varies considerably between species as a result of evolutionary development to fit a habitat. It is known that parathyroid glands are present in tetrapods but absent in fish. This led to the hypothesis that parathyroid cells were unique to terrestrial and amphibious life – first emerging in land based creatures. While aquatic life has an abundant external source of calcium, terrestrial life needs to be able to mobilize calcium from the skeleton. This is reflected by the importance of hypocalcemic hormones, such as calcitonin, in aquatic mammals. Recently, genes homologous to the human *parathyroid hormone gene (PTH)* were found in fish. (Danks et al., 2003, Gensure et al., 2004)

Studies of syndromes presenting with congenital hypoparathyroidism have revealed nine genes whose expression is required for parathyroid development in the pharyngeal pouches. (Brandi, 2011) Using a phylogenetic approach, the expression of one of these genes - *Gcm2* - was demonstrated in Actinopterygii (bony fish, e.g. zebrafish) and Chondrichthyan (cartilaginous fish, e.g. sharks) species. (Figure 5) Interestingly, the *Gcm2* expression was localized to the gill buds, co-localizing with the expression of two genes homologous to human *PTH* and the *calcium sensing receptor gene (CASR)* in fish. This suggests an evolutionary relationship between the gills and parathyroid glands. (Okabe and Graham, 2004) The evolutionary perspective of parathyroid gland formation may contribute to the understanding of parathyroid physiology and tumorigenesis in man.



**Figure 5.** Phylogenetic tree showing the occurrence of the parathyroid glands during the evolutionary development of vertebrates.

## Parathyroid physiology

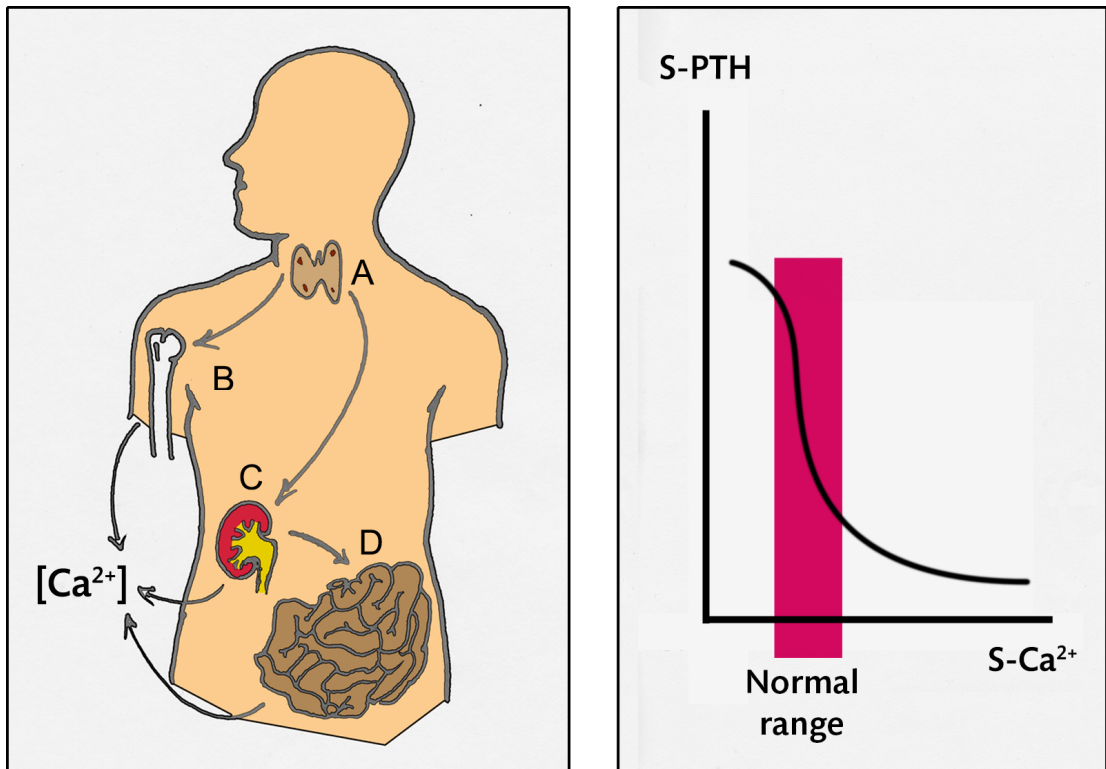
Calcium ( $\text{Ca}^{2+}$ ) is an ion that has a multitude of roles in cellular physiology, e.g. subcellular signaling, exocytosis and enzymatic regulation. Since it is such a crucial ion for normal cellular function an intricate network of regulatory systems tightly controls its extracellular concentration. Specialized cells in multicellular organisms may also be dependent on calcium as a regulatory element for their functionality e.g. muscular contraction, intravascular coagulation, neuron signaling, and bone formation. Indeed, it is hypothesized that ossified bone developed to store calcium and other minerals, rather than to provide mechanical stability. One of the most important regulators of calcium homeostasis is the parathyroid hormone (PTH).

Calcium is provided from external (dietary) sources, and the uptake is achieved by active and passive transport across the small intestinal microvilli. The kidneys continuously excrete and resorb calcium through the urinary system. The main depot is the bone, where approximately 99% of the body's calcium is stored in the form of calcium phosphates. In addition to the parathyroid hormone-family, many regulatory systems may affect calcium metabolism including e.g. calcitonin, vitamin D and estrogens. Based on the clinical presentation of hyperparathyroidism (HPT), we tend to think that PTH is solely a mediator of calcium increase. However, the actions of PTH are more diverse in normal physiology.

### ***PTH and PTH receptors***

Parathyroid cells exert their function through their unique production of PTH, an 84 amino acids hormone. It is synthesized as a prepro-hormone, and stored in intracellular vesicles after protein cleavage. Through a cascade of subcellular signaling, the vesicles are emptied into the extracellular space through exocytosis. The hormone is then rapidly distributed throughout the body, and undergoes further cleavage in the liver and kidney. (Habener et al., 1984) Its half-life is estimated to around 4 minutes *in vivo*. (Bieglmayer et al., 2002)

Parathyroid hormone related protein (PTHrP) is another member of the parathyroid hormone family. It is produced by the placenta, and is thought to mainly act through auto- and paracrine mechanisms. PTHrP has the same N-terminal domain as PTH and



**Figure 6.** *Left:* Target organs of PTH. PTH is released from the parathyroid glands in response to low serum calcium (A), which increase osteoclast activity and osteoclastogenesis in the bone (B), and increase calcium reabsorption and vitamin D activation in the kidney (C). Vitamin D increase calcium uptake in the intestine (D). The net effect of the events in B-D is an increase in serum calcium. *Right:* The normal relationship between serum calcium and PTH. The red field indicates the normal range of serum calcium i.e. 2.2-2.6 total serum calcium or 1.1-1.4 mmol/L ionized serum calcium.

may exert PTH-like effects. Generally, small amounts are present in the circulation. It has a characterized function in e.g. chondrocyte differentiation, placental calcium transport during pregnancy and calcium mobilization during lactation; but is more commonly known for its part in paraneoplastic syndrome. (Broadus et al., 1988, Wysolmerski 2012)

PTH binds to the parathyroid hormone receptors PTH1R and PTH2R that are both members of the secretin family of 7-transmembrane (7TM) receptors. PTH1R is mainly expressed in bone and kidney tissues and is able to bind both PTH and PTHrP. It activates the GTP-binding proteins  $G\alpha_s$  and  $G\alpha_q$ , thus increasing adenylyl cyclase and phospholipase C activity. The cellular response to PTH can vary dramatically depending on the ligand concentration, intensity, pulsatility, and subcellular constitution. (Juppner, 1994, Usdin et al., 1995, Mannstadt et al., 1999) PTH2R do not bind to PTHrP, but have affinity for PTH and tuberoinfundibular peptide of 39 residues

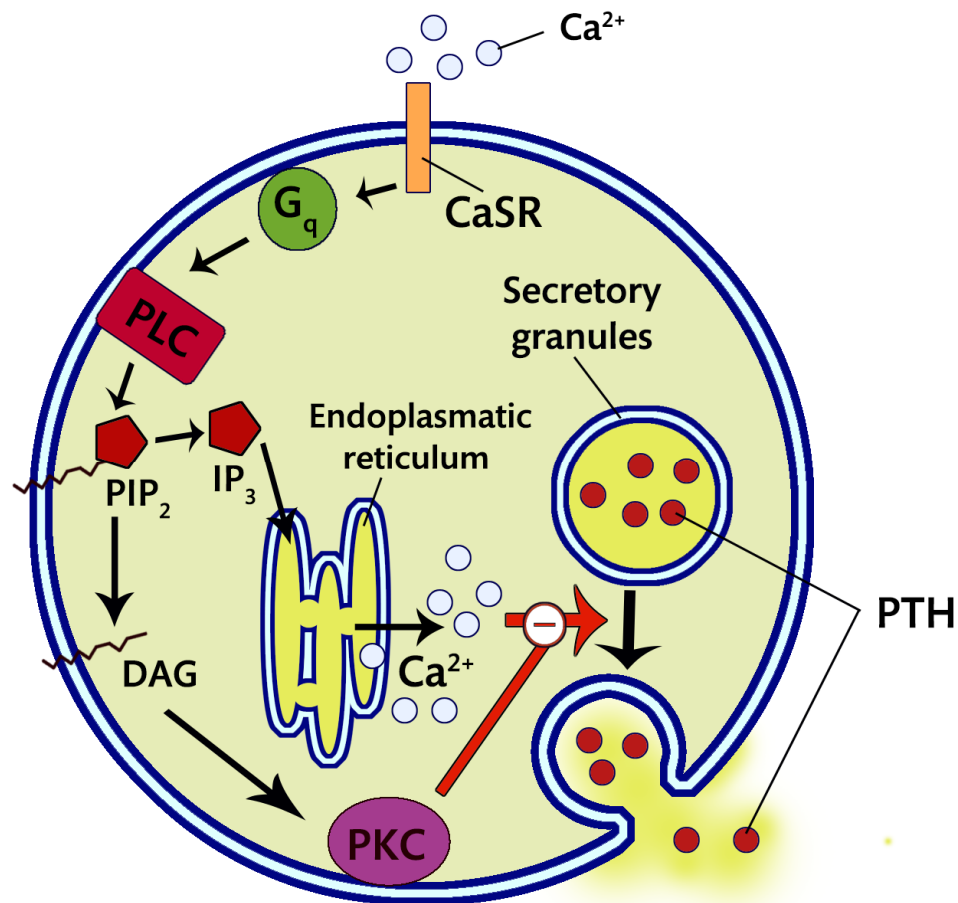
(TIP39), another member of the PHT gene family. (Usdin et al., 1999) PTH2R receptor is abundantly expressed in the nervous system where TIP39 is also expressed, and has been suggested to regulate pituitary and nociceptive functions. (Dobolyi et al., 2012)

### ***PTH & calcium homeostasis***

The bone and kidneys are considered the principal target tissues of PTH. (Figure 6) In bone PTH have both anabolic and catabolic functions depending on the secretory level and pattern. At intermittent high levels, anabolic effects in the bone are predominant. A combination of osteoblast activity and a cascade of growth factors, cytokines and eicosanoids are thought to mediate this effect. (Hock and Gera, 1992, Esbrit and Alcaraz, 2013) Therefore, administration of intermittent PTH is used in osteoporotic patients to increase bone density. (Neer et al., 2001) At continuously high levels PTH indirectly stimulate osteoclast activity and osteoclastogenesis resulting in the catabolic release of calcium and phosphate from the bone. (Fuller et al., 1998) In the kidney, PTH acts on the distal tubules and thick ascending limb by increasing the reabsorption of calcium and decreasing the reabsorption of phosphate, the latter resulting in a decrease of serum phosphate. PTH also increase the activation of cholecalciferol in the kidney by up regulating the 25-hydroxy D<sub>3</sub> 1-alpha-hydroxylase. (Henry et al., 1974, Kumar et al., 2012) In turn, vitamin D acts on the intestine to increase calcium uptake. (Norman, 1979) Recent studies have identified PTH receptors in the intestine, suggesting also a direct effect of PTH in the intestine. (Nemere and Larsson, 2002) Moreover, PTH has vasodilator and hypotensive effect in the rat vascular system as well as ionotropic and chronotropic effects in the heart. (Mok et al., 1989, Ogino et al., 1995)

### ***Regulation of PTH***

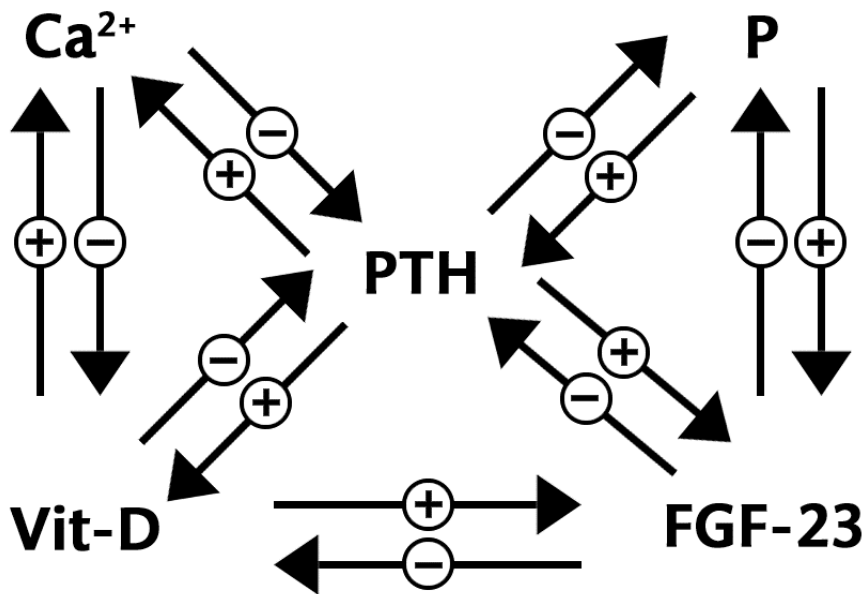
The main regulator of PTH secretion is the extracellular calcium level, and the calcium sensing receptor (CaSR) is regarded as the principal mediator of this regulatory relationship. CaSR is a G-protein coupled receptor, whose activity increase with increased levels of extracellular calcium. (Brown et al., 1993) Downstream signaling has been coupled to phospholipase C (PLC), A<sub>2</sub> (PLA<sub>2</sub>), D (PLD), mitogen-activated protein kinase (MAPK) and cAMP. (Brown and MacLeod, 2001) An increase in serum calcium will cause CaSR signaling to increase the activity of PLC, increasing inositol



**Figure 7.** Schematic model of intracellular signaling pathway resulting in PTH secretion. DAG = Diacyl-glycerol; PKC = Protein kinase C; PIP<sub>2</sub> = Phosphatidylinositol 4,5-bisphosphonate; IP<sub>3</sub> = Inositol triphosphate.

triphosphate (IP<sub>3</sub>) and diacyl glycerol (DAG) levels, and resulting in intracellular release of calcium from the intracellular calcium storages (endoplasmic reticulum). In parathyroid cells, contrary to all other cell types, intracellular calcium levels negatively regulate exocytosis. Thus an increase in extracellular calcium results in a decreased PTH secretion. (Coburn et al., 1999) (Figure 7) For any given serum Ca<sup>2+</sup> level there is a corresponding level of PTH secretion. The sensitivity to calcium of the parathyroid glands is reflected by the *calcium-PTH set point*, defined as the serum calcium level required to induce half of the maximum PTH secretion. (Figure 6) This set point may be altered by the quantity of CaSR expressed in the cellular membrane, as well by modulation of CaSR activity by e.g. magnesium or calcimimetics. (Cetani et al., 2000, Drueke, 2004)

Since calcium levels are regulated by multiple systems, it is not straightforward to establish whether a given substance has a direct effect on PTH secretion or not in an *in vivo* setting. However several regulatory systems have been proposed, such as negative



**Figure 8.** Relationships between PTH and serum calcium (Ca<sup>2+</sup>), serum phosphate (P), vitamin-D (Vit-D) and fibroblast growth factor 23 (FGF-23). Picture inspired by Imanishi et al. (Imanishi et al., 2009)

feedback through cholecalciferol/vitamin D receptor (VDR) and FGF23/Klotho, and positive feedback through phosphate. (Nielsen et al., 1996, Ben-Dov et al., 2007, Meir et al., 2009) (Figure 8) Additional effects mediated by glucocorticoids, estrogens and progesterone have been suggested. (Duarte et al., 1988)

## HYPOPARATHYROIDISM

Hypoparathyroidism is a state defined by insufficient amounts of biologically available PTH. This is most commonly caused by accidental iatrogenic removal or damage of parathyroid glands during surgery or neck irradiation. Several genetic causes are described including the 22q11.2 deletion syndrome, polyglandular autoimmune syndrome type 1, hypoparathyroidism-dysmorphism-retardation syndrome and familial isolated hypoparathyroidism. Hypoparathyroidism has also been described as part of mitochondrial disorders. (Neufeld, 1980, Finnish-German APECED Consortium, 1997, Brandi, 2011) It should be noted that pseudohypoparathyroidism could mimic hypoparathyroidism. It is a condition where patients exhibit resistance to PTH together with normal or elevated PTH levels.



# HYPERPARATHYROIDISM

Hyperparathyroidism (HPT) is defined as an abnormal increase in PTH levels. Primary hyperparathyroidism (PHPT), the most common form of HPT, is a state of inadequately high PTH as compared to serum calcium levels. It is caused by the faulty regulation of PTH secretion in one or more of the parathyroid glands. In PHPT, the continuously high levels of PTH have considerable hypercalcemic effects, with a marked increase in bone turnover. Secondary hyperparathyroidism (SHPT) is a physiological responsive state. It may be caused by hypocalcemic states, vitamin-D deficiency or inability to activate vitamin D, e.g. liver or kidney failure. In SHPT a reactive hyperplasia of the parathyroid glands may often be observed. After a long period of SHPT, a reversible autonomous secretion of PTH may develop, referred to as tertiary hyperparathyroidism (THPT). Some environmental factors for the development of HPT have been described such as lithium treatment (Garfinkel et al., 1973) and ionizing radiation. Pharmacological treatments lowering urine calcium secretion, e.g. thiazide diuretics, cause a mild hypercalcemia and may unmask underlying PHPT, but whether they affect PHPT development is not known.

## PRIMARY HYPERPARATHYROIDISM (PHPT)

### Clinical presentation

In 1957 Walter St. Goar introduced a well-known description of PHPT: “Stones, bones, abdominal groans and psychiatric moans” referring to signs and symptoms from:

- Stones: nephrolithiasis, nephrocalcinosis and diabetes insipidus
- Bones: osteitis fibrosa cystica and osteoporosis
- Abdominal groans: constipation, dyspepsia, acute pancreatitis and peptic ulcer
- Psychiatric moans: fatigue, depression, dementia, delirium and coma

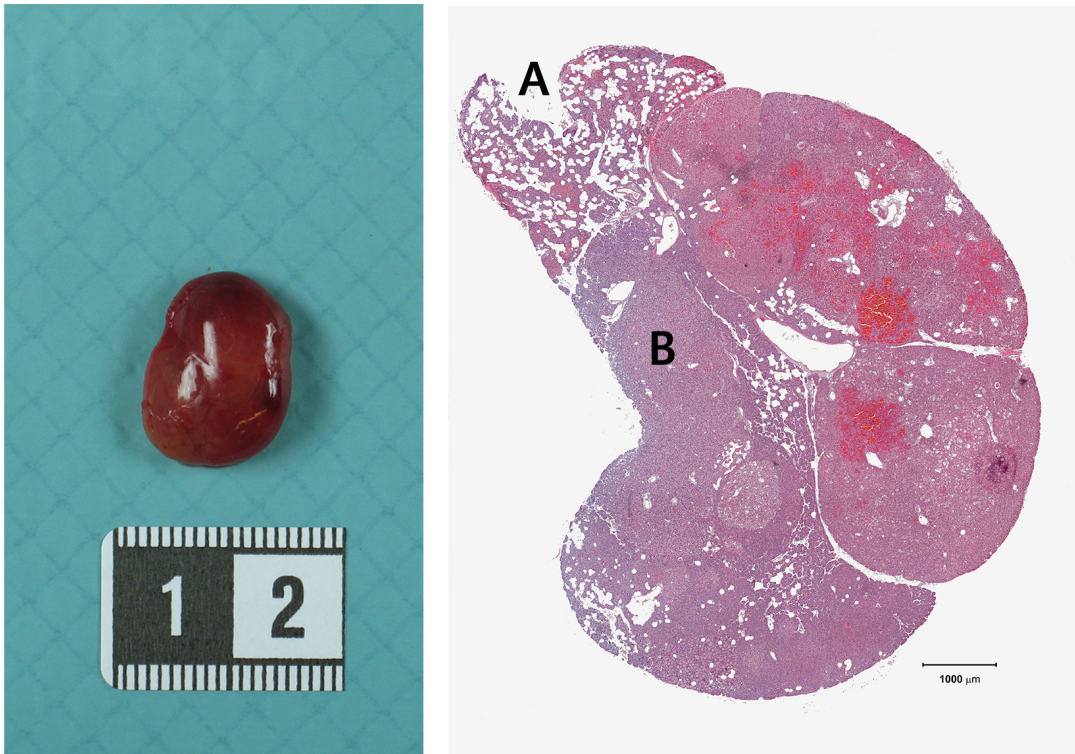
Since the introduction of routine measurement of serum calcium during the 70's, the clinical presentation of PHPT has dramatically changed. Pronounced symptoms are getting increasingly rare as the diagnosis is often made at an early stage. Nowadays the most common presentation is nephrolithiasis or none of the classic PHPT symptomatology (including nephrolithiasis, renal failure and osteoporosis). (Fraser, 2009) Nevertheless, patients may suffer from increased morbidity and overall mortality, including peripheral polyneuropathy, decreased kidney function and osteopenia,

cardiovascular- and cerebrovascular events as well as cancer. (Ronni-Sivula, 1985, Palmer et al., 1987, Hedback et al., 1990, Ljunghall et al., 1991, Hedback and Oden, 2002, Nilsson et al., 2002, Nilsson et al., 2007, Diniz et al., 2013) Some patients with PHPT do not have elevated calcium, which is designated normocalcemic PHPT. It is presently unclear what is the best clinical management of this patient group, especially regarding the benefits of parathyroid adenectomy. (Shlapack and Rizvi, 2012)

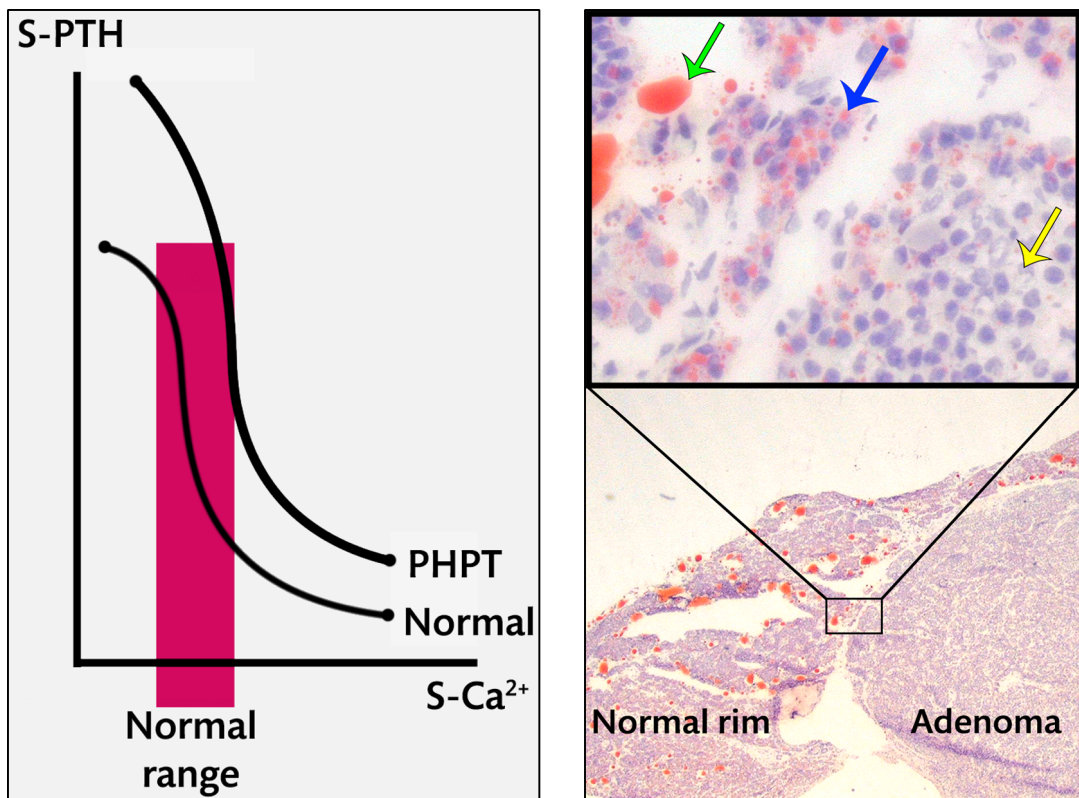
## **Parathyroid adenoma**

Tumorous growth in one or more parathyroid glands and pathological change in calcium-PTH set point are two hallmarks of PHPT. Histopathologically three main types of parathyroid tumors are classified i.e. adenoma, atypical adenoma and carcinoma. (DeLellis, 2004) In addition the disease may be uniglandular or less commonly multiglandular. Thus, the histopathological diagnosis can be established only if a biopsy from another gland is found to be normal. A single parathyroid adenoma is most common, constituting ~85-90% of all PHPT. (Figure 9) In 10-15% the disease is multiglandular, and <1% have parathyroid carcinoma. (Marx, 2000)

Several studies have tried to characterize the clonality of uniglandular and multiglandular PHPT; however, different results have been obtained suggesting both mono- and polyclonal origins. (Arnold et al., 1988, Noguchi et al., 1994, Arnold et al., 1995, Tominaga et al., 1996, Sanjuan et al., 1998, Shan et al., 1999) Histopathologically the adenoma presents a fat depleted neoplasia of parenchymal cells. In 50-60% remnants of normal tissue is present adjacent to the tumor. (Figure 9) Most adenomas have a majority of chief cells and may also include a minority of oxyphilic cells (so called chief cell adenomas). Oxyphilic tumors are less common, constituting ~3% of all adenomas. The pathological increase in PTH release is due to a change in the calcium-PTH set point, which is shifted to the right and up. (Figure 10) It is yet to be proven whether sporadic parathyroid tumors harbor primary changes in set point altering molecules that could cause the tumorous growth.



**Figure 9.** Parathyroid adenoma. *Left:* Macroscopic appearance. *Right:* Microscopic overview of a parathyroid adenoma, showing normal parathyroid rim (A) and tumor tissue (B).

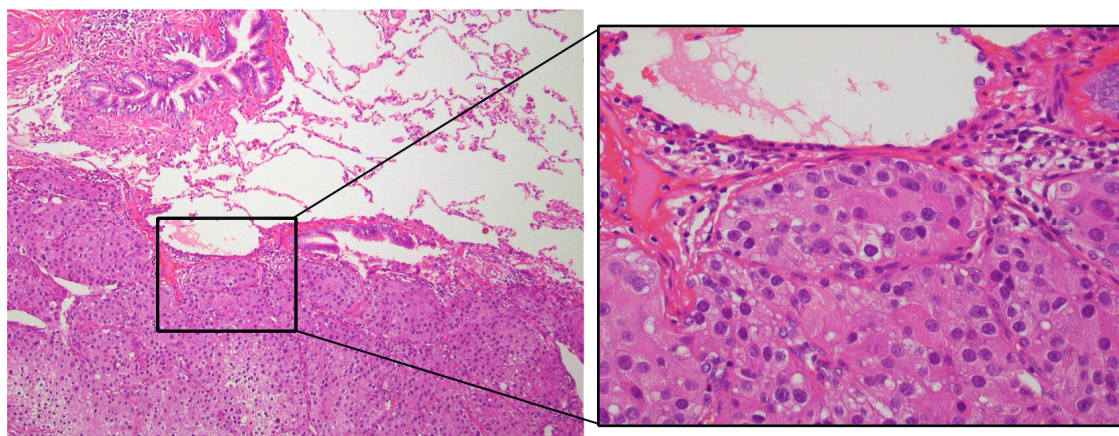


**Figure 10.** *Left:* Set point in PHPT, shifted to the right and up. *Right:* Oil red and hematoxylin stained fresh frozen section of a parathyroid adenoma. Oil red binds to lipids and triglycerides reflecting the functional activity of the parathyroid cells. Hyperfunctioning parathyroid adenoma with reduced lipid droplets (yellow arrow). Resting normal parathyroid cells with increased number and size of lipid droplets (blue arrow). Normal adipocyte present in normal parathyroid rim (green arrow).

## Parathyroid carcinoma and atypical adenoma

Parathyroid malignancy is a rare entity. Parathyroid carcinomas primarily metastasize to adjacent neck tissue, however both hematogenic and lymphogenic spreading occur. The clinical manifestation is a combination of tumor growth and pronounced hypercalcemia, which may present with hypercalcemic crisis. Preoperatively, signs of malignancy include a palpable neck mass, severe HPT or hypercalcemia. (Schulte and Talat, 2012) In the absence of metastases, the diagnosis constitutes a challenge for the pathologist. Indirect signs of malignancy are macroscopic findings of a hard mass, adherence to adjacent tissues or a dense fibrous capsula; or microscopic findings of fibrous trabeculae, capsular invasion, vascular or perineuronal invasion or mitosis.

According to the current WHO classification, the only definite criteria of parathyroid tumor malignancy are tissue invasion or distant metastasis. (Figure 11) Tumors with indirect signs of malignancy, but lacking metastases are classified as atypical adenomas of unknown malignant potential. (DeLellis, 2004) This entity was previously referred to as equivocal carcinomas as opposed to unequivocal carcinomas that exhibit metastases. Recent genetic studies suggest that parathyroid adenomas and carcinomas follow genetically different pathways. (Sulaiman et al., 2012, Costa-Guda et al., 2013a) These observations would suggest that parathyroid carcinomas develop either *de novo* or through clinical progression of predisposed tumors first appearing as adenomas without metastases. The advancement in molecular genetics has identified several immunohistochemical markers that may aid in the diagnostics of parathyroid malignancy. (Tan et al., 2004, Juhlin et al., 2010b, Wang et al., 2012)

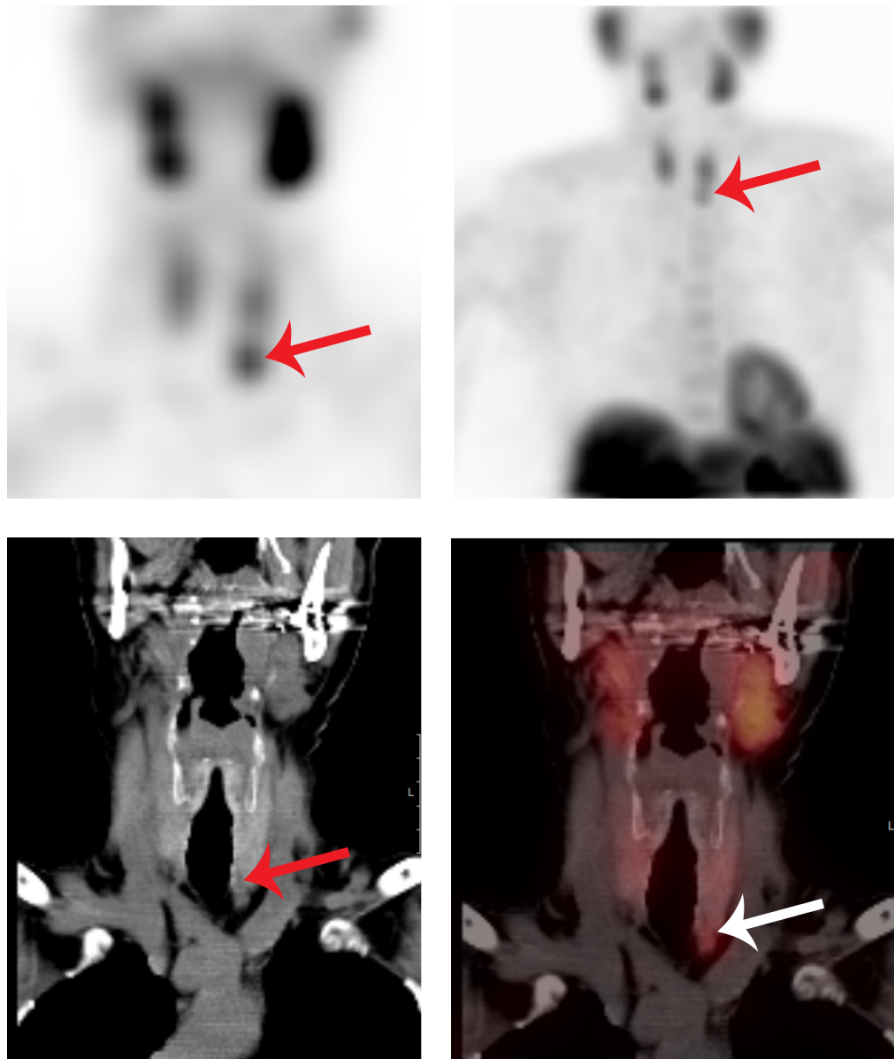


**Figure 11.** Histopathological appearance of a parathyroid carcinoma metastasized to the lung.

## Treatment of PHPT

### *Surgical treatment*

PHPT is commonly cured by surgical removal of the diseased gland(s). Since multiglandular disease occurs, bilateral neck exploration with inspection of all glands was the common surgical approach. (AACE/AAES Task Force on Primary Hyperparathyroidism, 2005) Today the use of preoperative tumor mapping by ultrasound and <sup>99</sup>technetium-labeled Sestamibi radionuclear examinations, (Figure 12) have led to increased application of focused neck explorations. Furthermore, PTH has a short half-life, and therefore successful removal of all pathological glands can be confirmed *per operatively* by hormone measurement. In multiglandular disease, the approach is to remove nearly all parathyroid tissue, leaving the equivalency of a single normal gland.



**Figure 12.** Preoperative detection of a parathyroid adenoma. *Upper left and right:* Sestamibi scintigraphy in detail and overview. *Lower left:* CT-scan of the neck region. *Lower right:* Combination of the upper and lower left pictures. A red or white arrow indicates the parathyroid adenoma. Image courtesy of Dr. Anders Sundin.

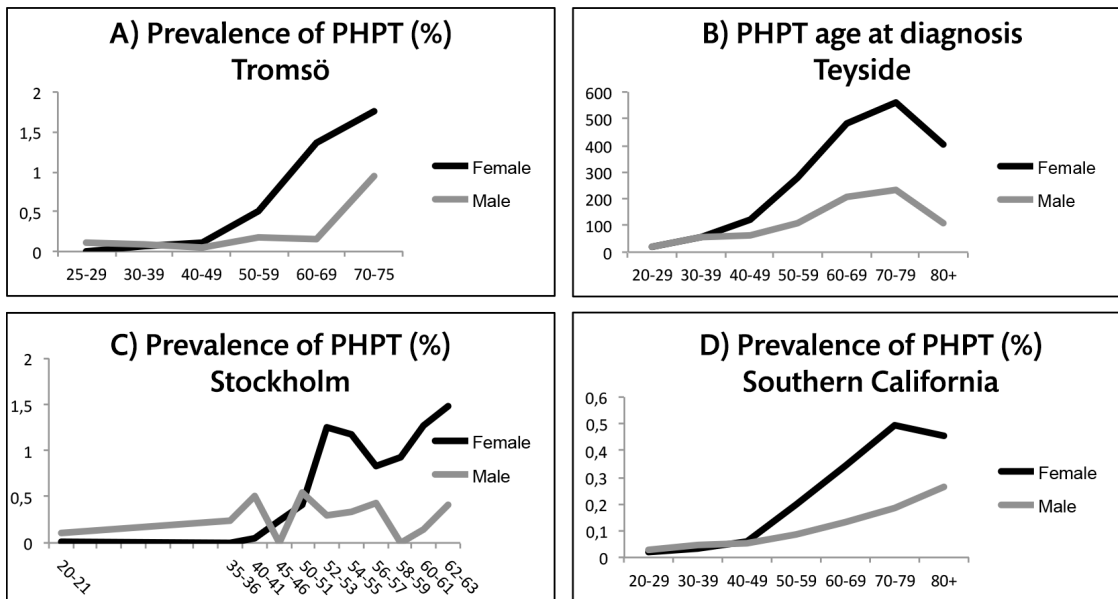
## ***Medical management of PHPT***

Surgery is the gold standard of PHPT treatment. In some cases however, it may not be applicable. In such situations a medical regime may be necessary to reduce symptomatology or complications. In postmenopausal women, hormone replacement therapy or raloxifene may be considered. (AACE/AAES Task Force on Primary Hyperparathyroidism, 2005) Bisphosphonates, especially alendronate, have been found to improve bone structure. (Rossini et al., 2001, Chow et al., 2003) Finally, the calcimimetic cinacalcet directly lower PTH and calcium through agonistic effects on CaSR. (Peacock et al., 2005) All pharmacological regimes carry side effects and are not always compatible with the treatment of a chronic disease.

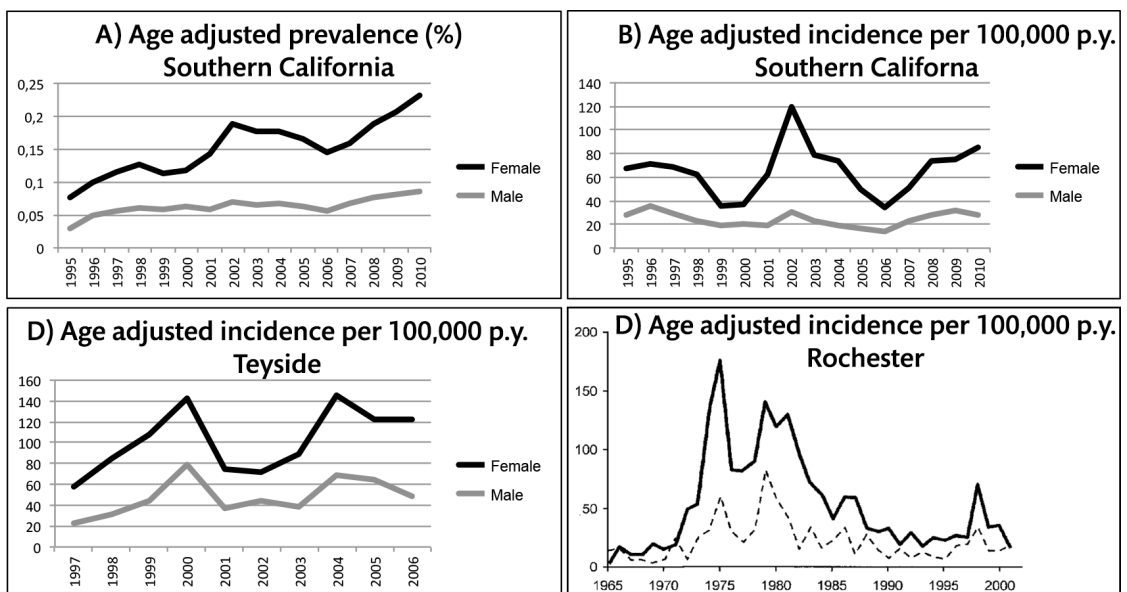
## **Epidemiology**

The prevalence and incidence of PHPT is difficult to estimate. Screening of populations in the USA, Sweden and Scotland has tried to identify the true prevalence of the disease. (Boonstra and Jackson, 1965, Christensson et al., 1976, Palmer et al., 1988, Yu et al., 2009) Estimates vary depending on the age of the population, the method of screening, cut-offs for increased calcium or PTH and the laboratory methodology used. However, the true prevalence of PHPT is thought to be around 1% in the general population. Studies coherently report the highest prevalence in post-menopausal women, with estimations from 1.3 to 13.9%. (Christensson et al., 1976, Sorva et al., 1992, Lundgren et al., 1997, Jorde et al., 2000) (Figure 13)

Both Yu et al. (Yu et al., 2009) and Yeh et al. (Yeh et al., 2013) reported a threefold increase in incidence from 1997 to 2006 and in prevalence from 1995 to 2010, respectively. (Figure 14) The cause of this increase remains to be determined, but is speculated to be due to increased diagnostics of mild PHPT. The reported incidence of PHPT is highly dependent on the number of calcium measurements performed. Reports have estimated the incidence around 27-30 per 100,000 person years in the general population, (Fraser, 2009) and greatly increased in females aged 70-79 (95-196 per 100,000 person years). (Yeh et al., 2013) The sex distribution is equal before 50 years of age, after which a stark increase in female incidence occur.



**Figure 13.** Age distribution of PHPT prevalence in Tromsø, Norway (A); Teyside, Scotland (B); Stockholm, Sweden (C); and Southern California, USA (D). An increased incidence of PHPT is noted in women aged >50 years. The x-axes show the ages in years and the Y-axis the prevalence of PHPT (A, C and D) or the number of cases (B).

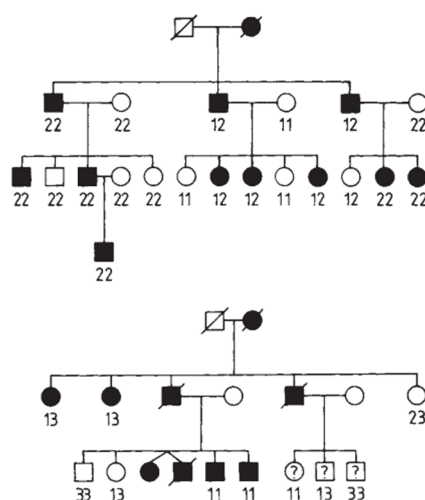


**Figure 14.** Changes over time in age adjusted PHPT concerning prevalence in Southern California, USA (A); incidence in Southern California, USA (B); and incidence in Teyside, Scotland (C). Wermers et al. published changes in age-adjusted incidence per 100,000-person years of definite plus possible PHPT (y-axis) among Rochester women (solid line) and men (dashed line) (D). Reprinted with permission from John Wiley and Sons. (Wermers et al., 2006) The X-axes show the years under study and the Y-axes age-adjusted prevalence of PHPT (A), age-adjusted incidence per 100,000-person years (B, C and D). p.y. = person years

The Mayo Clinic in Rochester has followed the PHPT incidence from 1966 to date. (Figure 14) The introduction of routine calcium measurements during the 1970's resulted in a striking increase in incidence (i.e. diagnosed cases) both in Rochester, MN and Verona, Italy. (Adami et al., 2002, Wermers et al., 2006) The continuous fall in incidence of PHPT since the 1970's suggesting a change in an etiological factor. Wermers et al. proposes that this may be attributed to a decreased use of head and neck irradiation, changes in dietary calcium and vitamin D or hormone replacement therapy. Another disease which incidence is dependent on head and neck irradiation is thyroid cancer. However, the incidence of these entities did not correlate in the Rochester population. Additionally, no evidence supports an improved calcium or vitamin D nutrition during this time course. Hence, there is no support for head and neck irradiation or calcium / vitamin D nutrition being the responsible factor for these changes in incidence. (Wermers et al., 1997) It is unknown whether altered usage of hormone replacement treatment after the Women's Health Initiatives report will affect PHPT incidence. (Lawton et al., 2003) The incidence of PHPT seems to fluctuate over time, but no evident factor has been able to explain this. (Figure 14)

## Familial forms of PHPT

The majority of PHPT cases are considered to be sporadic. The study of families with a genetic predisposition to PHPT has led to the identification of genes and signaling pathways that are also involved in subsets of sporadic tumors.



**Figure 15.** Pedigrees of two families affected by MEN 1 showing linkage to a DNA marker in chromosomal region 11q13. Figure from Larsson et al. (Larsson et al., 1988)



### ***Multiple endocrine neoplasia type 1 (MEN 1) and the MEN1 gene***

Multiple endocrine neoplasia type 1 (MEN 1), or Wermer's syndrome, is an autosomal dominant disorder caused by mutations in the *Multiple Endocrine Neoplasia (MEN1)* gene. The disease locus was originally mapped to 11q13 by LOH and linkage analysis in affected families, (Larsson et al., 1988) (Figure 15), and isolated by positional cloning. (Chandrasekharappa et al., 1997, Lemmens et al., 1997) The syndrome has a high penetrance of parathyroid adenomas, often with multiglandular engagement. Additionally, patients may develop tumors of the endocrine pancreas and duodenum, anterior pituitary and less commonly adrenal cortex, thymus, gastrointestinal tract, bronchi and soft tissues. The *MEN1* shows frequent inactivating mutations in affected patients, and the 11q13 region is frequently lost in MEN 1-related parathyroid tumors. Mutations in the *MEN1* gene are also present in 25-35% of sporadic parathyroid adenomas. (Friedman et al., 1989, Heppner et al., 1997, Carling et al., 1998, Farnebo et al., 1998, Imanishi and Tahara, 2001) Additionally, MEN 1 is estimated to constitute 1-18% of all cases of PHPT, and is believed to be the most common hereditary tumor syndrome that engages the parathyroid glands. (Brandi et al., 1987) The *MEN1* gene encodes the protein menin, coupled to numerous molecular functions including wntless (Wnt) signaling, JunD, SMAD interaction and genome methylation. (Agarwal et al., 1999, Hughes et al., 2004, Hendy et al., 2005, Chen et al., 2008) Menin is considered to be a tumor suppressor but oncogenic properties have also been reported. (Yokoyama et al., 2005) A recent study reported *MEN1* intragenic deletions in 13/24 parathyroid adenomas using FFPE material, but this alteration remains to be reproduced. (Alvelos et al., 2013)

### ***Multiple endocrine neoplasia type 2 (MEN 2) and the RET gene***

Mutations in the *REarranged during Transfection (RET)* gene located at 10q11 cause the autosomal dominant syndrome multiple endocrine neoplasia type 2. (Mulligan et al., 1993) In the most common form MEN 2A, or Sipples's syndrome, the patients present with medullary thyroid carcinoma, pheochromocytoma and parathyroid adenoma. (Steiner et al., 1968) In addition isolated familial medullary thyroid carcinoma and the aggressive form MEN 2B occur. The *RET* gene encodes a tyrosine kinase receptor, activating PI3K/Akt, ERK, JNK, MAPK and Wnt signaling cascades. (Ichihara et al., 2004, Tartari et al., 2011) There is a distinct genotype-phenotype correlation as the location of the mutation determines the MEN2 presentation. (Witt et

al., 2013) Studies have failed to identify *RET* aberrations in sporadic parathyroid tumors. (Padberg et al., 1995, Williams et al., 1996)

### ***Multiple endocrine neoplasia type 4 (MEN 4) and the CDKN1B/P27KIP1 gene***

In recent years, the occurrence of spontaneous multiple endocrine tumors in a rat colony were accidentally discovered. (Piotrowska et al., 2004) No mutations were present in the *RET* or *MEN1* genes. The colony developed anterior pituitary adenomas, bilateral pheochromocytomas and paragangliomas, thyroid C-cell and pancreatic islet cell hyperplasia. Since this phenotype resembled, but did not constitute either MEN 1 or MEN 2 syndromes, it was named MEN X. After identifying mutations in the *Cdkn1b/p27<sup>Kip1</sup>* gene in mouse, the same gene was screened in *MEN1* mutation negative patients presenting with MEN 1 syndrome. Indeed several *CDKN1B/P27KIP1* mutations were discovered, and the syndrome was named MEN 4. (Pellegata et al., 2006) In human it is characterized mainly by PHPT followed by pituitary adenomas and other neuroendocrine lesions.

The involvement of cyclin D1 (described below) and cyclin dependent protein kinases in parathyroid tumorigenesis motivated Costa-Guda et al. to sequence cyclin-dependent kinase inhibitor genes in sporadic parathyroid adenomas. Mutations in *CDKN1A*, *CDKN2B* and *CDKN2C* (p21, p15 and p18) were found in 6% of all examined cases, at least half of these harboring germline mutations. (Costa-Guda et al., 2013b)

### ***Hyperparathyroidism-Jaw Tumor Syndrome (HPT-JT) and the HRPT2/CDC73 gene***

Mutations in the *Hyperparathyroidism 2 (HRPT2) / Cell division cycle 73 (CDC73)* gene cause the autosomal dominant hyperparathyroidism-jaw tumor (HPT-JT) syndrome. (Carpten et al., 2002) The *HRPT2/CDC73* gene is located in 1q31.2. Patients have a high risk of developing parathyroid tumors, which carries increased risk of malignancy, fibro-osseous jaw tumors, renal and uterine lesions. (Teh et al., 1996, Fujikawa et al., 1998) *HRPT2/CDC73* encodes parafibromin, a component of the human Paf1/RNA polymerase II complex. (Rozenblatt-Rosen et al., 2005) Parafibromin has both tumor suppressor and oncogenic properties, and its function is coupled to Wnt and hedgehog signaling and H3K9 methylation. (Mosimann et al.,

2006, Mosimann et al., 2009, Yang et al., 2010, Takahashi et al., 2011) Mutations of *HRPT2/CDC73* frequently occur in apparently sporadic parathyroid carcinomas and are also detected in a small subset of parathyroid adenomas. (Carpten et al., 2002, Shattuck et al., 2003) Loss of parafibromin immunoreactivity has been proposed as a marker for malignancy in parathyroid tumors. (Tan et al., 2004, Juhlin et al., 2007)

### ***Familial hypocalciuric hypercalcemia (FHH) and the CASR gene***

FHH types 1-3 are caused by mutations in the *CASR* at 3q13.3, the *Adaptor protein-2* at 19p13.3, or an unknown still elusive gene mapped to 19q13, respectively. (Pollak et al., 1993, Lloyd et al., 1999, Nesbit et al., 2013) Patients present with low urinary calcium, elevated serum calcium and high or normal levels of PTH, but lack hyperplastic or adenomatous changes in the parathyroid. Thus these patients are not helped by parathyroidectomy. One family with a *CASR* mutation exhibited hypercalcemia, hypercalciuria and parathyroid tumors, i.e. features of both FHH and familial isolated hyperparathyroidism. (Carling et al., 2000) No mutations or other genetic variants in *CASR* have been found in sporadic parathyroid adenomas. (Hosokawa et al., 1995, Cetani et al., 1999, Brown, 2002, Cetani et al., 2002, Vierimaa et al., 2009) In sporadic parathyroid adenomas the expression of *CASR* is decreased, and its expression levels have an inverse relationship to the calcium-PTH set point. Down-regulation of CaSR has also been suggested as a prognostic marker in parathyroid carcinoma. (Farnebo et al., 1997, Cetani et al., 2000, Witteveen et al., 2011)

### ***Familial isolated hyperparathyroidism (FIHP)***

Families presenting with PHPT as their sole symptom are designated FIHP, which is thus an exclusion diagnosis. FIHP has been attributed to mutations in the *MEN1*, *HRPT2/CDC73* and *CASR* genes. (Teh et al., 1998, Kassem et al., 2000, Simonds et al., 2004, Warner et al., 2004) An additional locus has been assigned by linkage to 2p13.3-14, but a putative disease gene in this locus (*HRPT3*) remains to be identified. (Warner et al., 2006)

## MOLECULAR BACKGROUND OF PHPT

A constrain in elucidating the molecular background of parathyroid tumorigenesis is the limited possibility of culturing parathyroid cells. Both cytogenetic data from tumor cell metaphases and functional data from established cell lines are limited. Moreover, the comparison of tumor findings to normal parathyroid tissue is done in a small number of cases only.

Our understanding of parathyroid tumorigenesis in many aspects stems from the field of molecular genetics. As outlined above, studies of hereditary forms of PHPT have identified disease genes involved in parathyroid tumorigenesis. These genes are also mutated in subsets of PHPT adenomas and carcinomas. However, a large proportion of tumors (especially adenomas) lack an identified genetic etiology. To identify new mutations in sporadic parathyroid adenomas, two studies have used a *whole-exome sequencing* approach. In both studies, few mutational events were identified in the majority of tumors. Except for *MEN1* mutations, no mutation was frequently occurring when reproduced in a larger cohort. (Cromer et al., 2012, Newey et al., 2012) A whole-genome sequencing of a single parathyroid carcinoma, including a recurrent metastasis was recently published. (Kasaian et al., 2013) Besides loss of heterozygosity (LOH) and a missense mutation in *CDKN2C* (encoding p18), no mutations were found in genes with known association to PHPT. Several mutations in known cancer genes were identified including *MLL2*, *mTOR* and *PIK3CA*. Additionally two translocations and one inversion were identified. A logic follow-up to this study would be an analysis of these variants in additional parathyroid tumors.

By sequencing the *mitochondrial DNA* of parathyroid tumors, genetic variants were detected in 6/18 chief cell and 9/12 oxyphilic adenomas, while 8 primary chief cell hyperplasias and corresponding normal tissue were wild-type. (Costa-Guda et al., 2007) While it is debated whether mutations in mitochondrial DNA constitutes a significant event in tumorigenesis, (Taylor and Turnbull, 2005) these variants may in the future prove to be of importance.

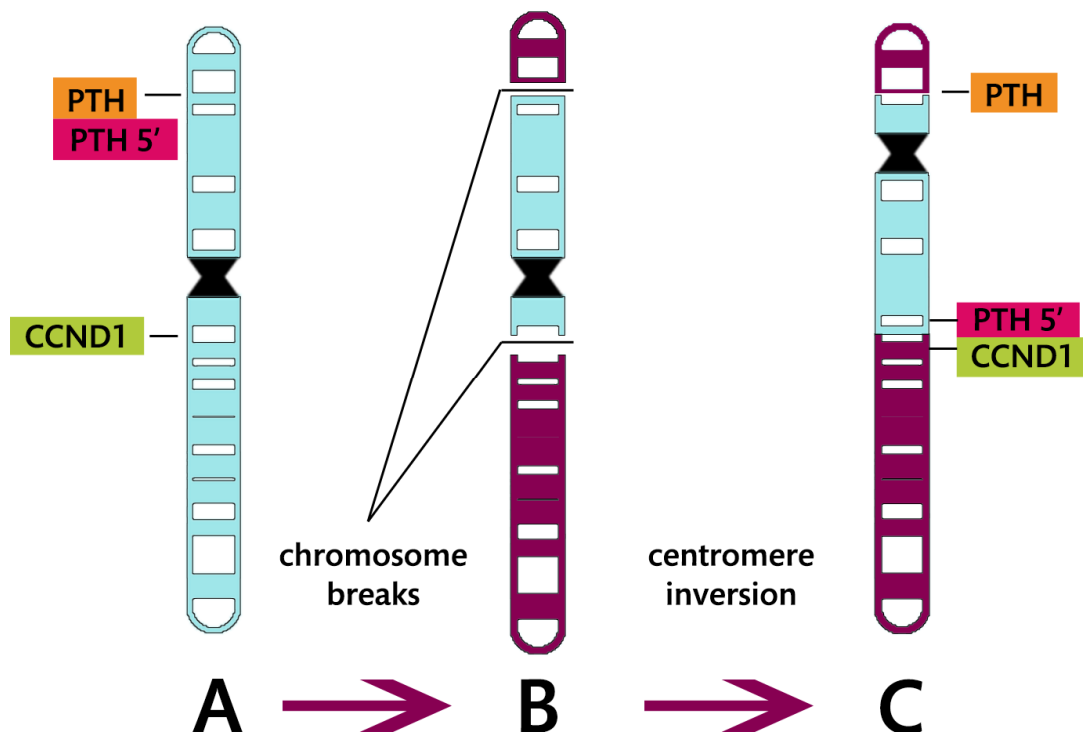
*Epigenetic modifications* of the genome are increasingly recognized in tumor development. Parathyroid tumors have so far been characterized with regard to CpG-island methylation of candidate genes, (Knutson et al., 2000, Carling et al., 2003,

Hewitt et al., 2007, Masi et al., 2008, Hahn et al., 2010, Juhlin et al., 2010a, Andreasson et al., 2012, Sulaiman et al., 2012, Svedlund et al., 2012) as well as using a DNA methylome approach. (Starker et al., 2011) The coupling of menin function to H3K4 methylation implied that parathyroid tumors may develop due to aberrant methylation; however global H3K4 methylation was not found altered in *MEN1*-related parathyroid adenomas. (Dreijerink et al., 2009) Parafibromin may recruit SUV39H1, a histone methyltransferase, to induce H3K9 - but not H3K4 - methylation. (Yang et al., 2010) A study of plant homologues of parafibromin suggested a function also in H3K27 methylation. (Park et al., 2010) Histone methylation is yet to be studied in parathyroid tumors with *HRPT2/CDC73*/parafibromin alterations. By measuring methylation of LINE-1 repeats, Juhlin et al. suggest that the global methylation levels are comparable in parathyroid adenomas and normal parathyroid tissue. (Juhlin et al., 2010a) Overall, additional data is required to assess if epigenetic events are important for parathyroid tumor development.

**Copy number alterations** (CNAs) as well as LOH of genetic material have been reported for a limited number of regions in parathyroid tumors. CNAs were first described using conventional metaphase based comparative genomic hybridization (CGH) and later by array-based CGH or SNP-based arrays. Loss in chromosome 11 is the most common CNA known in parathyroid adenomas, being present in approximately half of the cases. Other CNA losses are observed in 1p, 6q, 9, 13q, 15q, 18q, 22q and X in adenomas, while parathyroid carcinomas may present loss in chromosomes 1p and 13q. Gains are commonly found in 7, 13q, 16p, 19p and 20q in adenomas; and 1q and 16p for carcinomas. (Agarwal et al., 1998, Palanisamy et al., 1998, Farnebo et al., 1999, Kytola et al., 2000, Yi et al., 2008, Sulaiman et al., 2012) While this would indicate that some parathyroid tumor suppressor genes and oncogenes remain to be discovered, the general significance of these CNAs is yet to be elucidated.

The difficulty in culturing parathyroid cells has restrained the usage of classical cytogenetic experiments. Nonetheless, a few **chromosomal rearrangement** events have been identified in parathyroid tumors. Most notably may be the *inv(11)(p15;q13)*, resulting in the insertion of the *CCND1* (encoding cyclin D1) oncogene in front of the *PTH* promoter resulting in *PTH/CCND1* and *CCND1* over-expression. (Arnold et al., 1989) (Figure 16) At the time of this discovery the function of *CCND1* was unknown, and since then the role of cyclin D1 in cell cycle progression has been uncovered.

While this rearrangement is uncommon, cyclin D1 is over-expressed in 20-40% of parathyroid adenomas. (Arnold et al., 2002) Interestingly, transgenic mice with a *PTH/CCND1* develop biochemical HPT and parathyroid adenomas with decreased levels of CaSR. (Imanishi et al., 2001)



**Figure 16.** The *PTH / CNND1* rearrangement. A normal chromosome 11 (A) has two chromosome breaks (B) leading to an inversion around the centromere (C). This moves the *PTH* promoter in front of the *CCND1* gene forming *PTH/CCND1* (*PRAD1*).

Endocrine glands under trophic-hormone control (e.g. the hypothalamus-pituitary-adrenal axis) often react with hyperplasia when subjected to an increase in the stimulatory signal. Since the parathyroid glands are considered autonomous in function, a different feedback mechanism for trophic control should exist. The finding of decreased CaSR levels in these tumors suggests a regulatory relationship between proto-oncogenes or proliferative signals and modulators of the endocrine set point.

Using a cytogenetic approach, two additional translocations have been identified:  $t(1;5)(p22;q32)$  and  $t(4;13)(q21-24;q14-21)$ . However the involved genes or the frequency of these translocations have not been further explored. (Orndal et al., 1990, Sammarelli et al., 2007)

An *autoimmune* background has also been considered, in analogy with Morbus Graves-Basedow where activating autoantibodies target the thyrotropin receptor in the thyroid gland. Some data would support an autoimmune component in PHPT, possibly similar

to Morbus Graves-Basedow. Bjerneroth et al. detected anti-parathyroid antibodies in patients with PHPT. Additionally, serum from patients with PHPT was able to induce MHC class II expression in parathyroid adenomas transplanted in nude mice. (Bjerneroth et al., 1998) Furthermore, autoantibodies targeting the extracellular domain of CaSR were found in 5/75 patients with PHPT, but autoantibodies were neither coupled to non-curative parathyroid adenectomy nor to lymphocytic tumor infiltration. (Charrie et al., 2009)

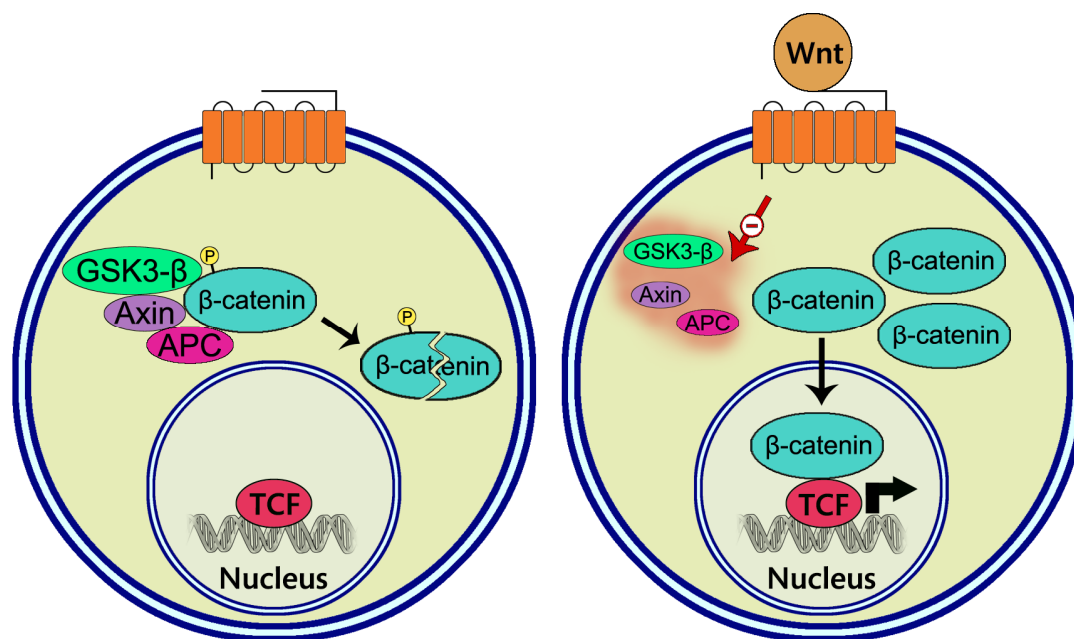
In addition to the above-described *CCND1/PTH* transgenic mice, PHPT has also been suggested in some **genetically modified mice**. These include modified knockouts of *MEN1*, *cyclooxygenase 2 (COX2)*, *prolactin receptor (PRLR)*, combined *CDKN1B-CDKN2C* (p27 and p18) and *CASR*. (Ho et al., 1995, Franklin et al., 2000, Crabtree et al., 2001, Kedzia et al., 2005, Xu et al., 2005)

Using **gene expression profiling** genetically distinct subgroups of HPT have been identified. By supervised clustering Morrison et al. showed distinct expression patterns for secondary hyperplasia, non- and familiar primary hyperplasia, parathyroid adenomas and normal parathyroid glands. Unsupervised clustering was not able to distinguish adenomas from hyperplastic tissue, although the parathyroid adenomas clustered into two groups, one being more alike hyperplastic disease. (Morrison et al., 2004) Moreover, Haven et al. identified three clusters including 1) *HRPT2/CDC73* associated tumors and parathyroid carcinomas, 2) Parathyroid hyperplasias, a pooled sample of normal parathyroid, a lithium associated adenoma, a MEN2A associated adenoma and three sporadic adenomas, and 3) *MEN1* and FIHP associated adenomas, tertiary hyperplasias and the majority of sporadic adenomas. (Haven et al., 2004) Finally, Forsberg et al. compared sporadic adenomas to normal glands, and identified two distinct groups of adenomas based on the presence or absence of LOH at 11q13. (Forsberg et al., 2005)

### ***The Wingless/integration1 (Wnt-) signaling pathway and the CCND1 gene***

Since menin and parafibromin have been coupled to the Wnt signaling cascade, (Mosimann et al., 2006, Chen et al., 2008) several studies have examined Wnt signaling in parathyroid tumors.

Wnt signaling is an evolutionary conserved pathway known to regulate proliferation, segment polarization and differentiation during embryogenesis. In addition it is highly associated to cancer development. (Klaus and Birchmeier, 2008) Wnt signaling is divided into a canonical (i.e.  $\beta$ -catenin-dependent), or non-canonical form, of which the former is best characterized.



**Figure 17.** Canonical Wnt signaling. *Left:* Absence of Wnt signaling resulting in destruction of  $\beta$ -catenin. *Right:* Active Wnt signaling resulting in  $\beta$ -catenin accumulation, translocation and initiation of transcription. TCF = T-cell factor; APC = Adenomatous Polyposis Coli; GSK3- $\beta$  = Glycogen synthase kinase 3- $\beta$ .

Canonical signaling is mediated by Wnt peptides binding to the Frizzled/LRP/Dishevelled membrane receptor complex. By activating the complex, the Dishevelled protein is disassociated and inhibit a destruction complex in the cytosol. This destruction complex, consisting of GSK3- $\beta$ , APC and Axin continuously phosphorylates  $\beta$ -catenin on multiple sites encoded by exon 3. Phosphorylated  $\beta$ -catenin is in turn is ubiquitinated by  $\beta$ -TrCP and subsequently degraded in the proteasome. Upon inhibition of the destruction complex, non-phosphorylated  $\beta$ -catenin accumulates in the cytoplasm and is transported into the nucleus where it interacts with the TCF/LEF (T-cell factor / Lymphoid enhancer factor) family of transcription factors. (Rao and Kuhl, 2010) (Figure 17) There is also the less studied non-canonical pathway, where Frizzled signaling is independent of  $\beta$ -catenin. Additionally, many proteins inherent of the Wnt pathway (including  $\beta$ -catenin) have other functions dependent on protein association and subcellular localization. (Cole and Sutherland, 2008)



The mapping of the Wnt pathway in combination with the implication of cyclin D1 in parathyroid tumorigenesis spurred Ikeda et al. and Semba et al. to investigate  $\beta$ -catenin in parathyroid adenomas. Sequencing of exon 3 of *CTNNB1* (encoding  $\beta$ -catenin) revealed no mutations, and immunohistochemical analysis only showed nuclear accumulation of  $\beta$ -catenin in 1/33 cases. (Semba et al., 2000, Ikeda et al., 2002) In contrast Björklund et al. reported a homozygous S37A *CTNNB1* mutation in 3/20 parathyroid adenomas as well as nuclear accumulation of  $\beta$ -catenin in 37/37 Swedish cases. (Bjorklund et al., 2007) Subsequently, the same group described nuclear accumulation of  $\beta$ -catenin in 104/104 adenomas, and S37A mutations in 6/104 adenomas, giving a mutational frequency of 7.3% in Swedish PHPT patients. (Bjorklund et al., 2008b) Five other studies, including American, Italian and Swedish (Paper II) cohorts, did not detect the S37A mutations in a total of 570 parathyroid tumors. (Costa-Guda and Arnold, 2007, Cetani et al., 2010, Haglund et al., 2010) Two of these studies reported a single *CTNNB1* S33C mutation (resulting in a frequency of 2/722, or <0.3% in parathyroid tumors, including unpublished data from Paper II). (Guarnieri et al., 2012, Starker et al., 2012) Finally, immunohistochemical analysis by Juhlin et al. (Paper I) and Cetani et al. (including a total of 63 adenomas, 3 atypical adenomas and 18 parathyroid carcinomas) described no aberrant nuclear expression in parathyroid tumors as compared to normal rim. (Juhlin et al., 2009, Cetani et al., 2010) While exon 3 mutations in *CTNNB1* are very infrequent, there is no consensus regarding the potential nuclear accumulation of  $\beta$ -catenin in parathyroid adenomas.

### ***Aberrant protein expression***

The need for a malignancy marker in parathyroid tumors has been a major drive for protein studies in parathyroid tumors. Hence, a number of aberrantly expressed proteins have been identified. Parathyroid carcinomas and adenomas show deviant expressions of the Rb1, p53, Ki-67, mdm2, bcl-2, p27 and cyclin D1 proteins. (Cryns et al., 1994, Lloyd et al., 1995, Farnebo et al., 1999, Stojadinovic et al., 2003) While these proteins differ between adenomas and carcinomas, their expression has not been exclusive enough to be utilized for diagnostic purposes. As mentioned above, loss of parafibromin, the protein product of *HRPT2/CDC73*, has been proposed as a marker of parathyroid malignancy. In addition, we report in Paper I that GSK3- $\beta$  and APC expression is altered in malignant parathyroid tumors.

In addition the proteins Klotho, CaSR, Calveolin-I and VDR have been shown aberrantly expressed in parathyroid adenomas. (Cetani et al., 2000, Kifor et al., 2003,

Bjorklund et al., 2008a, Latus et al., 2013) While the altered expression of these proteins has helped characterize the molecular constitution of parathyroid tumors, much work remains to identify their role in the development of PHPT.

## HORMONAL ASPECTS ON PHPT

Dr. Ernest Starling coined the term *hormone* in 1905. It is derived from the ancient Greek word ὁρμῶν (Hormo – “to set in motion”). He utilized the term to describe a substance carried from a producing organ to a target organ by the blood stream in order to convey a physiological response. Today the term has been expanded to include other types of signaling, and depends on how one defines inter-, intra-, para- and autocrine signaling. While a hormone originally was an endogenous substance, it may also be an exogenous synthetic chemical compound with similar mechanism of action. Hormones may be divided into the following classes: peptides, steroids, eicosanoids and monoamines. Specialized cells organized into endocrine tissues or glands commonly produce hormones, and many of these utilize feedback loops in axis organizations to regulate the expression, e.g. the hypothalamic-pituitary-adrenal axis. Since no tropic- or releasing-hormone has been identified for the parathyroid glands, it is considered autonomous. However, as mentioned above, several factors have been suggested to modulate parathyroid function.

### Female hormones and receptors in PHPT

Estrogens have established roles in bone turnover and tumor development. Since postmenopausal women are more commonly affected by PHPT, studies on estrogen signaling in parathyroid tumors began early. Greenberg showed in 1987 that estrogen and progesterone were able to increase PTH secretion in bovine parathyroid cells *in vitro*, and clinical studies revealed that estrogens could alter the calcium-PTH set point. (Greenberg et al., 1987, Boucher et al., 1989) Estrogen receptors (ER) however, proved difficult to find in the parathyroid glands, and after several reports with negative findings it was concluded that the parathyroid glands did not express ERs. (Prince et al., 1991) It was believed that the observed estrogenic effects on the parathyroid glands were indirect through other pathways. However, this conclusion was drawn before the discovery of additional ER isoforms.

Epidemiological data suggests an increased risk of PHPT for women with high parity. (Rastad et al., 2001) Factors coupled to female endocrine systems are plausible candidates for a possible common underlying etiological factor for breast cancer and PHPT.

During pregnancy the female endocrine system undergoes massive changes. With regard to calcium homeostasis, the development of fetal bones requires the recruitment of large amounts of calcium. Consequently the maternal bones risk leaching and osteopenia, especially during the third trimester when the majority of calcium recruitment takes place. To decrease the risk of bone leaching, an increased amount of calcium needs to be absorbed from the diet. Changes in calciotropic and pregnancy hormones have been proposed as explanations for these changes. During pregnancy the levels of PTH decrease, while 1,25-dihydroxycholecalciferol and E2 increase. Throughout the first two trimesters bone resorption increase, while in the third trimester it decreases in association with an increase in IGF-I. (Moller et al., 2013) By contrast, in women from Gambia and Brazil with a low dietary calcium intake, increased PTH levels were found during pregnancy. And while the urinary secretion of calcium decreased in these women, the impact of increased PTH on bone turnover is ambiguous. (Fairweather-Tait et al., 1995, Ritchie et al., 1998, Vargas Zapata et al., 2004)

Addition calcium is required for milk production during lactation. Postpartum, increased prolactin levels cause a time-dependent increase of bone turnover, with ensuing increase of PTH levels as well. (Moller et al., 2013) However, this relationship was not observed in postpartum women living in rural areas. (DeSantiago et al., 2002) As described bellow, prolactin has an expanding repertoire of functions. This includes induction of L-type calcium transporters in the intestine, facilitating calcium absorption during pregnancy. (Dorkkam et al., 2013) While prolactin may affect PTH secretion during lactation, this relationship during pregnancy is unclear.

As mentioned above, PTHrP is produced by the placenta and increases calcium transport across the placental circulation by paracrine signaling. And while mammary PTHrP production is greatly increased during lactation and thought to regulate bone turnover, the serum levels are equivocal. (Wysolmerski 2012, Moller et al., 2013,)

Historically it has been disputed if calciotropic hormones may vary across the menstrual cycle. Recent studies argue that this is actually the case. Studies have independently reported cyclic fluctuation of PTH during the menstrual cycle, with the levels peaking during the follicular phase with a correlation to E2 levels. (Finkelstein

and Schoenfeld, 1999, Chiu et al., 2000, Zittermann et al., 2000, Thys-Jacobs et al., 2007)

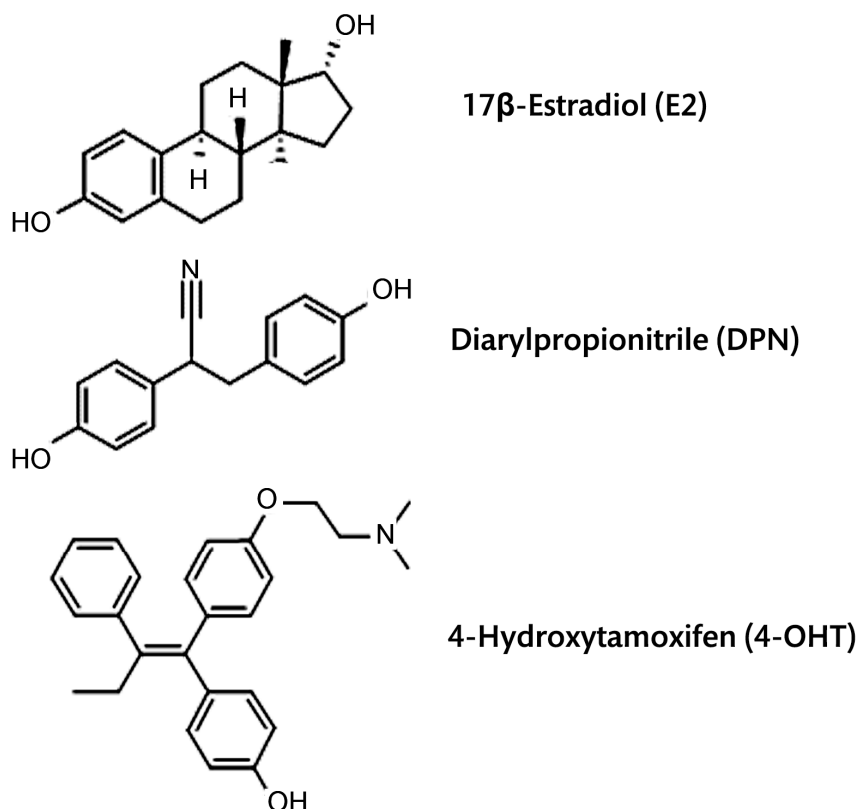
## Estrogens

Estrogens constitute a group of steroid hormones, originally found important for the estrous cycle. Based on the physiological context, different estrogens are predominant.  $17\beta$ -estradiol (E2) (Figure 18) is produced by the ovaries and considered the most potent estrogen during adult female life. (Simpson et al., 2002) After menopause and prior to adolescence estrone (E1) is more prevalent. Estrogens have numerous physiological functions, including but not limited to reproduction, coagulation, calcium homeostasis, metabolic changes and salt and water balance. (Nilsson et al., 2001) Hence defects in estrogen signaling can lead to multiple pathological conditions.

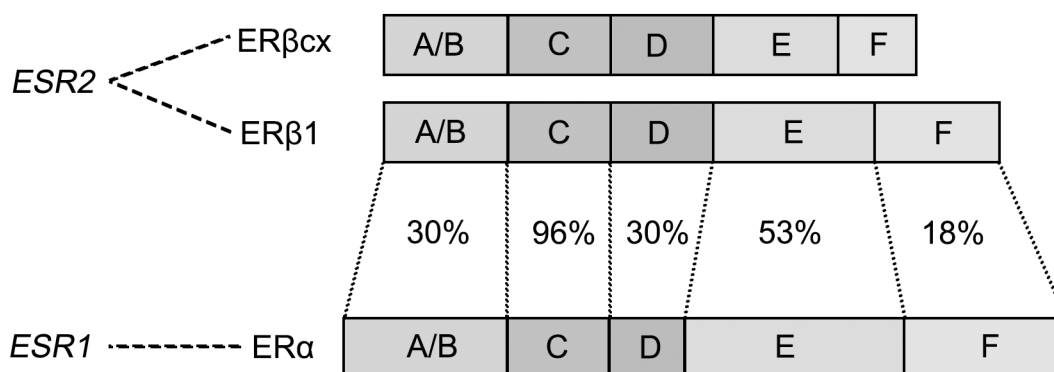
Estrogen signaling is mediated through estrogen receptors (ERs). To date, two genes encoding nuclear estrogen receptors have been identified, i.e. ER $\alpha$  encoded by *ESR1* and ER $\beta$  encoded by *ESR2*. ER $\alpha$ , identified in 1958, has been detected in ovaries, endometrium and hypothalamus, and ER $\beta$ , identified in 1996, has a high expression in e.g. the ovaries and testis. (HI, 1960, Kuiper et al., 1996) Both genes have multiple promoters and complex 5'-UTR structures, and alternative splicing gives rise to a variety of isoforms. The most well characterized isoform of *ESR2* is ER $\beta$ 2/ER $\beta$ cx, holding a unique C-terminus. This isoform is unable to bind ligands or active estrogen responsive elements (EREs), and is considered a negative inhibitor of ER $\alpha$  function. (Zhao et al., 2010) The ERs have structural similarities and functional domains involved in receptor dimerization, and ligand-, DNA- and transcription factor binding. (Figure 19) Variations in these domains result in different receptor subtype functionality. (Leung et al., 2006, Thomas and Gustafsson, 2011)

After ligand binding ERs dimerise and bind to ERE genomic motifs, which affect transcriptional activity. Additionally, ERs are involved in cross-talking with other transcription factors and affect non-EREs. Estrogen may also bind to the G-protein coupled estrogen receptor 1 (GPER/ GPR30) resulting in non-genomic signaling. (Bjornstrom and Sjoberg, 2005, Schultz et al., 2005, Rosano et al., 2012) (Figure 20)

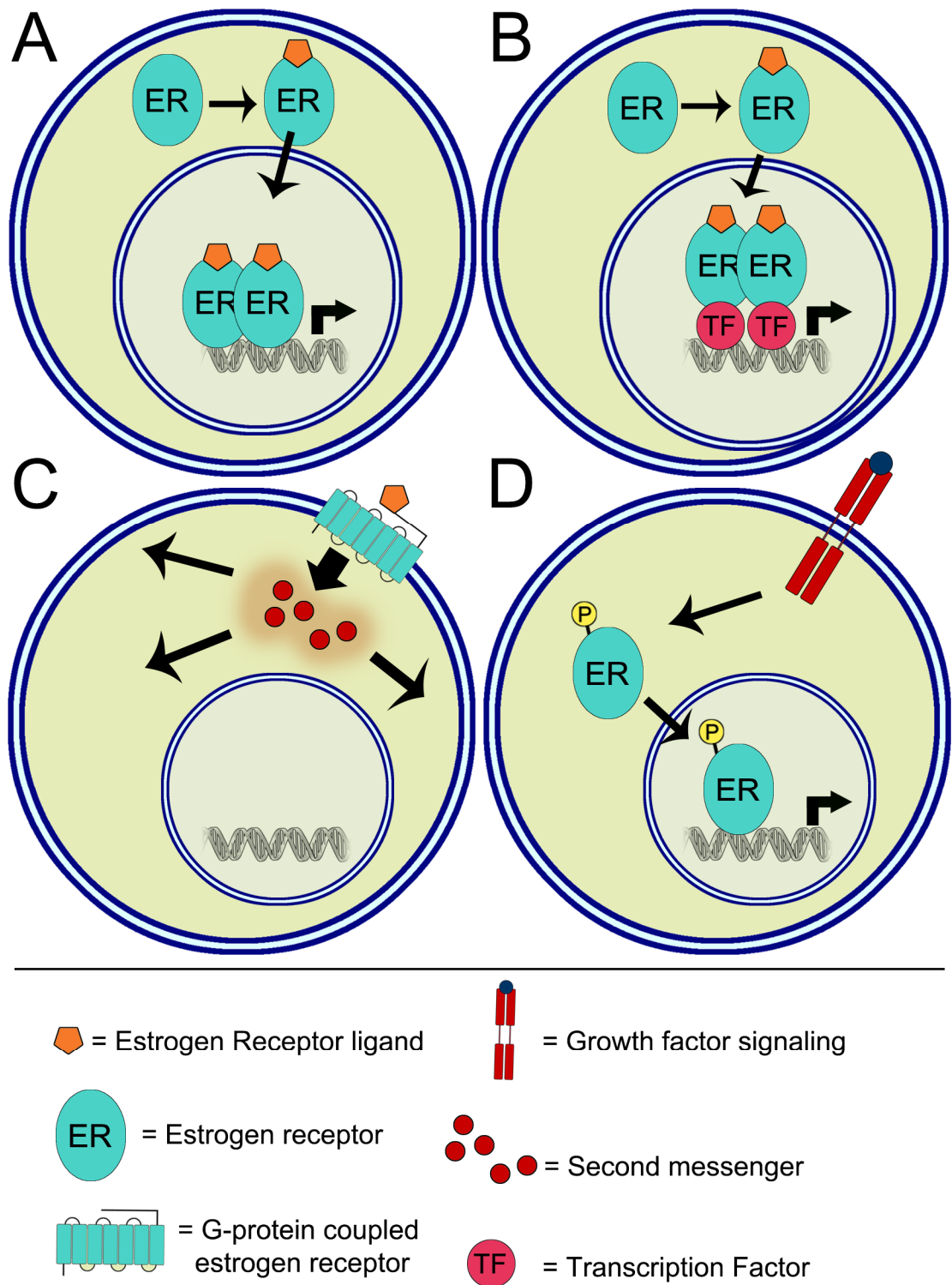
In Paper IV, we utilized selective estrogen receptor modulators (SERMs), with modulated affinity for estrogen receptors. (Figure 18) Currently there is a growing number of SERMs, with increasing number of indications for clinical use. In our study we have used diarylepropionitrile (DPN), an ER $\beta$ 1 selective agonist; and 4-OHT, the active metabolite of tamoxifen behaving both as an agonist and antagonist dependent on the context.



**Figure 18.** Chemical structures of estradiol (E2) and the two selective ER modulators (SERMs) diarylpropionitrile (DPN) and 4-Hydroxytamoxifen (4-OHT) used in Paper IV.



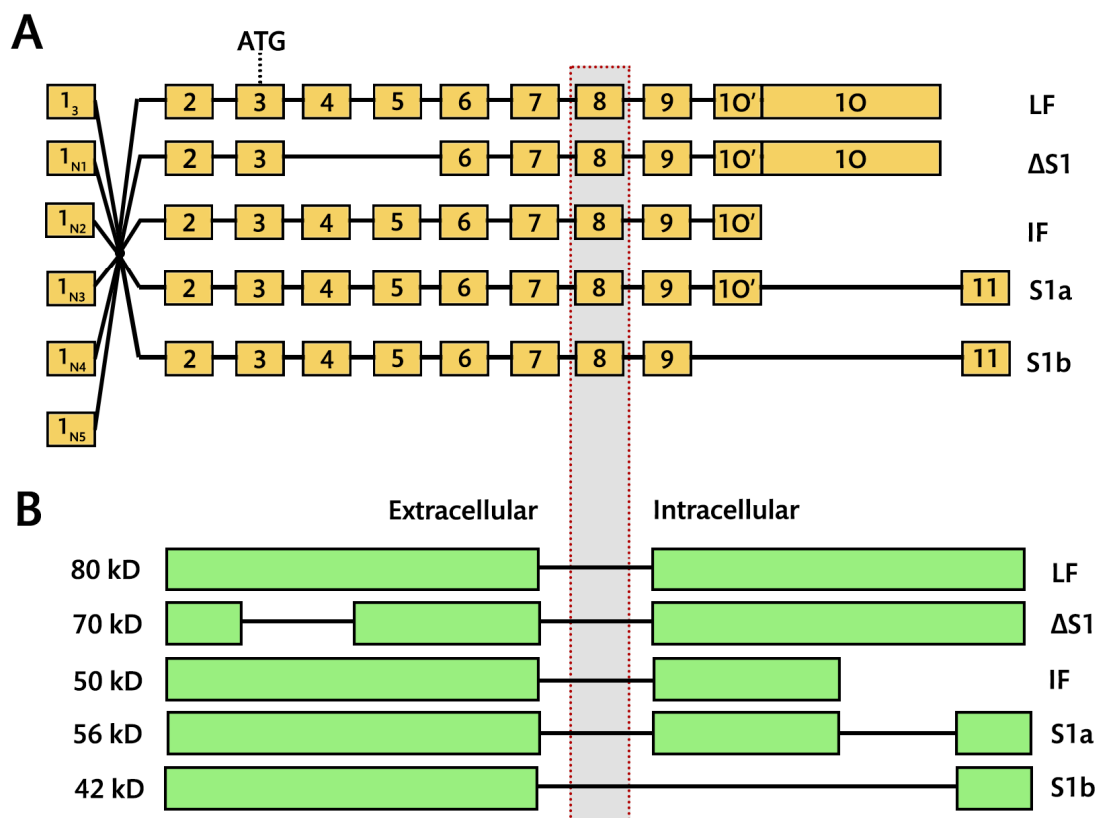
**Figure 19.** Schematic illustration of domains and homologies between ER $\alpha$ ,  $\beta$ 1 and  $\beta$ 2. The domains are involved in transcriptional activity (A/B), DNA-binding (C), structural hinge (D), ligand binding (E) or agonistic/antagonistic distinction (F). Figure inspired by Klinge. (Klinge, 2000)



**Figure 20.** Estrogen receptor (ER) signaling. In classical nuclear ER signaling ligand-ER complexes bind to EREs (A). In the ER-TF associated pathway the ligand-ER-TF complex binds to non-ERE (B). In membranous-ER signaling ligand activated GPERs transduce cytoplasmic signaling (C). Ligand independent ER signaling is caused by phosphorylation of ERs. Figure inspired by Zhao et al. (Zhao et al., 2010)

## Prolactin

Prolactin is a 198-aa peptide hormone secreted from acidophilic cells in the anterior pituitary. Its secretion is regulated through inhibitory signaling from dopaminergic neurons in the hypothalamus. Thyrotropin-releasing hormone may increase, but is not considered required, for prolactin secretion. (Benker et al., 1990) As the name imply, prolactin was originally considered mainly a lactotropic hormone, since its levels increase ten-fold during pregnancy and lactation. In fish it is believed to be of osmoregulatory function. (Auperin et al., 1995) The prolactin receptor (PRLr) is expressed in immune cells, liver, prostate, ovary and adipocytes. Not surprisingly additional functions have been identified, including cytokine-like signaling, cellular growth, development and neuromodulatory effects. (Dogusan et al., 2001, Ignacak et al., 2012) Recently this repertoire has been expanded to calcium homeostasis. PRLr has been shown to up-regulate L-type calcium channels in the intestine, increase bone resorption and increase calcium levels in lactated milk. (Powe et al., 2011, Wongdee et al., 2011, Dorkkam et al., 2013, Wongdee and Charoenphandhu, 2013)

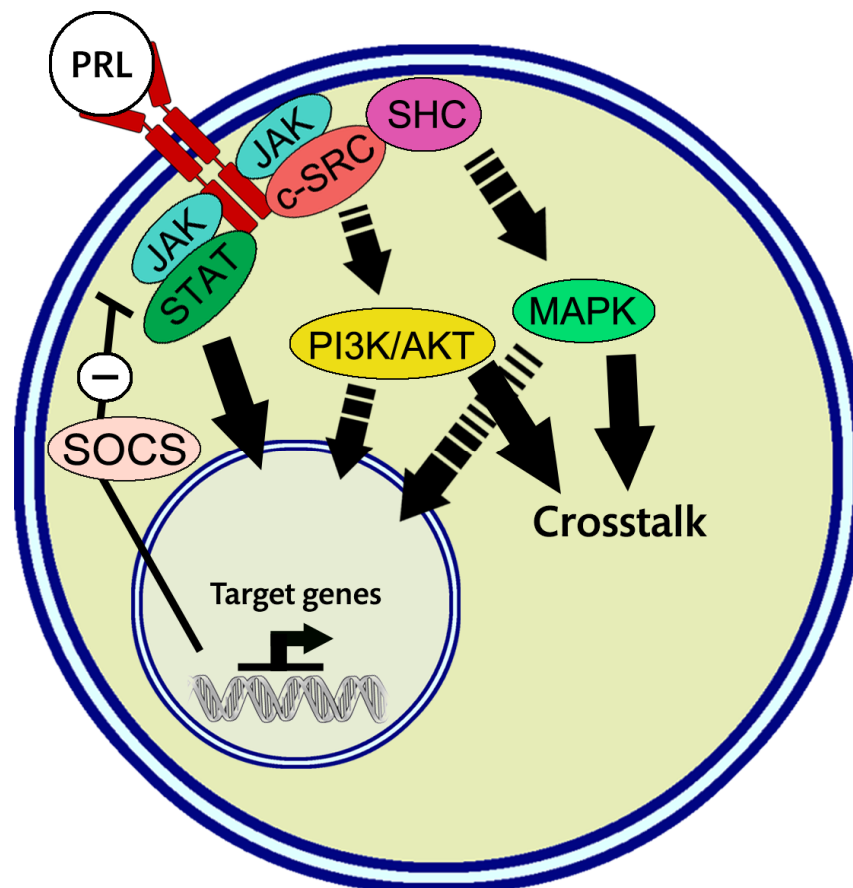


**Figure 21.** The *prolactin receptor gene (PRLR)* transcripts (A) and their encoded isoforms (B). The boxed area corresponding to exon 8 includes the intramembranous part. Alternative exons 1 are used in different tissues, and downstream splicing further generates different transcripts. Additional isoforms exist, including a soluble form consisting of the extracellular domain (not depicted in figure).



PRLr is a type I cytokine receptor with multiple isoforms produced by alternative splicing. In human, five identified isoforms are functional, excluding the soluble isoform mainly made up by the extracellular domain. (Figure 21) While downstream signaling depends on the isoform expressed and the cellular constitution, several signaling pathways have been identified, including JAK-STAT/Fyn/Tec, SHP-2/VAV, MAPK and IP3K. (Clevenger and Kline, 2001, Brooks, 2012) Upon stimulation, transcription of suppressors of cytokine signaling (SOCS) is induced and represses JAK-STAT signaling in a negative feedback manner. (Pezet et al., 1999) (Figure 22)

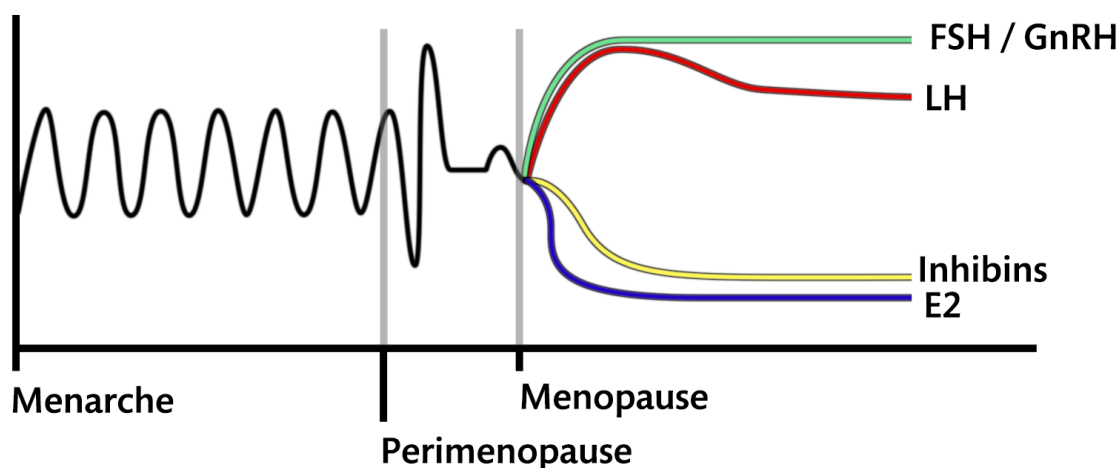
Prolactin signaling has been implicated in human disease, including breast and prostate cancer, lymphangioliomyomatosis and autoimmune disease. (El-Garf et al., 1996, Terasaki et al., 2010, Jacobson et al., 2011)



**Figure 22.** Schematic illustration of the PRLr signaling and SOCS feedback system. Depending on the subcellular constitution and the PRLr isoform several signaling pathways can be activated, including indirect crosstalk. PRL = prolactin; JAK = Janus kinase; STAT = Signal Transducer and Activator of Transcription; c-SRC = v-src avian sarcoma (Schmidt-Ruppin A-2) viral oncogene homolog; SHC = Src homology 2 domain-containing transforming protein 1; SOCS = Suppressor of cytokine signaling; PI3K/AKT = Phosphatidylinositide 3-kinase / Protein kinase B; MAPK = Mitogen-activated protein kinase.

## Menopause

At menopause the female monthly periods stop due to a naturally occurring alteration of the hormonal homeostasis. The median age of onset is 51 years in a Caucasian population. (McKinlay, 1996) Menopause is defined by a primary failure of the ovaries, resulting in amenorrhea and infertility. The ovarian failure is considered to be primarily caused by a depletion of viable oocytes. This leads to an increase in circulating follicle stimulating hormone (FSH) and luteinizing hormone (LH), and since no vital oocytes or follicles exist, estrogen (E2) levels will fall. (Figure 23) Prior to amenorrhea, the hormonal imbalance may cause peri-menopausal symptoms, which may be treated medically. Epidemiological studies suggest that women suffering from PHPT enter menopause several years earlier. (Christensson, 1976) The causality behind this observation is unknown.



**Figure 23.** Changes in circulating hormonal levels at menopause. FSH = follicle stimulating hormone; GnRH = gonadotropin-releasing hormone; LH = luteinizing hormone; E2 = estrogen.

# AIMS OF THE THESIS

The overall aim of this thesis was to further characterize the molecular background of parathyroid tumors, focusing on certain members of the Wnt pathway and female sex hormone receptors. The aim of each included paper is given below:

- I To characterize expression of key proteins in the Wnt signaling cascade in parathyroid carcinomas in comparison to adenomas. Additionally, to evaluate APC as a potential diagnostic marker for parathyroid malignancy.
- II To evaluate if *CTNNB1* S37A mutations are present in parathyroid tumors from Swedish patients by sequencing 98 parathyroid tumors with diverse clinical features.
- III To evaluate the potential involvement of prolactin signaling in PHPT by analyzing the expression and functionality of the prolactin receptor in parathyroid tumors.
- IV To re-evaluate the expression of estrogen receptor subtypes in parathyroid adenomas. Additionally to evaluate the functionality of any estrogen receptor present.

# MATERIAL AND METHODS

## GENETIC ANALYSIS

*“It has not escaped our notice that the specific pairing we have postulated immediately suggests a possible copying mechanism for the genetic material.”*

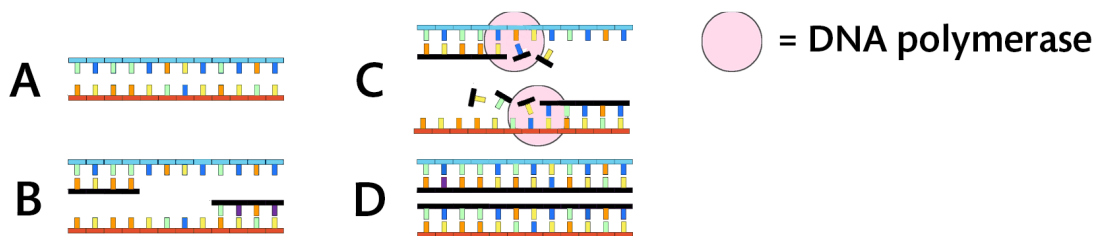
– Francis Crick and James D. Watson; Nature 1953

*“Conclusion: Big helix in several chains, phosphates on outside, phosphate-phosphate inter-helical bonds disrupted by water. Phosphate links available to proteins.”*

– Rosalind Franklin; Lecture notes 7<sup>th</sup> February 1952

## Polymerase chain reaction (PCR)

A fundamental methodology in molecular biology is the polymerase chain reaction (PCR), developed by Mullis in 1983. The method is based on the enzymatic replication of DNA strands, utilizing thermal cycling to repeatedly anneal region-specific primers and a DNA polymerase to exponentially amplify a target region. (Figure 24)



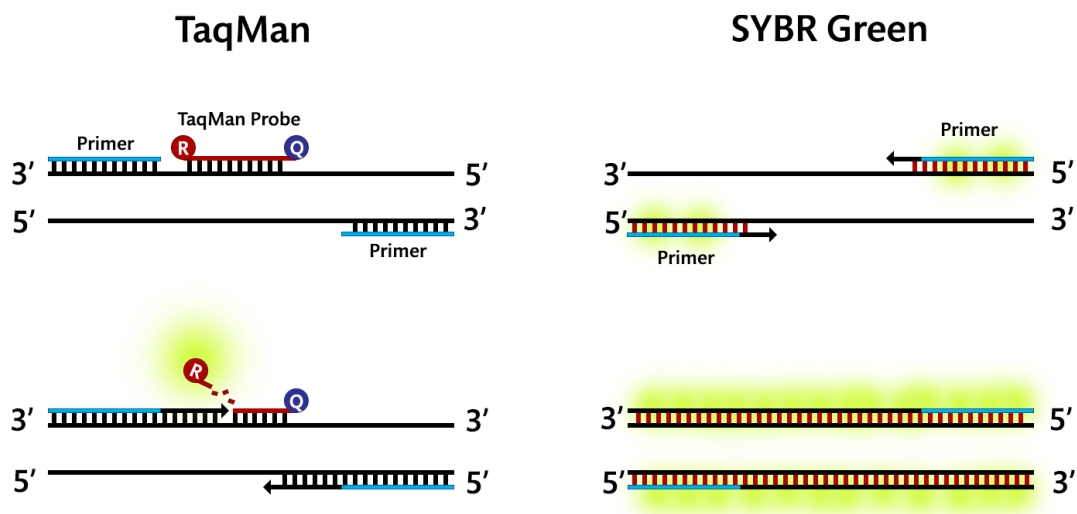
**Figure 24.** PCR amplification cycle starting from double stranded DNA (A), including denaturation and annealing of primers (B), and addition of deoxynucleotides by DNA-polymerase (C), generating new DNA copies (D), whereafter the cycle is restarted. Black strands represent new copies.

## Reverse transcription and quantitative real-time PCR

Reverse transcription (RT) is the conversion of ribonucleic acids (RNA) to a complementary DNA (cDNA) strand. There are a number of benefits for using cDNA, since the natural stability of DNA is superior to that of RNA. The cDNA is often analyzed by PCR, and the product can either be visualized by gel electrophoresis or Sanger sequenced. The cDNA is not amplified during its synthesis, and hence

quantitatively equal to the number of RNA strands, allowing for indirect quantification. Quantitative real-time PCR (qRT-PCR) can use either DNA-binding dyes or incorporated probes as quantitative reporters. (Figure 25) The reporter signal intensity is corresponding to the number of strand copies during the PCR.

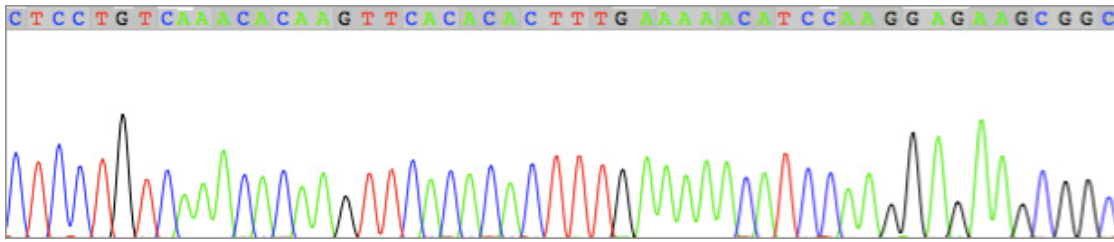
One must ensure that the PCR is run with accurate efficiency not to introduce bias in the quantification. With the parallel analysis of quantified housekeeping genes the relative or absolute RNA levels can be estimated. In this thesis, qRT-PCR was used to estimate RNA expression of genes in Papers III and IV.



**Figure 25.** qRT-PCR using the TaqMan and SYBR green platforms. TaqMan uses a hybridization probe, consisting of a reporter (R) and a reporter quencher (Q). When the probe is cleaved, the reporter is separated from the quencher and emits fluorescent light. In the SYBR green protocol fluorescent SYBR green binds to double stranded DNA as dNTPs are incorporated.

## Sanger sequencing

In 1977 Frederick Sanger developed an effective way of sequencing DNA. (Sanger et al., 1977) Today this involves PCR amplification of a DNA region of interest. In the subsequent sequencing reaction fluorescent dideoxynucleotides (ddNTP) are mixed together with deoxynucleotides (dNTPs). ddNTPs holds the practical quality of terminating the polymerases reaction, resulting in a premature stop upon incorporation. As the ddNTPs are randomly incorporated, the result is an accumulation of DNA fragments of different length. Fragments are then run through a capillary system to sort



**Figure 25.** Chromatogram after Sanger sequencing. The peaks represent the nucleotides G (black), A (green), T (red) and C (blue).

them according to size, and exposed to an argon ion laser. As each type of ddNTP holds different fluorescent properties, the resulting fluorescence can be interpreted as a chromatogram (Figure 25) – each curve representing a nucleic base in the DNA sequence. In this thesis Sanger sequencing was used to screen for the *CTNNB1* S37A mutation in Paper II.

## DNA Pyrosequencing

*“Unfortunately, I obtained financing only for the old project. The committee did not share my enthusiasm for the new idea, reasoning that ATP is a substrate for DNA synthesis and therefore would interfere with the luciferase assay. However, this point is wrong: ATP is not a substrate for DNA polymerase.”*

– Pål Nyrén, 2007

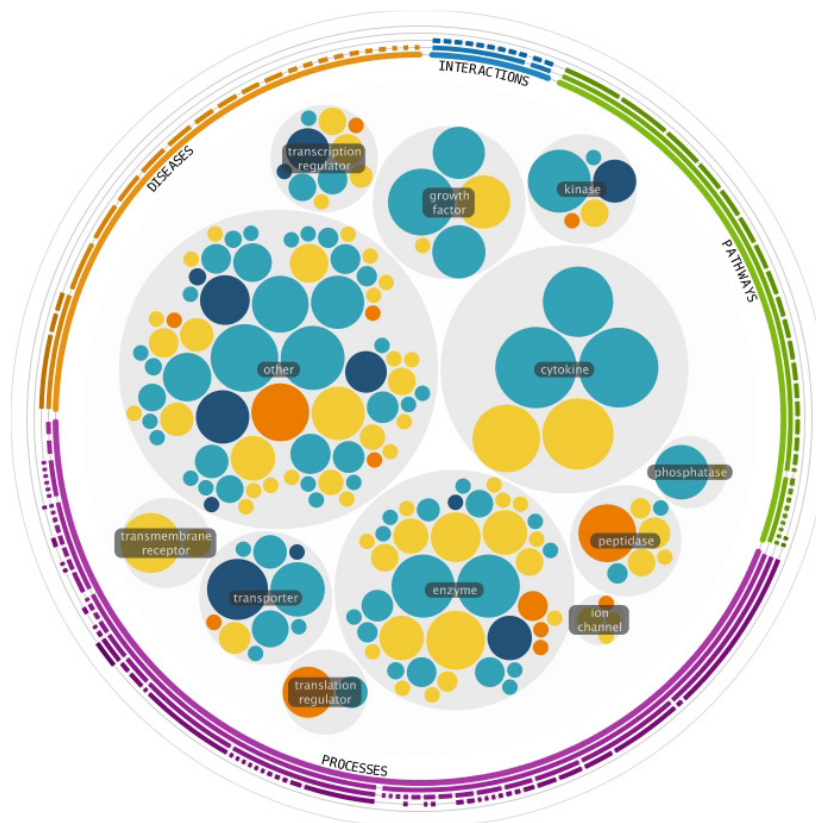
Pyrosequencing has many similarities to Sanger sequencing, but differs in the way it identifies nucleotide sequences. First, a PCR amplify the DNA region of interest. During a sequential PCR a single type of dNTP is added at the time. The incorporation of dNTP releases a pyrophosphate that is converted to ATP by ATP-sulfurylase. This ATP is converted to light by a luciferase assay, whereby the emission is quantitatively equal to the number of consecutive equal bases. Residual dNTPs and ATP are cleaved by apyrase. We employed this method to quantify methylation densities at CpG-sites of selected promoter regions. Before the first PCR the DNA was treated with bisulfite, converting non-methylated cytosine to uracil, while methylated cytosine remains unchanged. The first PCR converts uracil into thymine. The target sequence is programmed into the pyrosequencer, including CpG-site locations. During the second PCR, thymine and cytosine will be added sequentially for all CpGs, resulting in two separate reactions. By comparing the resulting light emission between thymine and cytosine, a quantitative estimation of the DNA methylation is given.

## Gene expression microarray

Expression microarray is a method to analyze levels of multiple genes simultaneously. Sample RNA is converted to cDNA by RT-PCR, introducing fluorescent labeling into the cDNA. The sample cDNA is hybridized to complementary oligonucleotides at specific positions on a chip. The fluorescent light from the cDNA can be quantified for each probe, and based on this signal strength the relative expression of a gene is calculated. A number of normalization and qualitative control steps are performed prior to the bioinformatic analysis. In Paper III we utilized expression arrays from the Affymetrix platform to compare changes in gene expression in parathyroid cells after treatment with recombinant prolactin.

## Whole transcriptome shotgun sequencing

With the completion of the Human genome project, new ways of genome-wide screenings became possible. We employed second-generation whole transcriptome sequencing based on the Illumina platform to analyze changes in gene expression. To sort the coding RNAs from abundant rRNA, mRNA was purified using their poly(A) tail. RNA was then hydrolysed into fragments of 2-300 nucleotides and converted into cDNA. The cDNA fragments bind to random primers covalently attached to a chip surface, where they are amplified into colonies. During the sequencing reaction, fluorescent single dNTPs are incorporated into the fragment colonies. After its incorporation it inhibits further elongation due to an inhibitory terminator. The fluorescence is captured, and the terminator cleaved from the sequence. This is repeated in a cyclic manner. Since the amplified colonies are bound to the surface, the consecutive capturing of fluorescence allow for massive parallel capturing of all fragment sequences. The resolution depends on the number of reads (sequence depth). Fragments can be aligned to a reference genome, or assembled into a transcriptome *de novo*. Based on the read length and number of sequences read to the reference genome, the FPKM factor (fragments per kilobase of exons per million fragments mapped) can be used to describe the relative quantity of a specific read.



**Figure 27.** Illustration of gene ontology analysis after expression profiling. Genes are sorted according to function (grey circles), with each gene represented by a color circle where color represents increased or decreased expression. The size of each gene circle represents the number of known interactions. Lines surrounding the circle represent different functional annotations.

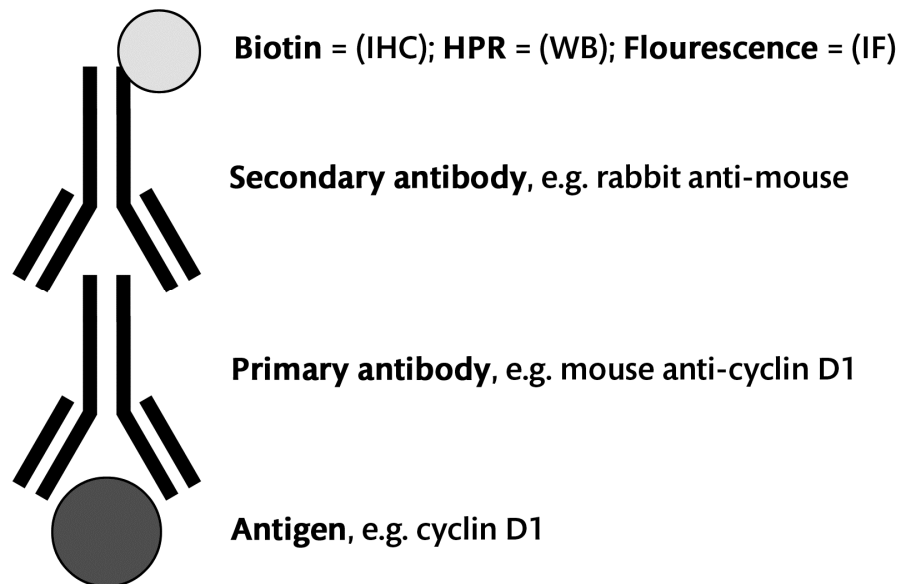
## Data analysis

Analysis of gene expression data is performed in a number of steps. First, a quality control is done to check for possible methodological bias and compare raw data signatures to expected ones. Secondly, a comparison between the different data sets will identify differentially expressed genes that fulfill requirements with regard to the magnitude of expression change and significance level. One issue with biological repeats is the considerable difference between individual tumors, which has to be considered in the analysis. Since multiple genes are tested for significance, the false discovery rate must be calculated. Genes considered significant can be analyzed with regard to their putative function. One can compare gene expression patterns with previously published gene-sets or enrichments in pathways or functions based on the gene ontology. (Figure 27)



# PROTEIN ANALYSIS

Antibodies are immunoglobulins produced by the immune system to bind and neutralize antigens. Molecular biology has exploited this ability in numerous experiments, often to identify a specific protein. (Figure 28)



**Figure 28.** Principle of antibody usage to identify a target antigen. A primary antibody binds the target antigen. A detection system coupled-secondary antibody targets the primary antibody. Depending on the detection system, different protocols can convey the immunological binding. IHC = Immunohistochemistry; WB = Western blot analysis; IF = Immunofluorescence.

## Immunoblotting / Western Blot

*“With due respect to Southern, the established tradition of “geographic” naming of transfer techniques (“Southern,” “Northern”) is continued; the method described in this manuscript is referred to as “Western” blotting.”*

– W. Neal Burnette 1981

In Western blot analyses peptides are separated by electrophoresis and the protein of interest is identified using a specific antibody. The primary antibody can then be detected using several methods, e.g. radio- or reporter enzyme linked. We used secondary antibodies coupled to horseradish-peroxidase – a reporter enzyme that may cleave chemiluminescent agents, thus allowing capturing of the signal using a photographic film. Proteins can be extracted from cell cultures, fresh or fresh frozen tissue. During the electrophoresis, denatured proteins are separated according to size.

Proteins are then transferred onto a membrane, which is incubated with the primary antibody. Subsequent incubation with a control antibody is done as a control of protein quality and quantity. The methodology is highly dependent on adequate controls and detailed knowledge about the antibody and antigen. We applied Western blot analysis in Paper I and III to identify potential isoforms and validate the specificity of the antibodies used.

## **Immunoprecipitation**

Immunoprecipitation isolates an antigen from a solution. Antibodies coupled to beads or other components are added to a solution where it binds to its antigen. Thereafter, the antigen-antibody complex is immobilized in a column. Through a series of buffers, the solution is cleaned and the target antigen eluted separately. Using a solution containing native proteins, this method is able to precipitate protein complexes. In Paper III we utilized regular immunoprecipitation to increase the specificity of antigen detection. By using an antibody to elute a protein of interest, and then apply a second antibody to the same protein, increased specificity can be achieved.

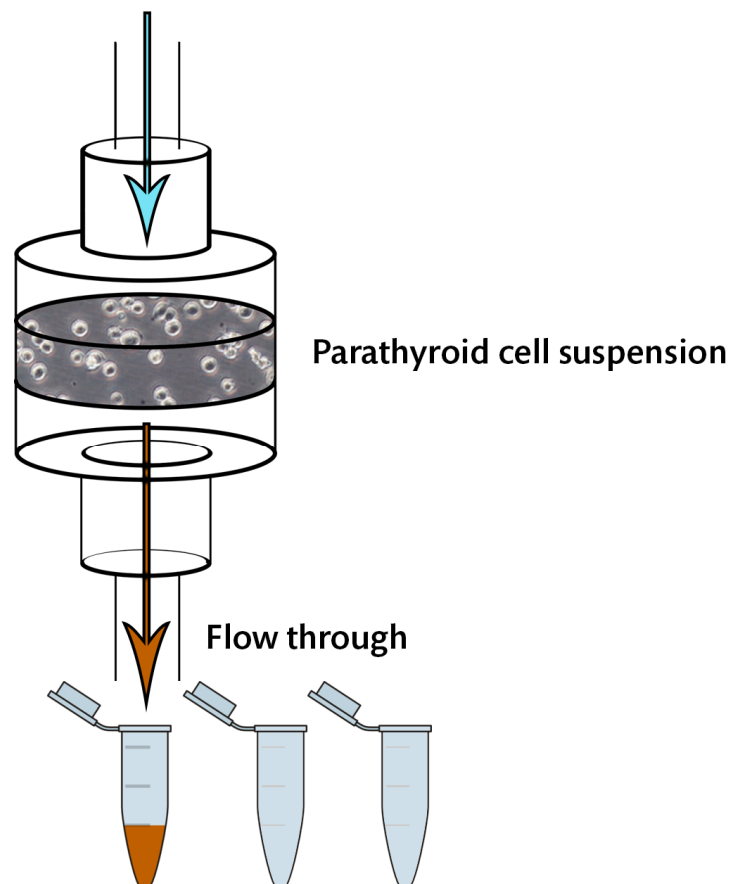
## **Immunohistochemistry and Immunofluorescence**

The immunological principle of protein detection can also be applied to histologically intact tissues. Tissues are fixated and cut into slides, approximately 4  $\mu\text{m}$  thick. Much like immunoblotting, a primary antibody detects the antigen of interest, followed by a reporter linked secondary antibody. If the visualization technique is based on fluorescent dye it is commonly referred to as immunofluorescence (IF), as opposed to immunohistochemistry using color. Fresh frozen or FFPE samples may be used. Depending on the antibody, antigen retrieval techniques may be necessary, such as boiling samples in citrate. Slides can be manually viewed using a brightfield light microscopy or in the case of IF, a confocal microscope. While the signal amplification is inferior in IF, the use of confocal microscopy allows a higher resolution. In addition, we utilized a scanner system to capture the full slides. The digitalization of samples made it possible to measure the staining intensity. While this method is highly dependent on the replicative accuracy of the staining, it allows for statistical comparisons not possible with the naked eye. We used immunohistochemistry to identify immunoreactivity at histological resolution of specific proteins in Papers I, III

and IV. In addition IF was applied to increase image resolution, making protein localization possible on a subcellular level.

## Cultures and PTH measurements

For studies of parathyroid cell function *in vitro* parathyroid tumor samples were collected directly from surgery. The tumor tissue was minced and treated with collagenase before culturing on a plate. These primary cultures were then mounted in a column and suspended between gel filters and perfused with medium. (Figure 29) This allowed for intermittent measurements of PTH concentrations in the perfused medium. Although cell viability decreases over time the functionality of the parathyroid cells can be controlled using calcium starvation. For analysis of gene expression changes, samples were cultured on plates under different conditions after which RNA was extracted. This method was utilized in Paper III. Additionally, cells were incubated with Fura-2, a fluorescent indicator of intracellular calcium levels. The fluorescent emission characteristic of this molecule is altered when bound to calcium, making the estimation of free calcium in a solution possible.



**Figure 29.** Perifusion experiments. Parathyroid cell suspension is continuously perfused with medium (blue arrow). Intermittent collection of flow through (red arrow) is used for analysis.

# RESULTS AND DISCUSSION

## Parathyroid carcinomas exhibit aberrations in the Wnt signaling pathway (Paper I)

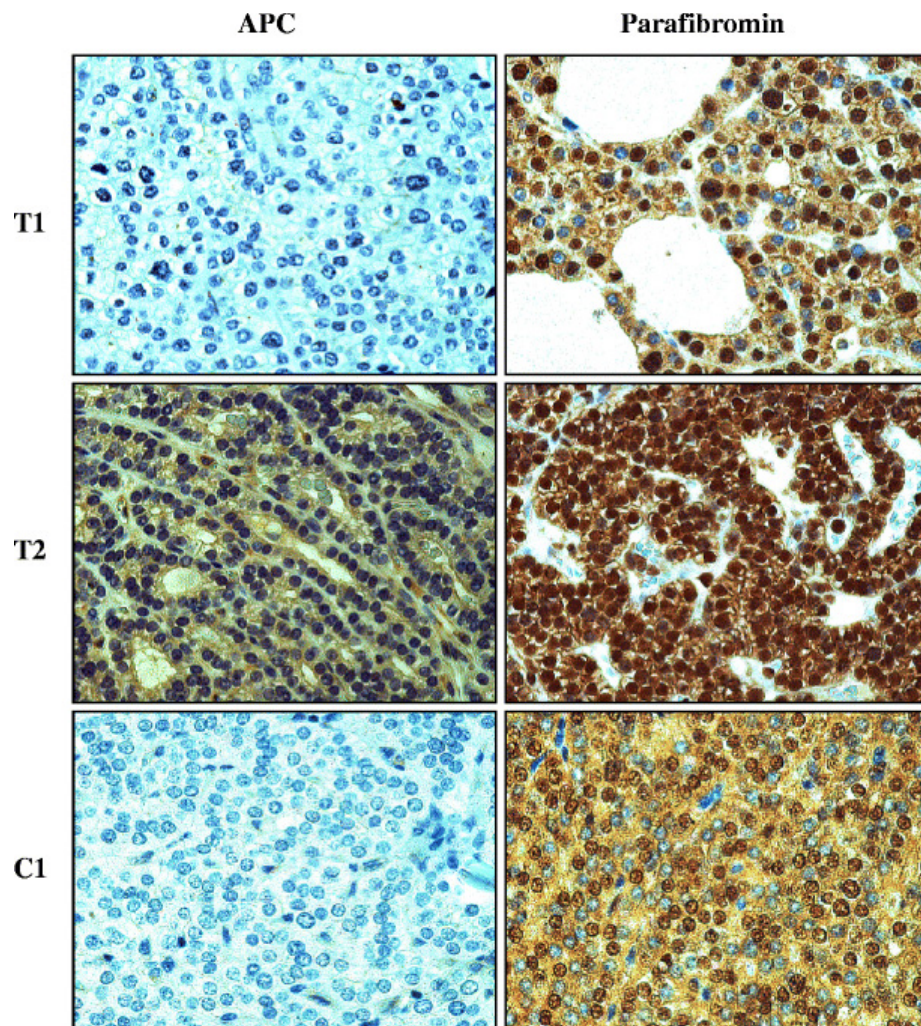
Inactivating mutations in the *HRPT2/CDC7* gene encoding parafibromin are present in the majority of parathyroid carcinomas. At the time of study design, parafibromin was identified as a member of the Paf1-complex and shown to activate Wnt-target gene transcription by binding to  $\beta$ -catenin. We hypothesized that other aberrations in the Wnt signaling pathway could be found in parathyroid malignancy. Parafibromin had also been proposed as a diagnostic marker in parathyroid tumors. However, the value of parafibromin for clinical application was disputed, and the discovery of additional diagnostic markers was highly warranted. Thus we sought to characterize protein expressions within the Wnt pathway to potentially ascertain a malignancy marker.

Using immunohistochemistry we evaluated protein expression and localization for the total and active form of  $\beta$ -catenin (a central mediator of canonical Wnt signaling), APC and GSK3- $\beta$  (members of the destruction complex), and Cyclin D1 (a known target for Wnt signaling and known proto-oncogene in parathyroid tumors). The FFPE specimens included 12 parathyroid carcinomas, 18 parathyroid adenomas and normal parathyroid rims present in 12 of the adenomas.

Cyclin D1 was increased in tumor tissues, but was not significantly different between adenomas and carcinomas. The levels of total and non-phosphorylated  $\beta$ -catenin were comparable in normal parathyroids, adenomas and carcinomas as measured by Western blot and immunohistochemistry. The staining pattern was cytoplasmic and membranous, with the addition of a mixed pattern of positive and negative nuclei for the antibody targeting non-phosphorylated  $\beta$ -catenin. GSK3- $\beta$  expression was lost in 4/12 carcinomas and 1/18 adenomas. APC immunoreactivity was abolished in 9/12 carcinomas, retained in all adenomas and normal rim. Using an additional antibody directed towards C-terminal APC, we observed loss in 6/12 carcinomas and retained expression in all adenomas. Parathyroid carcinomas with either loss of APC or GSK3- $\beta$  immunoreactivity did not differ in their  $\beta$ -catenin levels. If loss of APC and/or GSK3- $\beta$  causes the malignant phenotype, it may be mediated by  $\beta$ -catenin independent mechanisms. For example as ~20% of the proteome is estimated to have GSK3- $\beta$

phosphorylation sites, this may reflect a change in global protein stability of parathyroid carcinomas intracellular milieu. (Taelman et al., 2010)

These data indicate frequent molecular aberrations within the Wnt-pathway components in parathyroid carcinomas, and to a lesser extent, parathyroid adenomas. The highly specific loss of APC immunoreactivity in malignant lesions suggests its usage as a marker in histopathological diagnosis. The N-terminal APC antibody held a specificity of 100%, a sensitivity of 75%, a negative predictive value of 99.5% and positive predictive value of 100% for parathyroid carcinoma. Since the publication of Paper I, additional studies have found usage of APC as a diagnostic marker in parathyroid tumors. (Juhlin et al., 2010b, Juhlin and Hoog, 2010, Kauffmann et al., 2011, Iacobone, 2012)



**Figure 30.** Comparison of APC and parafibromin immunoreactivity in atypical adenomas (T1-2) and a parathyroid carcinoma (C1). T1 and C1 show negative APC immunoreactivity, and a mixed pattern of positive and negative nuclei for parafibromin. T2 is positive for both APC and parafibromin. Reprinted with permission from Springer. (Juhlin et al., 2010b)

## Mutations in *CTNNB1* S37A constitute a rare event in primary hyperparathyroidism (Paper II)

*“Positive findings are around twice as likely to be published as negative findings. This is a cancer at the core of evidence based medicine.”*

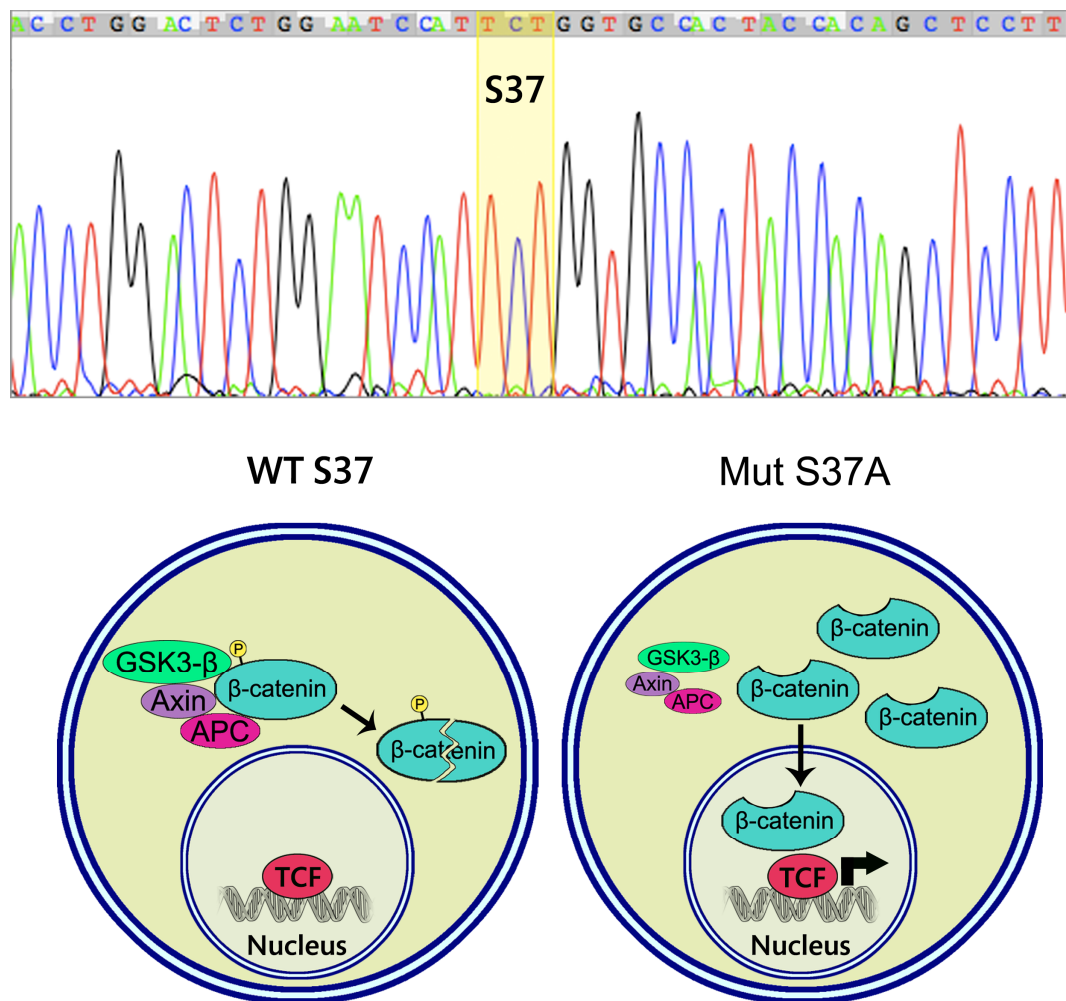
- Ben Goldacre

The amino acid substitution S37A of the *CTNNB1* gene, encoding  $\beta$ -catenin, has been described in different tumor types e.g. liver and endometrial carcinomas and gastrointestinal carcinoids. (Terris et al., 1999, Fujimori et al., 2001, Saegusa et al., 2001) The mutation is of special interest since it stabilizes  $\beta$ -catenin and leads to its nuclear accumulation. (Saegusa and Okayasu, 2001) (Figure 31) In parathyroid tumors the mutation has been studied by several groups with both positive (Bjorklund et al., 2007, Bjorklund et al., 2008b) and negative (Semba et al., 2000, Ikeda et al., 2002, Costa-Guda and Arnold, 2007, Cetani et al., 2010) findings. In two Swedish studies S37A was found to be relatively frequent and often present in homozygous form but was not detected in patients constitutional DNA suggesting that it was a somatic event. Nuclear accumulation of  $\beta$ -catenin was also observed in the Swedish population. (Bjorklund et al., 2007, Bjorklund et al., 2008b) In reports from Japan, USA and Italy the S37A mutation was not detected, and nuclear  $\beta$ -catenin accumulation was not observed by us (Paper I) or others. Thus, it was suggested that the S37A mutation predominantly occurred in the Swedish population. This could possibly be due to the presence of an associated clinical subgroup in this geographical region, which would be of interest to determine for the etiology of PHPT in the Swedish population.

We studied a total of 98 parathyroid adenomas from Swedish patients. Sequencing of exon 3 of the *CTNNB1* gene and subsequent inspection of the S37 region failed to identify any S37A mutations. This study ruled out the possibility of a Swedish phenomenon, as well as excluded *CTNNB1* S37A mutations as an important genetic event in sporadic parathyroid tumors.

Since our publication, Guarnieri et al. and Starker et al. have published two studies of the *CTNNB1* mutational status in PHPT, respectively. These studies report absence of S37A mutations, but in each cohort a single S33C mutation was identified. (Guarnieri et al., 2012, Starker et al., 2012) While Guarnieri reported no difference in  $\beta$ -catenin

expression levels, Starker et al. report nuclear accumulation compared to a single non-related normal parathyroid gland. While we only reported amino acid position 37 in our cohort, a secondary review of the data did not reveal any variant in position 33 (data not published). Furthermore two studies using exome sequencing of parathyroid adenomas have been published in which *CTNNB1* mutations were not detected. Altogether 9 PHPT tumors with S37A have been reported together with 722 negative cases. This would correspond to a S37A mutation frequency of less than 1,25%. In all, an S37A mutation in the *CTNNB1* gene seems to have little impact on parathyroid tumor development.



**Figure 31.** Above: Chromatogram illustrating the wild-type sequence in an excerpt of *CTNNB1* exon 3, encompassing nucleotides encoding amino acid 37. Below: *CTNNB1* wild-type serine 37 facilitates GSK3- $\beta$  mediated phosphorylation and degradation of  $\beta$ -catenin (left). Mutated alanine 37 hinder GSK3- $\beta$  phosphorylation of the amino acid, thus stabilizing  $\beta$ -catenin mimicking canonical Wnt-signaling (right).

## **Prolactin signaling may be involved in primary hyperparathyroidism and normal parathyroid function (Paper III)**

The rationale for investigating prolactin signaling in parathyroid tumors was multifaceted. Except for mainly affecting women, epidemiological data suggest a link between PHPT development and childbearing. During pregnancy, prolactin signaling in pancreatic  $\beta$ -cells suppresses menin levels, causing  $\beta$ -cell proliferation. Previous parathyroid tumor gene expression studies independently suggested high levels of the *PRLR* in parathyroid adenomas; (Haven et al., 2004, Forsberg et al., 2005) additionally supported by public EST profiling data, showing that parathyroid adenomas have even higher *PRLR* expression than breast tumors. *PRLR* knockout mice show phenotypic alterations in calcium, PTH levels and bone. Prolactin has been shown to increase PTH secretion *in vitro* in bovine parathyroid cells, and some clinical correlations between PTH and prolactin levels exist. Recently prolactin has been proposed to be an important calciotropic hormone during pregnancy. (Charoenphandhu et al., 2010) With this background we choose to investigate the presence of PRLr in parathyroid tumors.

We found high levels of the *PRLR* as compared to breast cancer cell lines and different normal tissues. The long isoform of PRLr was found only in parathyroid tumor samples and the breast cancer cell line T74D. This isoform has proliferative properties in breast cancer cells. The long PRLr isoform is regulated by a free pool of GSK3- $\beta$ , unbound to the destruction complex responsible for  $\beta$ -catenin destruction. The activity of this pool of GSK3- $\beta$  is mediated by phosphorylation on serine residue 9. As analyzed by Western blot, GSK3- $\beta$  serine 9 phosphorylation was strong in 29, weak in 6 and barely negative in 2 of the parathyroid tumors, while it was negative in normal parathyroid. Tumors without GSK3- $\beta$  serine 9 phosphorylation generally had lower levels of the long PRLr isoform.

While PRLr was uniformly expressed in cytosol and cytoplasmic granule in the normal rims, the subcellular staining pattern varied between tumors. Tumor staining patterns were characterized by weaker cytoplasmic staining, membranous localization and absence of cytoplasmic granule. Additionally, equivocal ring-like staining was seen in some tumors. Using IF we showed that the granule and ring-like structures corresponded to lysosomes. Granular staining was almost never seen together with



membranous staining, suggesting a possible connection between membranous and lysosomal localization of the receptor.

To test the receptor functionality, primary cultures of parathyroid tumors were exposed to prolactin. Perfusion experiments were used to determine PTH secretion in physiological levels of prolactin. Treatment with 200 ng/mL prolactin increased PTH secretion as compared to 100 ng/mL prolactin. However, these changes were not statistically significant, possibly because of high variability between tumors. Changes in gene expression following prolactin treatment were examined using microarray based gene expression profiling, and a similarly high variability was observed between biological samples. Significantly increased expression of genes in the RIG-I like receptor, JAK-STAT and Type II interferon signaling pathways were detected. The effects of gene expression changes remain to be investigated. The fact that PRLr is a cytokine receptor can explain the apparent similarity to cytokine signaling. With regard to the high variability in PTH secretion and gene expression after prolactin treatment one could speculate that the individual tumor responsiveness to prolactin is variable.

This study provides further evidence to the expanding repertoire of prolactin functionality, specifically its potential role in calcium homeostasis. While the receptor expression seems to be altered in parathyroid tumors, additional research is required to determine the cause and effects of these molecular alterations.

## Estrogen receptor beta is expressed and may convey tumor suppressor like signaling in parathyroid tumors (Paper IV)

*“Absence of evidence is not evidence of absence.”*

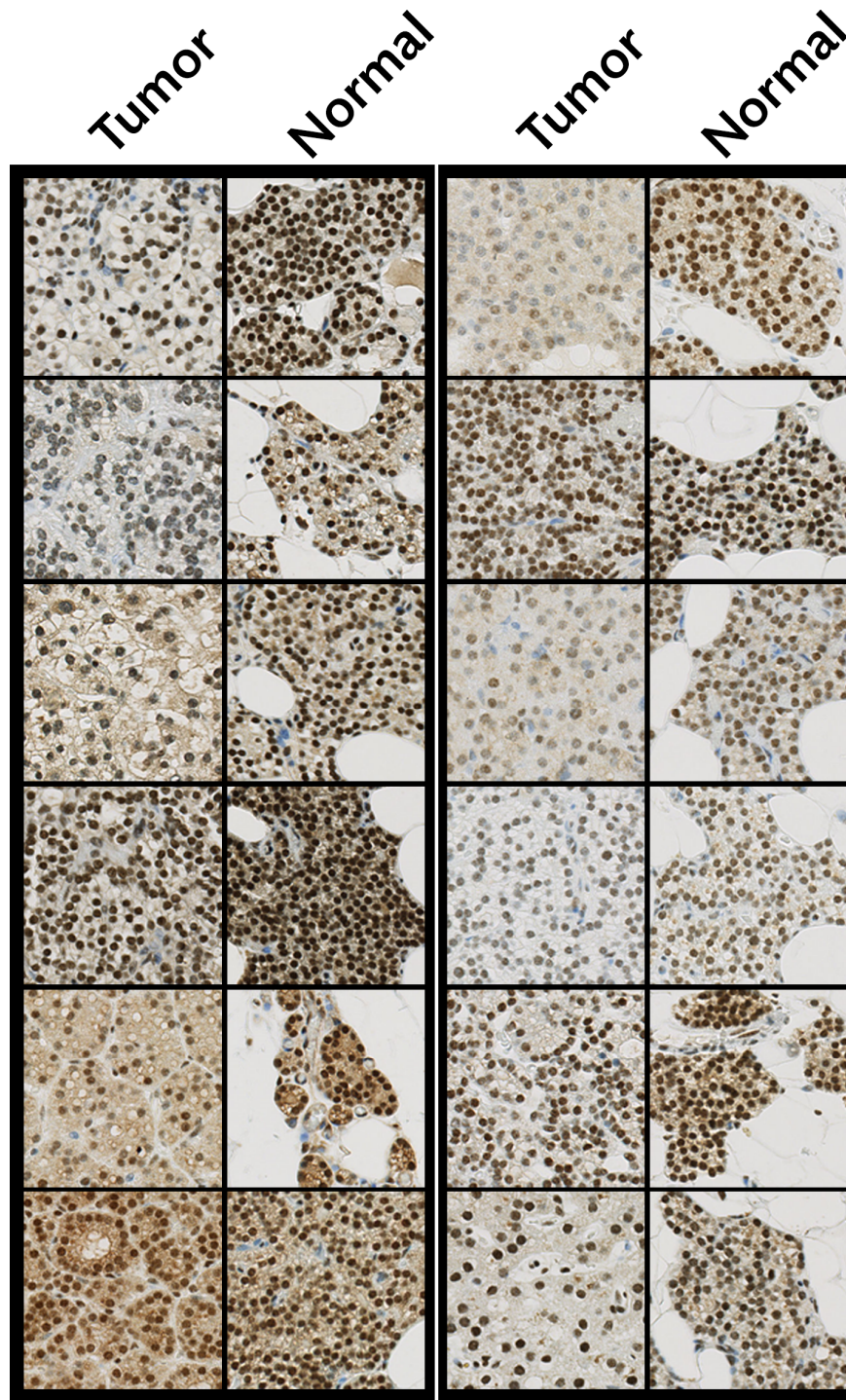
- Carl Sagan

Since postmenopausal women represent the predominant group of PHPT patients, estrogen has been investigated as a pathogenic factor. As described above, administration of estrogen has proven effects on parathyroid function *in vitro* and *in vivo*, but ERs have not been identified in parathyroid tissue. (Duarte et al., 1988, Prince et al., 1991) Hence these effects have been attributed to indirect pathways. The recent discovery of ER $\beta$  motivated us to reassess this postulate.

We investigated a panel of parathyroid tumors for ER $\beta$  and  $\alpha$  subtypes, and identified a wide expression of the ER $\beta$ 1 and ER $\beta$ cx isoforms. Expression analysis showed that *ESR2* gene expression levels were comparable between breast cancer cells and parathyroid tissue, and tumors originating from female patients showed higher levels of *ESR2*. These data were later reproduced using Taqman based qRT-PCR yielding comparable results (data not published). The staining pattern and intensity of ER $\beta$ 1 was changed in parathyroid tumors as compared to normal parathyroid tissue, (Figure 32) while the expression of ER $\beta$ cx was uniform. ER $\beta$ 1 immunoreactivity was weaker in larger or histopathologically adverse tumors. If loss of ER $\beta$ 1 signaling is important for parathyroid tumor development one could argue for two principal mechanisms, 1) loss of estrogen levels; and 2) loss of receptor signal transduction. If this hypothesis is valid, we would expect ER $\beta$ 1 levels to be less decreased in tumors from postmenopausal women. Indeed, ER $\beta$ 1 expression was lower in premenopausal women; however there is a substantial risk of selection bias in this comparison. The loss of E2 levels in postmenopausal women may also induce ER expression in a feedback dependent manner. Nonetheless, the expression levels varied between normal and tumor tissues, arguing for a tumor specific event.

ER $\beta$ 1 functionality was tested by treating primary parathyroid tumor cultures with either DNP or 4-OHT, and measuring changes in gene expression. Observed changes included parathyroid specific genes e.g. *VDR*, *CASR* and *ORAI2*. Gene expression profiling of significantly changed genes after DNP treatment were most significantly

associated with apoptosis of cancer cells, while 4-OHT was mostly associated to inhibition of angiogenesis. Additional analysis using Genemania (Warde-Farley et al., 2010) revealed that treatment with DPN (48h) most significantly correlated to “ER nucleus signaling pathway” ( $p < 0.0133$ , unpublished data).



**Figure 32.** Comparison of ER $\beta$ 1 immunohistochemical expression in parathyroid adenomas and corresponding normal rims.

Comparing genes differentially expressed in parathyroid adenomas (Haven et al., 2004) to genes significantly altered by DPN treatment revealed an interesting overlap. Among the 10 genes differentially expressed in parathyroid adenomas, the following 3 genes had their expression significantly changed by DNP: 1) *PRLR* that is comprehensively covered above. Estrogen has been shown to up-regulate *PRLR* mRNA in human endometrial stroma cells. (Tseng and Zhu, 1998) In the parathyroid tumor panel, levels of *ESR2* and *PRLR* inversely correlated ( $p=0.019$ ,  $r = -0.433$ ), suggesting a possible functional relationship. However, both receptors correlated to patient gender, which may have been a confounding factor. 2) *PVALB* encoding parvalbumin, a calcium binding protein co-localizing with PTH in normal and neoplastic parathyroid tissue. Its expression is increased in parathyroid adenomas, and hypothesized to play a part in PTH regulation. (Pauls et al., 2000) 3) Ephrin A1 encoded by *EphA1*, is a tyrosine kinase receptor with unknown function, (Hirai et al., 1987) and public EST profiling data show the highest expression in parathyroid tissue.

To elucidate how ER $\beta$ 1 mediate transcriptional changes, we analyzed whether transcriptional factor binding sites and ERE elements were present in differentially expressed genes. Binding sites of several transcription factors with known interaction with ER $\beta$  (Specificity protein 1 (SP1), Activator protein 1 (AP1), cAMP response element-binding (CREB), nuclear factor kappa-light-chain-enhancer of activated B cells (NF $\kappa$ B), Signal Transducer and Activator of Transcription 5A (STAT5A), tumor protein 53 (p35), Jun and Myc) as well as ERE elements were identified close upstream of target genes. These data suggest that both direct and indirect signaling (Figure 20) regulate the observed transcriptional changes. The role of ER $\beta$ cx in parathyroid tissue is unknown. Previous data suggest a dominant negative effect against ER $\alpha$  transcriptional activity, but the absence of this receptor in normal and tumorous parathyroid could suggest another role for ER $\beta$ cx.

This is the first study to identify a functional estrogen receptor in parathyroid tumors. The decreased proteins levels in tumors and transcriptional effects on genes in apoptosis suggest a tumor suppressive role for the receptor in the parathyroid.

## CONCLUDING REMARKS

Although a common entity, the etiology of PHPT remains clouded. Since gender and menopause constitute strong risk factors for PHPT, female endocrine systems have been implicated in the pathogenesis. Additionally, progress in the molecular genetic characterization of the HPT-JT syndrome suggested the involvement of the Wnt signaling pathway in malignant parathyroid tumors. This thesis aimed to characterize parts of the Wnt signaling pathway and female endocrine receptors in parathyroid tumors.

For Paper I-IV, the general conclusions are outlined below:

Parathyroid tumors exhibit aberrations within the Wnt signaling pathway. No changes in the levels or subcellular localization of  $\beta$ -catenin were observed as compared to normal parathyroid, suggesting that the changes within the Wnt pathway are involved in processes other than canonical Wnt signaling. Absence of APC immunoreactivity was a distinguishing feature of parathyroid carcinomas, suggesting APC as a marker for malignancy in parathyroid tumors. (Paper I)

The *CTNNB1* S37A mutation is a rare event in parathyroid tumors. (Paper II)

The PRLr is highly expressed in normal parathyroid. Parathyroid tumors show an altered expression level and subcellular localization of the PRLr. Primary cultures of parathyroid adenoma cells responded to physiological levels of prolactin with changes in gene expression and PTH secretion. These data suggest a role of prolactin signaling in normal parathyroid function and primary hyperparathyroidism. (Paper III)

Parathyroid tumors express Estrogen Receptor  $\beta$ 1 and  $\alpha$  isoforms. ER $\beta$ 1 expression was altered in parathyroid tumors, inversely correlating to tumor weight. Primary cultures of parathyroid adenoma cells treated with an ER $\beta$ 1 selective agonist resulted in differentially expressed genes, whose expression profiling suggested a tumor suppressive function of ER $\beta$ 1. (Paper IV)

Since menopause is a risk factor for aggravation of asymptomatic PHPT, (Silverberg et al., 1999) it is appealing to think of female hormone signaling (or lack thereof) as a mechanism to develop PHPT. Data from these studies suggest that functional female endocrine receptors are expressed in parathyroid tissue. While the altered expressions of these receptors may be an oncogenic drive in parathyroid tumors, it may also be a consequence of the tumor phenotype rather than a determinant of it. It is necessary that further studies are conducted to establish the role of these receptors in the normal and tumorous parathyroid glands.

# POPULÄRVETENSKAPLIG

## SAMMANFATTNING

Människan har normalt fyra bisköldkörtlar, vilka är belägna på halsen. Deras funktion är att producera parathormon, ett viktigt verktyg för kontroll av kroppens kalciumnivåer. Hormonet har flera funktioner i kroppen, bland annat ökar det både nedbrytningen av vårt skelett, njurarnas upptag av kalcium från urinen och aktiveringen av vitamin D – som bland annat ökar kalciumupptaget ifrån tarmen. Vid låga nivåer kalcium i blodet reagerar bisköldkörtlarna med att öka produktionen av parathormon, och återställer således kalciumnivån. Avsaknad av bisköldkörtlar leder omgående till allvarliga symptom såsom kramper och hjärtarytmier. Obehandlat kan det leda till döden.

Tumörer i bisköldkörtlarna är en relativt vanlig åkomma som drabbar ca 1 % av av den vuxna befolkningen. Hos kvinnor som passerat klimakteriet är förekomsten högre, och brukar uppskattas till ca 3 %. Dessa tumörer producerar ett överskott av parathormon, ett tillstånd som kallas hyperparathyreos (efter bisköldkörtlarnas latinska namn, *glandula parathyroideae*). Sjukdomen kan leda till diffusa symptom såsom trötthet, skeletturkalkning, njursten, nedstämdhet och förstoppning och är även förenad med ökad risk för hjärt-kärlsjukdom och cancer. I de flesta fall är dessa tumörer godartade, och patienten botas genom att tumören opereras bort. I vissa fall är dock sjukdomen elakartad, vilket leder till spridning av dottertumörer i kroppen. Efter operation undersöker en läkare (patolog) tumören i mikroskop. Trots lång erfarenhet hos patologen kan det vara svårt att identifiera elakartade (maligna) tumörer, och idag finns inga säkra tecken på malignitet innan spridning uppstått. Det har därför länge sökts efter ett diagnostiskt verktyg för att kunna identifiera elaka tumörer innan de sprids.

Eftersom sjukdomen oftast förekommer hos kvinnor efter klimakteriet har det länge misstänkts att kvinnliga könshormoner kan vara inblandade i sjukdomsuppkomsten. De molekylära strukturer (receptorer) som tar emot signaler från kvinnliga könshormoner i bisköldkörtlarna har inte kunnat påvisas, och därför har denna hypotes ifrågasatts.

Vid en ovanlig genetisk sjukdom drabbas patienten av maligna bisköldkörteltumörer. Molekylärgenetisk forskning har kunnat koppla den inblandade genen till den cellulära

signalvägen "Wingless" (flugor med genetiska fel inom Wingless saknar just vingar, därav namnet). Vi misstänkte därför att förändringar inom Wingless kunde ge upphov till maligna bisköldkörteltumörer. Inom ramen för denna avhandling har vi undersökt denna signalkaskad och förekomsten av kvinnliga könshormoners receptorer i bisköldkörteltumörer.

I ett första arbete har vi kartlagt proteiner i Wingless och funnit tumörspecifika förändringar. Ett av dessa proteins uttryck visade sig vara förlorat uteslutande i elakartade tumörer. Vår förhoppning är att detta i framtiden kan bli en viktig analys för patologens bedömning av bisköldkörteltumörens eventuella elakhet.

En central del av Wingless är proteinet  $\beta$ -catenin. Detta protein kodas av genen *CTNNB1*. Normalt bryts  $\beta$ -catenin kontinuerligt ned i cellen, men vid aktiverad Wingless signalering minskar denna nedbrytning och  $\beta$ -catenin ansamlas. Detta är ibland en normal process (vid exempelvis läkning av sårskador i huden), men om den sker okontrollerat kan det leda till tumörutveckling. Om genen *CTNNB1* har en särskild förändring (mutation) kan  $\beta$ -catenin inte brytas ned, vilket också tros kunna leda till tumörutveckling. Flera studier har letat efter dessa förändringar i bisköldkörteltumörer, dock har mutationer endast kunnat hittas i svenska patienter. Därmed uppstod frågan om denna mutation var en viktig mekanism för tumörutveckling i just svenska patienter.

Vi undersökte ett större antal tumörer ifrån svenska patienter, men kunde inte identifiera denna förändring. Genom denna studie kan vi statistiskt säkerställa att denna förändring är av mindre betydelse, även i den svenska populationen.

I de två sista studierna har vi kunnat påvisa receptorer för kvinnliga könshormoner i bisköldkörteltumörer. De identifierade är receptorerna för 1) *prolaktin*, en tillväxthormonliknande substans som är viktig vid amning, samt 2) *östrogen*, ett hormon som är viktig vid graviditet och mens, men har även många andra funktioner. Vi har kartlagt effekterna av deras funktion och hur receptorerna är uttryckta i bisköldkörtlarna. I bägge fallen verkar tumörens receptorer vara förändrade vid jämförelse med normal bisköldkörtel. Detta talar för att respektive molekyl kan vara viktig för tumöruppkomst i bisköldkörtlarna. Vi har också undersökt på vilket sätt bisköldkörtelcellen svarar på behandling av dessa hormoner. Medan signalförändringen



för prolaktin är svårvärderad, ser östrogensignalering ut att kunna skydda mot tumöruppkomst. Molekylärbiologiskt framstår en bild förenligt med att hormonsignalering kan ha en del i utvecklingen av bisköldkörteltumörer.

Sammanfattningsvis utgör dessa studier en grund för vidare forskning för ökad förståelse kring hormonsignaleringens roll i bisköldkörtlarna, både gällande dess normala funktion och i tumörer.

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Many people supporting me throughout the years are not mentioned below, thank you!

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**Anders Höög**, bihandledare. Endokrinpatolog med starkt engagemang för fältet. Jag

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