From the Department of Molecular Medicine and Surgery,
Karolinska Institutet, Stockholm, Sweden

IN SEARCH OF DIAGNOSTIC AND PROGNOSTIC MARKERS FOR THYROID CANCER: A PROTEOMICS APPROACH

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Dedicated to you

Tillägnat dig

<u>Thesis defense:</u>

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Friday the 30th of April 2010 at 09:00

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ABSTRACT

Early diagnosis of cancer can influence the therapeutic strategy increasing the chances of cure or, at least, prolonged survival. It is equally important to be able to detect prognostic signs, i.e. tumor features that characterize its behavior. In the case of thyroid tumors, which usually debut as palpable nodules, fine needle aspiration biopsy is that very stage when one should ideally set a secure diagnosis and gather prognostic information (such as tumor aggressiveness, metastatic potential, response to treatment). This sampling technique has improved its performance to a considerable degree, especially when guided by ultrasonography. Nevertheless, it still fails to conclusively diagnose certain thyroid tumors as the whole tissue structure, rather than just a few hundreds of cells, is necessary for diagnosis. Therefore, there is an imperative need for molecular diagnostic and prognostic markers, which could be easily traced on cellular material.

We investigated the potential of MIB-1, an index of cellular proliferation which is part of the cytological evaluation of other tumors, in facilitating the diagnosis or prognosis of thyroid tumors (Article I). Our conclusion was that MIB-1 is not particularly effective as a diagnostic marker for thyroid tumors, but it can serve as a marker of worse prognosis for patients with papillary thyroid carcinoma.

In an attempt to start exploring the field of thyroid proteomics, we began by developing a protein pre-fractionation protocol suitable for using archival frozen tissue and compatible with proteomics methodology, such as two-dimensional electrophoresis (2DE) and mass spectrometry (MS) (Article II). This protocol enabled us to obtain cytosolic and nuclear/nuclear membrane enriched protein fractions which comprised the starting material in our proteomics studies.

S100A6, a Ca^{+2}-binding protein evidently associated with p53 and β-catenin, was found to be significantly over-expressed in papillary thyroid carcinomas as compared to normal tissue or follicular thyroid tumors (Article III). Besides that and by applying 2DE-coupled MS, we were able to come up with two, partly overlapping sets of proteins which seem to be robust enough in distinguishing follicular thyroid carcinoma from its benign counterpart as well as from papillary thyroid carcinoma (Article IV).

This study gives new perspective in pursuing the discovery of novel thyroid cancer markers. We believe that by carrying out prospective studies particularly focused on the molecules presented in this thesis, thyroid diagnostics and prognostics can be greatly facilitated in the future for the sake of cancer patients. Such studies could also further elucidate the mechanisms behind thyroid tumor development.

Keywords: thyroid tumors, thyroid proteomics, S100A6, MIB-1, 2DE, MALDI-TOF-MS, SELDI-TOF-MS, protein pre-fractionation

LIST OF ARTICLES

The present thesis is based on the following articles. Throughout the text, these articles will be referred to by their Roman numerals.

I. **Sofiadis A***, Tani E, Foukakis T, Kjellman P, Skoog L, Höög A, Wallin G, Zedenius J, Larsson C
Diagnostic and prognostic potential of MIB-1 proliferation index in thyroid fine needle aspiration biopsy.
International Journal of Oncology, 2009, 35:369-374

II. Forsberg L, Larsson C*, **Sofiadis A,** Lewensohn R, Höög A, Lehtiö J
Pre-fractionation of archival frozen tumours for proteomics applications.
Journal of Biotechnology, 2006, 126:582-586

III. **Sofiadis A***, Dinets A, Orre LM, Juhlin CC, Foukakis T, Wallin G, Höög A, Zedenius J, Larsson C, Lehtiö J
Proteomic study of thyroid tumors reveals frequent up-regulation of the Ca^{+2}-binding protein S100A6 in papillary carcinoma.
(Submitted to Thyroid, under revision for resubmission)

IV. **Sofiadis A***, Becker S, Hellman U, Hultin-Rosenberg L, Zedenius J, Wallin G, Foukakis T, Höög A, Auer G, Lehtiö J, Larsson C
Novel candidate markers for thyroid follicular cancer: A proteomics approach
(Manuscript)

* Corresponding author

LIST OF ABBREVIATIONS

1D-/2D-PAGE	One-/Two-dimensional polyacrylamide gel electrophoresis
2DE	Two-dimensional electrophoresis
ACN	Acetonitrile
AMBIC	Ammonium bicarbonate
ATC	Anaplastic thyroid carcinoma
CID	Collision-induced dissociation
DAB	3,3-diaminobenzidine
DIGE	Difference gel electrophoresis
DNA	Deoxyribonucleic acid
EGFR	Epidermal growth factor receptor
ESI	Electrospray ionization
ETA	European thyroid association
FMTC	Familial medullary thyroid cancer
FNAB	Fine needle aspiration biopsy
FNMTC	Familial non-medullary thyroid cancer
FTA	Follicular thyroid adenoma
FTC	Follicular thyroid carcinoma
FT-ICR	Fourier transform ion cyclotron resonance
HRP	Horseradish peroxidase
IEF	Isoelectric focusing
IHC	Immunohistochemistry
IPG	Immobilized pH gradient
LC	Liquid chromatography
MALDI	Matrix assisted laser desorption/ionization
MEN	Multiple endocrine neoplasia
MI-FTC	Minimally invasive follicular thyroid carcinoma
MLR	Multiple linear regression

MS	Mass spectrometry
MS/MS	Tandem mass spectrometry
MTC	Medullary thyroid carcinoma
PAX8	Paired-box gene 8
PCA	Principal component analysis
PDTC	Poorly differentiated thyroid carcinoma
PLS(-DA)	Partial least squares (-Discriminant analysis)
PPARγ	Peroxisome proliferator-activated receptor gamma
PTC	Papillary thyroid carcinoma
PTM	Post-translational modification
RNA	Ribonucleic acid
RTK	Receptor tyrosine kinase
SELDI	Surface enhanced laser desorption/ionization
SNP	Single nucleotide polymorphism
(F)T3	(Free) Triiodothyronine
(F)T4	(Free) Tetraiodothyronine or thyroxine
TG	Thyroglobulin
TOF	Time-of-flight
TPO	Thyroperoxidase
TRH	Thyrotropin releasing hormone
TSH	Thyroid stimulating hormone or thyrotropin
TSH-R	TSH receptor
TSI	Thyroid stimulating immunoglobulin
VEGFR	Vascular epithelial growth factor receptor
WB	Western blot
WI-FTC	Widely invasive follicular thyroid carcinoma

TABLE OF CONTENTS

1. INTRODUCTION

1.1. The thyroid gland

1.1.1. Anatomy of the thyroid gland

The thyroid gland is a vascular, ductless gland situated in front of and around the trachea (Figure 1). It is named after the shield-shaped thyroid cartilage, which is the largest one of the larynx skeleton, protecting the vocal cords that lie directly behind it. "*θυρεός",* pronounced *theræos,* is the ancient greek word for "shield". The thyroid gland consists of two lobes – right and left – which are connected by a thinner tissue part in the middle (isthmus). Sometimes the isthmus gives rise to a third, middle lobe known as pyramidal lobe. Under normal conditions the gland's weight is approximately 10-25 grams. As far as its cellular composition is concerned, thyroid presents a great diversity consisting of follicular cells, parafollicular or C-cells, endothelial cells, fibroblasts, lymphocytes and adipocytes. Follicular cells are important for thyroid hormone synthesis and storage, while C-cells are responsible for the production of calcitonin, a hormone regulating calcium homeostasis. (Information from [1])

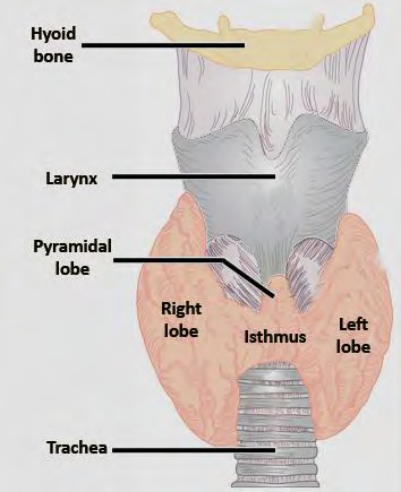

Figure 1. The thyroid gland and its anatomical relations. (Modified from: Barrett KE, Barman SM, Boitano S, Brooks H *Ganong's Review of Medical Physiology, 23rd Edition; www.accessmedicin.com*)

The superior and inferior thyroid arteries are responsible for supplying arterial blood to the gland, while the veins draining it form a plexus which gives rise to the superior, middle and inferior thyroid veins which, in their turn, drain to the internal jugular and innominate veins. The thyroid lymph vessels end in the right lymphatic and thoracic

trunks, while the gland's innervation is provided through the middle and inferior cervical ganglia of the sympathetic nervous system. (Information from [1])

1.1.2. Thyroid gland and thyroid hormones – Physiology and function

The basic structural and functional unit of the thyroid gland is the follicle, a large colloid-filled central cavity defined by a layer of follicular thyroid cells. Colloid comprises about 30% of the gland's mass and contains thyroglobulin (TG), a protein that holds a central role in the synthesis and storage of thyroid hormones. The latter are produced in the follicular thyroid cells which are both morphologically and functionally polarized – each side of the cell has specific functions pertaining to hormone synthesis and release – an important feature in the overall function of the cell. (Information from [2])

Thyroid hormones, triiodothyronine (T3) and tetraiodothyronine or thyroxine (T4), are produced by iodination of specific tyrosine residues located on thyroglobulin. The thyroid gland mainly releases T4 into the circulation (T4 concentration in plasma is about 90 nM, while for T3 it is only 2 nM). Most of the circulating T3 is, therefore, formed in the peripheral tissues by deiodination of T4 (a process occurring to a great extent in the liver). About 70% of T3 and T4 circulate bound to thyroxine-binding globulin (TBG), whereas a very small fraction circulates in its free form (0.3% of T3 and 0.03% of T4). (Information from [2])

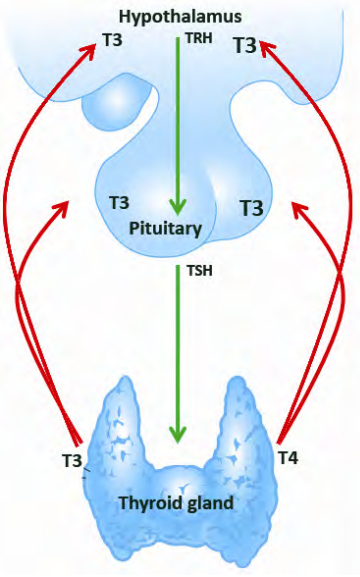

Figure 2. The hypothalamic-pituitary-thyroid axis regulating the production and release of thyroid hormones. Green arrows indicate stimulation (positive feedforward) and red arrows indicate inhibition (negative feedback). (Modified from: Molina PE, *Endocrine Physiology, 2nd edition; www.accessmedicin.com*)

TRH: Thyrotropin-releasing hormone
TSH: Thyroid stimulating hormone
T3: Triiodothyronine
T4: Tetraiodothyronine

Thyroid hormone synthesis and release are subjected to regulation by the hypothalamic-pituitary-thyroid axis (Figure 2). Thyrotropin-releasing hormone (TRH), a tripeptide synthesized in the hypothalamus, binds to membrane receptors of particular cells of the anterior pituitary gland resulting in stimulation of exocytosis and release of thyroid stimulating hormone (TSH) into the systemic circulation. TSH binds to TSH-receptors located on the basolateral membrane of the thyroid follicular cells activating a cascade of reactions which lead to thyroid hormone production and release. A regulatory negative feedback is provided mainly by T3 produced intracellularly in the hypothalamus and the anterior pituitary by deiodination of T4 (notably, the negative feedback effect of circulating T3 is weaker). TSH release by the pituitary is, thus, inhibited both directly and indirectly (by inhibiting the secretion of TRH from the hypothalamus). In addition, dopamine, somatostatin and glucocorticoids also exert a negative effect on TSH release. (Information from [2])

Thyroid hormones can affect multiple cellular events by binding to their receptors expressed in virtually all tissues. Thyroid hormone receptors are nuclear receptors (DNA-binding transcription factors) responding to hormone binding by functioning as molecular switches. Among others, thyroid hormone receptors mediate the following cellular events [2]:

- **i.** Expression of cell membrane Na^+/K^+-ATPase (increase in O_2 consumption)
- **ii.** Expression of uncoupling protein, enhancing fatty acid oxidation and heat generation without production of adenosine triphosphate (ATP)
- **iii.** Protein synthesis and degradation, contributing to growth and differentiation
- **iv.** Epinephrine-induced glycogenolysis and gluconeogenesis, affecting insulin-induced glycogen synthesis and glucose utilization
- **v.** Cholesterol synthesis and low-density lipoprotein receptor regulation

Controlling the rate of metabolism and, thus, the function of practically every organ in the human body, thyroid hormones are essential for normal growth and development. Some examples of the organ-specific effects they exert include [2]:

- **i.** Bone growth and development through osteoblast and osteoclast activity
- **ii.** Cardiac inotropic (force of heart muscle contraction) and chronotropic (heart rate) effects, increase of cardiac output and blood volume and decrease of systemic vascular resistance
- **iii.** White adipose tissue differentiation, induction of lipogenic enzymes, intracellular lipid accumulation and stimulation of adipocyte cell proliferation

iv. Liver cell proliferation and mitochondrial respiration, regulation of triglyceride and cholesterol metabolism, as well as lipoprotein homeostasis
v. Pituitary hormone synthesis regulation, stimulation of growth hormone production and inhibition of TSH secretion
vi. Axonal growth and development in brain

1.1.3. Goiter and thyroid nodular disease

An enlarged thyroid gland is referred to as "goiter". Hormone biosynthetic defects, iodine deficiency, autoimmune disease and nodular disease can each lead to goiter, through different mechanisms. [3]

Biosynthetic defects and iodine deficiency are associated with reduced efficiency of thyroid hormone synthesis which leads to increased TSH release. The latter stimulates thyroid growth as a compensatory mechanism to overcome the block in hormone synthesis. Graves' disease and Hashimoto's thyroiditis are also associated with goiter. TSH-R–mediated effects of thyroid stimulating immunoglobulins (TSI) are mainly responsible for inducing goiter in Graves' disease, while the goitrous form of Hashimoto's thyroiditis occurs because of acquired defects in hormone synthesis, leading to elevated levels of TSH and its consequent growth effects on the thyroid gland. Lymphocytic infiltration and immune system–induced growth factors also contribute to thyroid enlargement in Hashimoto's thyroiditis. Nodular disease is characterized by the disordered growth of thyroid cells, often combined with the gradual development of fibrosis. (Information from [3])

The management of goiter greatly depends on its etiology and, therefore, detecting a thyroid enlargement upon clinical examination calls for further evaluation to identify its cause. [3]

1.1.4. Thyroid tumors

1.1.4.1. Classification, epidemiology and molecular pathogenesis

According to the latest edition of the World Health Organization (WHO) Classification of endocrine tumors, thyroid tumors comprise follicular thyroid carcinoma (FTC), papillary thyroid carcinoma (PTC), medullary thyroid carcinoma (MTC), poorly differentiated thyroid carcinoma (PDTC), anaplastic thyroid carcinoma (ATC) and, the only benign tumor, follicular thyroid adenoma (FTA). [4] All thyroid tumors arise from the follicular cell, except MTC which originates from the C-cell. [5-7] Though the most frequent endocrine malignancy, thyroid cancer is a relatively uncommon neoplasm with a global incidence of 1.3 in men and 3.3 in women (Age Standardized Rates per

100,000 population and year). [8] In Sweden the incidence rates for men and women are 2.0 and 5.1 per 100,000 population, respectively. [9]

Follicular thyroid adenoma (FTA)

There are no certain figures on the epidemiology of FTA since distinguishing it from hyperplastic nodules is still difficult due to the lack of consistent criteria. Follicular adenomas do usually present as solitary, round nodules surrounded by a thin capsule. By definition, no capsular or vascular invasion should be present. [4]

Oncocytic adenoma (also known as oxyphilic or Hürthle-cell adenoma) is a common FTA variant, which is characterized by cells rich in eosinophilic, granular cytoplasm and large nuclei with very prominent nucleoli. [10] Oncocytic cell tumors account for approximately 3-10% of all thyroid neoplasms. [10] In addition, atypical FTA are characterized by high cellularity, atypical nuclei or unusual histopathology. [4]

A number of different genetic events have been reported to be related to FTA such as numerical chromosomal aberrations (usually trisomy 7 alone or concomitantly with other characteristic trisomies). [11, 12] The *PAX8-PPARγ* translocation or mutations in *RAS* oncogenes are also genetic events that, although typical for FTCs, they have been occasionally found in adenomas as well. [13-16]

Table 1. Overview of epidemiological characteristics of thyroid carcinomas. [17]

Tumor type	♀/♂	***Age (years)***	***Lymph-node metastasis***	***Distant metastasis***	***5-year survival rate***
Follicular thyroid ca	2:1-3:1	40-60	<5%	20%	>90%
Papillary thyroid ca	2:1-4:1	20-50	<50%	5-7%	>90%
Medullary thyroid ca	1:1	30-60	50%	15%	80%
Poorly diff. thyroid ca	0.4:1-2.1:1	50-60	30-80%	30-80%	50%
Anaplastic thyroid ca	1.5:1	60-80	40%	20-50%	1-17%

Follicular thyroid carcinoma (FTC)

FTC accounts for approximately 15% of all thyroid malignancies, it usually appears later in life than PTC and tends to metastasize mostly hematogenously giving lung, bone and, more rarely, liver metastases. The differential diagnosis between benign and malignant follicular neoplasms largely depends on evidence of vascular or capsular

invasion. The latter is generally observed in post-operative histopathology, but not in fine needle aspiration biopsy (FNAB). The FTC which presents with limited invasion of the tumor capsule or vessels is known as minimally invasive (MI-FTC) in contrast to the widely invasive (WI-FTC), which infiltrates the adjacent thyroid tissue and/or blood vessels in a greater scale. FTC, as well as its oxyphilic-cell variant (Hürthle cell carcinoma), have a worse prognosis than PTC. (Information from [4, 18])

FTCs are predominantly characterized by *PAX8-PPARγ* rearrangements, *RAS* point mutations and aneuploidy. [19] PAX8 (Paired-box gene 8) is a transcription factor essential for thyroid development, whereas PPARγ belongs to the superfamily of nuclear receptors and is expressed in low amounts in normal thyroid tissue. The *PAX8-PPARγ* fusion gene was first described by Kroll *et al* in 5 out of 8 FTCs and it was initially thought as a tumor-specific marker. [20] Although, a number of studies have also identified *PAX8-PPARγ* rearrangement in FTAs, as well as shown lower frequency in FTCs. [14-16, 21]

Activating point mutations of *RAS* oncogenes are also detected in FTC (most frequently involving *NRAS*). Ras are small GTPases playing a central role in oncogenic signal transmission. The fact that they are identified in FTA as well speaks for a possible role as an early event in thyroid carcinogenesis. [19] An interesting observation is that *RAS* mutations and *PAX8-PPARγ* fusion seem to be mutually exclusive genetic events, indicating that FTC develops following alternative pathways. [21] Moreover, it has been observed that FTCs harboring *RAS* mutations are associated with poor patient survival and might be the ones that ultimately develop into an ATC. [22-24]

Abnormalities in chromosome 3 are characteristic for FTC with the most common ones being losses at 3p. [25, 26] Deletions of 1p, 6p/q, 8p/q, 9p, 11q, 13q, 18q and 22q are among the copy number changes that have frequently been described in FTC patients. [12, 27, 28] Moreover, Dettori *et al* associated loss of chromosome 22 to an invasive FTC phenotype. [29]

Papillary thyroid carcinoma (PTC)

PTC is the most common thyroid malignancy which, nevertheless, has the best prognosis of all. It commonly spreads via the lymphatics and tends to grow slowly remaining confined to the thyroid and the regional lymph nodes for years. Characteristic cytological features, such as papillary structures, psammoma bodies, nuclear pseudoinclusions and large nucleoli giving “orphan Annie” appearance to the nuclei, facilitate the diagnosis of PTC either on FNAB or on histology after surgical removal of the gland. (Information from [3])

The genetic events being responsible for PTC development are well characterized. The *RET* proto-oncogene (chromosome 10q11.2), encoding for a membrane receptor tyrosine kinase (RTK), is under normal conditions expressed in neural and neuroendocrine cells as well as during embryogenesis. [30] Chromosomal rearrangements linking unrelated genes to the distal part of *RET* (called *RET/PTC* rearrangements) result in an illegitimate activation of *RET* and, thus, of the whole RET-Ras-Raf-MAPK oncogenic pathway. [31, 32]

RAS mutations – found in almost 1 out of 3 human malignant tumors – are also detected in PTC, though not as frequently as in FTC. Rearrangements of the *NTRK1* gene are also detected in no more than 12% of PTC cases. [33] The most common genetic alteration in PTC is the mutation of the *BRAF* gene (reported in nearly 50% of PTC cases, range 28-83%). [34] B-Raf belongs to a family of serine/threonine kinases (Raf family) and its V600E substitution is the mutation which is almost exclusively found in PTC. [4, 34] Interestingly, Soares *et al* detected no overlap between *BRAF* mutation and *RET/PTC* rearrangement, suggesting that the former might be an alternative event to the latter. [35] *BRAF* mutation poses a promising potential as a prognostic factor of PTC invasiveness, metastasis or recurrence. [34]

Medullary thyroid carcinoma (MTC)

MTC is the only thyroid malignancy arising from the parafollicular or C-cells and represents approximately 5-8% of all thyroid cancers. Although sporadic MTC is more common, up to 1/4 of all cases can occur as an heritable disorder representing either part of multiple endocrine neoplasia type 2 (MEN 2A or 2B) or familial MTC (FMTC). Activating *RET* mutations, both germ-line and somatic, are considered key events for the development of MTC. (Information from [36, 37])

As far as the Ras pathway is concerned, no *RAS* or *BRAF* mutations in MTC cases were reported in a number of studies, until Goutas *et al* presented opposing results. [37, 38]

Poorly differentiated thyroid carcinoma (PDTC)

Showing limited evidence of follicular cell differentiation and sharing morphological and behavioral characteristics of both differentiated and undifferentiated thyroid carcinomas, PDTC represents about 4-7% of all thyroid malignancies. [4] Most often it appears as a large, solitary thyroid mass with or without regional lymph node involvement. Lung and bone metastases are relatively frequent at the time of diagnosis. [39]

The molecular events behind the development of PDTC are not clearly understood, mostly owing to the inclusion or exclusion criteria which different authors have used as the histological features that define PDTC have been regularly revised. In a comprehensive review on the cytological and histological characteristics of PDTC, Bongiovanni *et al* recently reported *TP53* alterations ranging between 17-38% among the different studies and *RAS* mutations from 18% to 63%. Furthermore, *BRAF* mutations, *RET/PTC* rearrangement and *PAX8-PPARγ* fusion gene could only be detected in cases of PDTC with an associated FTC or PTC. (Information from [6])

Anaplastic thyroid carcinoma (ATC)

ATC accounts for only about 1-2% of thyroid cancers and it usually presents as a rapidly growing, irregular, solid mass infiltrating not only the gland itself, but neighboring structures as well (trachea, esophagus, larynx). Metastases to cervical lymph nodes or the lungs are very common and local recurrence after surgical excision is the rule. The treatment is almost exclusively palliative combining surgery, external radiation and chemotherapy. (Information from [18])

Smallridge *et al* recently reported a comprehensive review of the current status of studies on ATC across all different fields (genetics, functional genomics, cytogenetics, pre-clinical *in vitro* and *in vivo* studies). [7] Among others, mutations in *TP53*, *CTNNB1* or *PIK3CA* genes have been associated with ATC in numerous relevant studies. [7, 40]

In addition, there is an ongoing dispute whether this tumor arises from dedifferentiation of a well-differentiated carcinoma or not. *BRAF* mutations (predominant in PTC) and *RAS* mutations (mostly present in FTC) are also present in a proportion of ATC (ranging between 10-33% and 20-90% for *BRAF* and *RAS*, respectively). Interestingly, *RET/PTC* and *PAX8-PPARγ* rearrangements have not been detected in ATC. (Information from [41])

1.1.4.2. Risk factors

Exposure to ionizing radiation has been so far the only environmental risk factor evidently connected to thyroid carcinoma. Nevertheless, as the majority of thyroid cancer patients have no such known history, other risk factors like dietary, genetic or hormonal have been sought. [42]

A number of studies based on data from populations with acute environmental exposure to ionizing radiation (nuclear weapons or accidents) have confirmed the association of the latter to an increased risk of thyroid cancer, which is moreover inversely related to the age at exposure. It is very characteristic, for example, that in

parts of Belarus that were severely affected by the Chernobyl nuclear accident, the incidence of childhood thyroid cancer increased more than 100 times after the accident in 1986. [42, 43]

The effect of dietary factors like iodine, tobacco or alcohol is quite difficult to measure and relevant reports have been more or less contradictory. Although, for instance, iodine is necessary for thyroid hormone production and normal thyroid function, low-iodine diet has by some investigators been speculated to increase the risk of thyroid cancer, while others provide evidence towards the opposite. (Information from [42])

As far as gender is concerned, the incidence of thyroid carcinoma is 3 times higher in women than in men. Crude incidence rate ratios, though, reveal a more dramatic difference in women of reproductive to middle age. According to the latest version of SEER Program (Surveillance Epidemiology and End Results), the women/men incidence rate ratio for thyroid cancer is 8:1 at the age of 20, 4:1 at the age of 40 and 2:1 at the age of 60. [44] Therefore, a role of hormone-related factors in the development of thyroid cancer has been proposed, but the data from various cohort and case-control studies are inconsistent. [42]

At this point it should be mentioned that heredity can also play a decisive role in thyroid tumor development. As mentioned previously, heritable forms of MTC correspond to about 25% of all MTC cases and its genetic background (*RET* mutations) has been extensively studied and elucidated. A number of other genes are responsible for familial predisposition to thyroid cancer, such as *PTEN* in Cowden's syndrome. [45]

Additional susceptibility genes have been anticipated for familial forms of non-medullary thyroid cancer, but not yet identified. These tumors account for 3.2-6.2% of all thyroid cancers and PTC is the most common histological subtype. [45]

1.1.4.3. Diagnosis and treatment

The European Thyroid Association (ETA), along with the ETA Cancer Research Network (ETA-CRN), has issued a consensus statement on the diagnostic and therapeutical approach of a patient with thyroid nodule. Upon the discovery of a thyroid nodule, physical examination, complete medical history and appropriate laboratory tests are the first steps to a comprehensive evaluation of a patient. Today's diagnostic quiver includes also neck ultrasonography (US), FNAB, usually under ultrasonographic guidance, and thyroid scintigraphy. A descriptive flow-chart for the diagnostic approach of a thyroid nodule can be seen in Figure 3. (Information from [46])

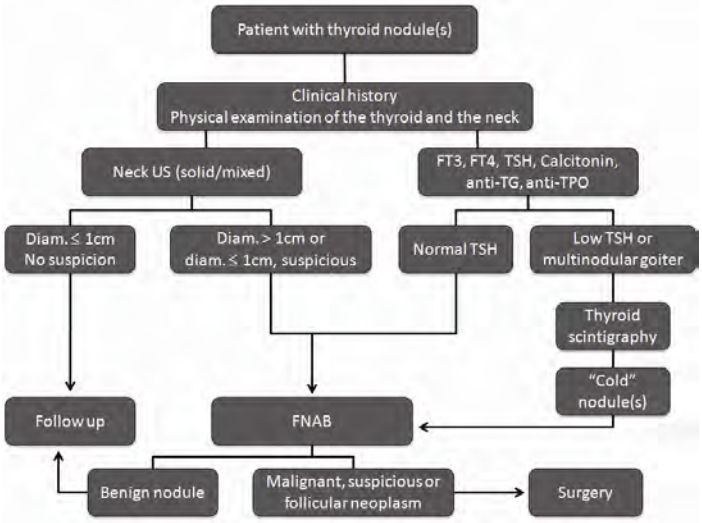

Figure 3. Diagnostic evaluation of patients with thyroid nodule(s) as recommended by ETA and ETA-CRN. (Modified from reference 45)

For comparison purposes, a simplified flow-chart of the management of thyroid nodules in daily clinical praxis in Sweden follows in Figure 4.

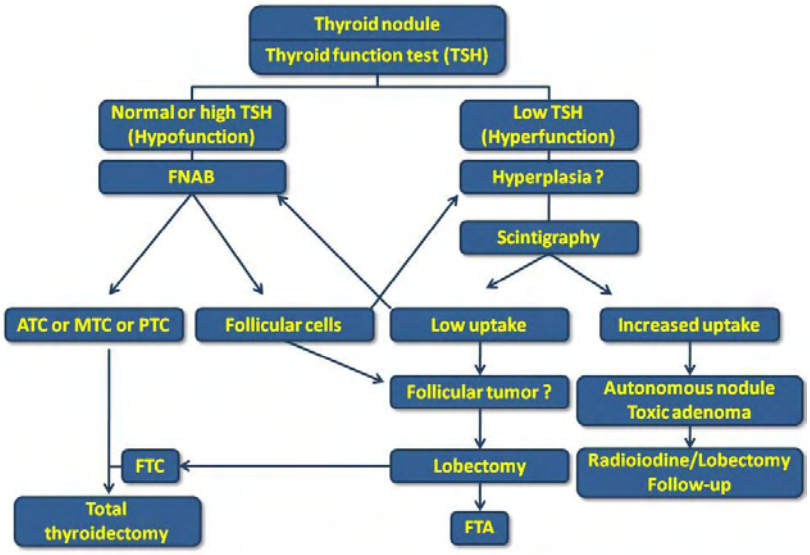

Figure 4. Diagnostic evaluation of thyroid nodule(s) in Sweden. (Modified from: Hamberger B, Zedenius J, Chapter 4. Endokrina Organ. In *Kirurgi 7th edition,* Hamberger B, Haglund U, Eds. Liber AB: Stockholm 2009.)

Surgical treatment and post-surgical radioiodine administration (thyroid ablation) are the fundamental treatment modalities used for differentiated thyroid cancer. With few exceptions, total or near-total thyroidectomy is the standard procedure, which minimizes the risk of local recurrence and is, under expert hands, performed with minimal morbidity. Besides that, it facilitates post-surgical thyroid ablation and allows for an adequate follow-up. (Information from [46])

There are several controversial issues when it comes to the treatment scheme of thyroid cancer. These issues are discussed in section 2.4 “Conclusions and Future perspectives" (page 48).

1.2. Tumor biomarkers

Tumor biomarkers are defined as those molecules that are often produced by the tumor itself or the host system as a response to the tumor. They constitute the biological material that can ideally determine the risk of getting cancer, detect and classify cancer or provide insight into prognosis and, consequently, a therapeutic advantage. Even if biomarkers can provide personalized information for the individual patient – information pertaining to diagnosis, prognosis and treatment – it is very crucial that straightforward algorithms are applied by the healthcare providers for a stepwise implementation of those markers. (Information from [47])

There are largely 3 general categories of analytes that can act as biomarkers, namely nucleic acids, proteins and metabolites. Nucleic acid-based markers include single nucleotide polymorphisms (SNPs), chromosome aberrations, DNA copy number changes, differential promoter-region methylation, over- or underexpressed RNA transcripts and regulatory RNAs (e.g. microRNAs). On the other hand, protein markers subsume tumor antigens (e.g. prostate specific antigen, PSA), cell-surface receptors or peptides released by tumors into various body fluids. (Information from [48])

Validating a candidate biomarker is equally important as its discovery itself. Before being considered for use in clinical routine, potential biomarkers need to be scrutinized and a number of practical obstacles ought to be overcome. Pepe *et al* proposed that biomarker development should take place in five phases: **a.** Preclinical exploratory, **b.** Clinical assay and validation, **c.** Retrospective longitudinal, **d.** Prospective screening and **e.** Cancer control. [49]

Currently, there is a number of candidate biomarkers for distinguishing thyroid tumors, although no consensus has been reached on their use in daily clinical practice. The *BRAF* and *RAS* point mutations as well as the *RET/PTC* and *PAX8-PPARγ*

rearrangements encompass the most common genetic alterations that characterize FTC and PTC and have a considerable impact in these tumors' diagnosis and prognosis.[50] In addition, a number of micro RNAs (miRNAs) that could also function as biomarkers have been identified and studied in thyroid carcinomas. [50] Finally, a panel of immunohistochemical markers, such as Ki-67, galectin-3, HBME-1 and cytokeratin 19 (CK19), also pose as a potential diagnostic or prognostic tool. [51, 52]

1.3. Proteomics

1.3.1. Overview

In 1996, and while reporting on his work on protein identification by 2DE and amino acid analysis, Marc Wilkins first came up with the term "proteome". [53] He defined it as "...the PROTEin complement expressed by a genOME...". However, as de Hoog and Mann commented more recently, the proteome is a very dynamic entity and, for that definition to be more complete, one should specify that it concerns all protein isoforms and modifications of a given cell at a specific time point. [54] In line with genomics (the study of DNA sequences) and functional genomics, the field of proteomics evolved to deal with protein structure, expression, localization, modifications and interactions.

Cancer research has until recently been devoted to extensive studies on the gene and gene expression levels. It has been realized, though, that in many cases there is very little correlation between protein concentration and number of mRNA transcripts and this owing to a certain level of biological regulation between transcript and protein (alternative splicing, epigenetic silencing, post-translational modifications). Taking this into account, it is of no surprise why a great deal of research has shifted towards proteomics as, in the long run, it is protein molecules that are mainly responsible for cellular biological phenomena.

1.3.2. Fundamental technology/methodology

Proteomics technologies can roughly be divided in the following categories [54]:

i. Gel-based, for separating complex protein mixtures

ii. Mass-spectrometry-based, for sensitive and comprehensive analysis of proteins and their identification

iii. Array-based, with similarities to gene dose and gene expression microarrays

iv. Structure- and imaging-related, meaning large scale investigation of the 3D structure, localization and dynamics of proteins

Before proceeding in outlining proteomics methods, particularly focusing on these used within the frame of the present thesis, it is worth mentioning that their performance relies heavily on the preparation of the starting material. Following tissue disruption and cell lysis – procedures equally important, where one is nowadays offered a great number of alternatives – protein pre-fractionation, *i.e.* separation of particular groups of proteins, constitutes a useful tool for better sub-proteome characterization (e.g. certain organelle proteins, membrane proteins, low-abundance proteins). Consequently, and depending on the applied methodology, a number of diverse issues have to be considered in order for that methodology to perform optimally. Averting protein degradation, ways of protein solubilization, avoiding all kinds of contamination, salt removal techniques, high-abundant protein depletion or mass-spectrometry compatibility are among those factors that can greatly influence both the experimental conditions and the final outcome.

1.3.2.1. Gel-based proteomics methods

The most commonly used method in this category and one of the cornerstone techniques in proteomics is the two-dimensional polyacrylamide gel electrophoresis (2D-PAGE), or two-dimensional electrophoresis (2DE) for short. 2DE is based on the separation of proteins first according to their isoelectric point[†] (1^{st} dimension, Isoelectric focusing or IEF) and then according to their size/molecular weight (2^{nd} dimension) (see Figure 5).

Since Patrick O'Farrell first developed the technique in 1975 [55], a lot of changes and improvements have been introduced. Probably one of the most revolutionary evolvements in the field was the introduction of immobilized pH gradient (IPG) gel strips which made it possible to create a truly steady IEF, with a much higher resolution and reproducibility. [56]

A relatively more recent modification was introduced by Ünlü *et al* in 1997, which, in brief, allows for the simultaneous separation of three protein samples in the same gel. [57] This technique, called difference gel electrophoresis (DIGE), utilizes three different fluorescent dyes (cyanine dyes or CyDyes) and by providing the experiment with an internal control, it has contributed to faster and reproducible detection of differences in protein expression patterns.

Once separated in the 2DE-gel, the proteins are stained and detected by either colorimetry or fluorescence (post-electrophoretic stains). Staining with silver nitrate or

[†] Isoelectric point (pI): The pH value for which a particular protein carries no net charge.

Coomassie blue are typical examples of the former, while SYPRO dyes are the most typical fluorescent dyes. CyDyes are an example of fluorescent pre-electrophoretic staining. The sensitivity and dynamic range of stains, the use of stains specific for particular post-translational modifications (PTMs) as well as the stains' compatibility with mass spectrometric methods are questions that need to be answered well in advance when setting up a 2DE experiment. (Information from [58])

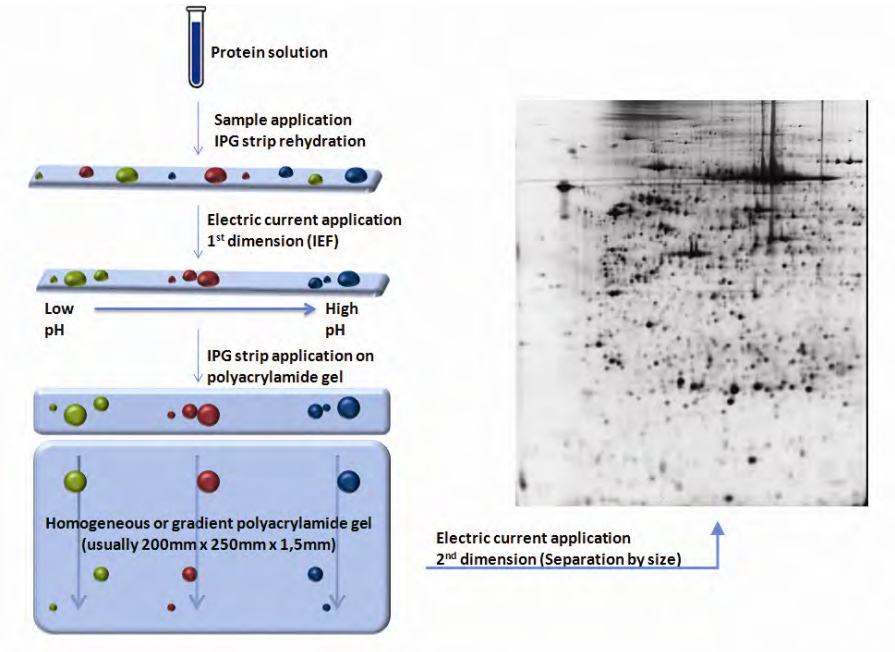

Figure 5. Schematic outline of two dimensional electrophoresis (2DE). A protein solution is applied on an immobilized pH gradient (IPG) gel strip. The IPG strip is rehydrated by absorbing the solution. Then, under the effect of electrical current, the protein molecules are migrating within the IPG strip until they reach their isoelectric point (pI) (isoelectric focusing or IEF). The strip is then placed on top of an acrylamide gel and an electric current is applied, this time to separate proteins according to their size. An example of a silver-stained 2DE-gel is shown to the right representing a cytosolic protein fraction from frozen thyroid tissue.

At last, but not least, a very crucial step in a 2DE experiment is that of image acquisition and analysis. In order to process the information from a 2DE-gel, one first needs to convert it to digital data. Depending on the protein stain used, this is carried out with the use of an appropriate imaging system, such as laser-based detectors, CCD cameras or flatbed scanners. Then obtaining useful biological information from the acquired complex data is done with the help of computerized analysis provided by software packages developed for this scope.

1.3.2.2. Mass spectrometry (MS)-based proteomics methods

The history of mass spectrometry (MS) is more than 100 years old. In 1899 Sir JJ Thompson (Nobel Prize in Physics, 1906) invented the first mass spectrometer [59] as cited in [60], but it was not until the 1980s that this discovery became a routine technique in protein chemistry studies. Lacking soft ionization methods for producing protein and peptide fragments, peptide sequencing was until then carried out by Edman degradation followed by UV-absorbance-based identification of the released amino acids. [60,61]

The course of events changed dramatically starting at the beginning of 1980s with the development of a series of ionization methods (such as soft desorption/ionization, electrospray ionization or ESI, soft laser desorption), which made it possible to volatize miniscule biomolecules into gas phase without destroying them. [60] Nowadays, protein identification by MS-based methodology has become a well-established, routine analysis.

The main components of a mass spectrometer are depicted in Figure 6. Since describing the fundamental notions of all available MS technology is beyond the scope of this thesis, special focus will be given only to the techniques used in our studies, namely matrix assisted laser desorption/ionization time-of-flight MS (MALDI-TOF-MS), surface enhanced laser desorption/ionization time-of-flight MS (SELDI-TOF-MS) and liquid chromatography-MS (LC-MS). These methods will at this point be briefly overviewed, but they will be more thoroughly presented in the "Materials and Methods" (section 2.2)

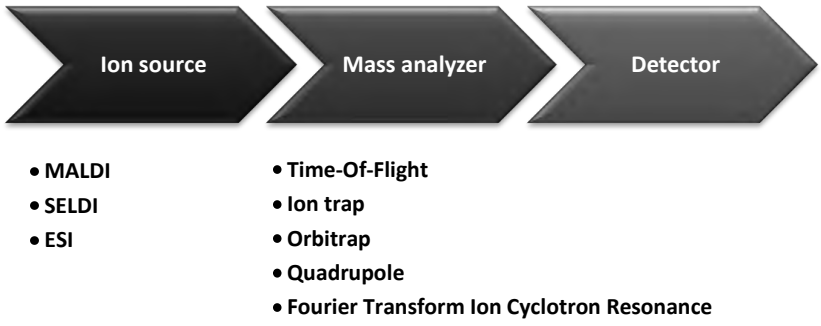

Figure 6. The main parts of a mass spectrometer.

MALDI-TOF-MS

In 1988, Karas and Hillenkamp realized that molecules with masses greater than 10 kDA could efficiently be desorbed by embedding them in a UV-absorbing matrix. [62] Since then, matrix-assisted laser desorption/ionization (MALDI) has become one of the most commonly used ion generating sources in MS (Figure 7).

Briefly, the analyte sample (be it proteins or peptides) is mixed with a matrix and this mixture is placed on a metal target to crystallize. After that, a pulsed UV laser beam transfers energy to the matrix crystals and they, in their turn, convert it to heat which leads to a localized "explosion" of the sample/matrix mixture. As the protein or peptide molecules enter the gaseous phase, they simultaneously get protonated (acquisition of H^+) and accelerated by an electric field into the mass analyzer.

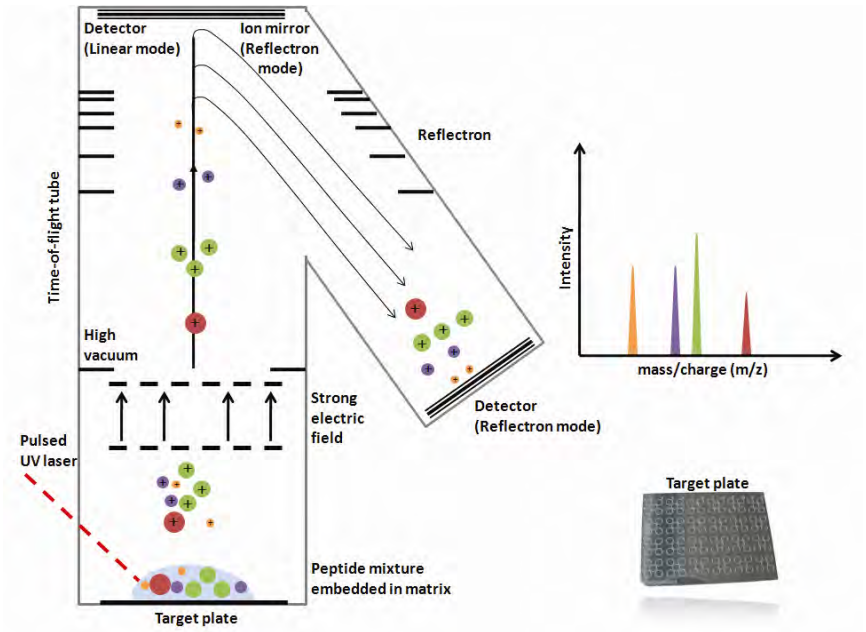

Figure 7. Schematic representation of MALDI-TOF-MS. Matrix crystals absorb energy from a UV laser source and transfer it to the analyte molecules, which in their turn get protonated and enter the gaseous phase. Analyte ions' acceleration by a strong electrical field is followed by their entrance in a vacuum chamber (time-of-flight tube). Reflectron-mode MALDI prolongs the ions' time-of-flight improving the instrument's mass resolution. To the left of the figure, a graphic example of the data output (spectrum) with the various peaks corresponding to the different groups of ions reaching the detector in different time points. (Modified from: Nijmegen Proteomics Facility - www.proteomicsnijmegen.nl/Maldi_pages/Maldi-about.htm)

Mass analysis is based on the time it takes for each ion to travel between the two ends of a field-free, vacuum chamber. The mass-to-charge (m/z) ratio of an ion is calculated based on the kinetic energy equation $E=½mv^2$ (E: kinetic energy, m: mass and v: velocity). At a constant energy, peptides with a greater mass will travel at a slower speed, thus taking them a longer time-of-flight before reaching the detector at the end of the flight chamber (Linear mode). In real conditions, though, a small spread of kinetic energy during ionization is unavoidable, thus making constant energy a mere assumption. This technical problem, however, is nowadays overcome by the inclusion of an ion mirror (reflectron) and the application of delayed extraction (*i.e.* time delay between ion generation and allowing the ions to enter the flight chamber, which minimizes their initial kinetic energy distribution), thus enhancing the instrument's mass resolution (reflectron mode). The resulting "spectrum" is the detector's output, where y axis corresponds to the amount of ions hitting the detector at a specific time point (*i.e.* signal intensity) and x axis corresponds to the "mass-to-charge" ratio (*i.e.* size).

SELDI-TOF-MS

Hutchens and Yip were the first ones to report the development of surface enhanced laser desorption/ionization (SELDI). [63] In this chip-based MALDI technique, the sample is analyzed directly on a solid-phase surface which is coated with various chemical or biological functional groups (e.g., hydrophobic, anionic, cationic, metal ion, antibody, receptor or DNA). The analyte sample is applied on the so called Protein Chip® Array allowing for binding of a specific group of analytes, depending on the affinity properties of the chip's surface. Sample incubation is followed by a washing step, which removes all non-specifically bound molecules as well as unwanted contaminants like salts, lipids or detergents. This sort of retentate chromatography is one of the major advantages of SELDI, as it provides a separation step which reduces the sample's complexity, facilitates the enrichment of low-abundant proteins or peptides and discards molecules that could eventually interfere with the MALDI analysis. (Information from [60, 64-66])

Ion generation, mass analysis and ion detection are carried out following the principles of MALDI-TOF-MS. SELDI-TOF-MS was initially widely implemented for biomarker discovery, aiming at producing proteomic patterns (based on different mass spectra) to distinguish between experimental populations. However, certain limitations have been noticed and questions have been raised concerning the actual potential of SELDI. Sample type, processing and storage, low dynamic range, decreased sensitivity, low ionization efficiency, lack of reproducibility and absence of evidence for the long-term robustness of the technology are some of the shortcomings of SELDI. (Information from [60, 64-66])

1.3.2.3. Array-based proteomics methods

Protein microarray is a recently developed technology which allows profiling the state of a signaling-pathway target. Moreover, a new type of protein microarray, namely reverse-phase array, has made it possible to analyze signaling pathways by using only a small number of cells, usually procured by laser-capture microdissection (LCM). Proteins are extracted from these cells, the lysate is arrayed onto nitrocellulose slides and the analyte(s) of interest is (are) detected by the application of labeled probe(s) (e.g. an antibody). (Information from [67])

One of the advantages of this sort of array is the ability to use both denatured and non-denatured protein lysates. In the case of denatured samples, antigen retrieval which is a common limitation in tissue arrays is no longer problematic, while processing non-denatured samples makes it possible to detect and characterize protein-protein or protein-nucleic acid interactions. Another advantage of this method is that each patient sample is arrayed in a low-scale dilution curve, thus providing an internal standard curve. At last, but not least, reverse-phase array does not require tagging of the protein of interest in order to obtain a clear readout, which enhances the method's reproducibility, robustness and sensitivity.(Information from [67])

1.3.2.4. Structure- and image-related proteomics methods

It has been realized that cellular processes are often carried out by macromolecular structures in which proteins can also be involved. It is, therefore, imperative to study and understand how proteins are oriented within complexes and how this feature affects their function. X-ray crystallography is the most common technique used for structural analysis in order to achieve comprehensive coverage of single-protein or domain structures. Two dimensional electron microscopy (2D-EM), electron tomography and nuclear magnetic resonance (NMR) are also implemented to acquire information regarding a protein's structure (even in subunit level), molecular partners and proximity. Moreover, dynamic measurement of a number of tagged proteins in live cells is nowadays possible by optical imaging using fluorescent fusion proteins, which allows us to study a protein's physiological state and cellular localization as well as to perform temporal experiments revealing permanent or transient interactions. (Information from [54])

1.3.3. MS-based protein identification

One of the main objectives of proteomics studies is to identify proteins of interest, (e.g. differentially expressed proteins between different conditions/treatments or

proteins functioning as key-factors in certain cellular processes). Protein identification follows nowadays two main strategies which can be described as the gel-based and the non-gel-based approaches. The former method utilizes chromatography for protein or peptide separation, while the latter depends on the use of gel electrophoresis (1D or 2D). [68] Both strategies finally converge in the use of mass spectrometry (MS or tandem MS) which will ultimately provide a long list of peptide masses. This list of experimental masses is matched against the calculated list of all theoretically expected peptides for each entry in a comprehensive protein database. Following a variety of sophisticated scoring algorithms, each peptide list is assigned to a list of ranked proteins with a different probability score for each protein.

2. PRESENT STUDY

2.1. Aims and objectives

The overall objective of this study was to discover novel diagnostic and/or prognostic markers for differentiated thyroid tumors of follicular origin focusing on the protein level.

More specifically, we aimed at:

i. Establishing a well-performing protocol for the extraction of protein fractions from frozen thyroid tissue

ii. Assessing the utility of MIB-1 as a diagnostic or prognostic marker for thyroid tumors, studying a large cohort of cytology specimens

iii. Discovering a panel of protein molecules that could eventually function as markers for distinguishing the different thyroid tumors

iv. Further elucidating the mechanisms and the phenomena that lie behind thyroid tumorigenesis and tumor progression

2.2. Materials and Methods

2.2.1. Materials

This thesis is entirely based on human thyroid tissue. In Article I, cytology specimens collected routinely between 1987 and 2005 were stained for MIB-1, while the final diagnosis for those patients who were operated on for their thyroid disease, was set on routine histopathology following the classification by WHO. [4]

The material used in Articles II, III and IV is frozen thyroid tissue (both normal and tumor tissue) provided by the Endocrine Biobank at the Karolinska University Hospital in Solna (Stockholm, Sweden). According to the current protocol, all tissues were collected in immediate connection to surgery, snap frozen in liquid nitrogen and then stored at -80°C. Of course, every sample was subjected to histopathological evaluation by experienced endocrine pathologists so that an accurate final diagnosis was established always according to the previously mentioned classification. Moreover,

tissue sample sections obtained for research purposes, were always controlled for representativity, ensuring a high proportion of tumor cells (approximately >80%).

2.2.2. Methods

2.2.2.1. Fine needle aspiration biopsy (FNAB) (Article I)

FNAB is a frequently used technique for the initial evaluation of a thyroid nodule discovered either upon palpation or by imaging. The commonly used 25- to 27-gauge needles can combine minimal pain and bleeding with adequate sampling in most of cases. Needles of wider lumen can be reserved for the drainage of cystic lesions. The use of needle with or without syringe and/or suction system can be individualized for each patient depending on the lesion's characteristics and the experience of the clinician performing the biopsy.(Information from [69])

The actual sampling is carried out by moving the needle back and forth within the nodule, where 2-5 passes are considered reasonable for adequate cell yield. The use of local anesthetic (usually 1% lidocaine or Lidocaine 2% Epinephrine 1:100,000) is an option that the clinician should consider based on the location of the nodule and the patient's preference. The FNAB specimen can be immediately smeared on a glass slide either for air-dried or alcohol-fixed preparation followed by the appropriate staining technique (Romanowsky and Papanicolaou, respectively). (Information from [69])

One of the key issues in FNAB is that the sample should be sufficient facilitating the setting of a secure diagnosis. Tangible definitions and guidelines as to what should be considered as "adequate fine needle aspiration" have recently been issued by thyroid associations around the world. [70, 71]

For the study described in **Article I**, aspirations were carried out as described previously by Zajicek *et al*. [72] The aspirates were divided into two parts, one for cytological evaluation and the other for MIB-1 index determination. The use of two different antibodies (Ki-67 and, after 1993, MIB-1) did not undermine the study's continuation as both antibodies were shown to give similar results in terms of proliferation. [73, 74] MIB-1 index was determined routinely by scoring 200 cells in 4 microscopic fields (x400 magnification).

2.2.2.2. Immunohistochemistry (IHC) (Articles I, III)

IHC is a method which allows for the localization of proteins within a cell. A tissue section which is supposed to contain the protein of interest is exposed to a solution containing an antibody against this particular protein. The antibody recognizes its

epitope and binds specifically to the protein and this phenomenon can then be visualized by either light-, fluorescence- or electron-microscopy, depending on the kind of labeling carried by the antibody.

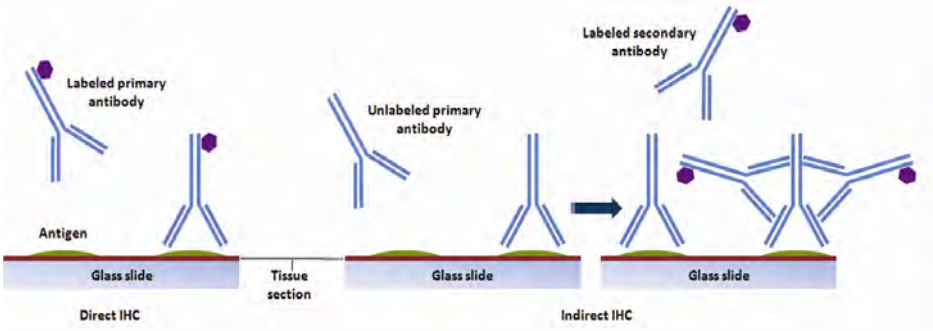

Figure 8. Direct and indirect immunohistochemistry. (Modified from: Mescher AL, *Junqueira's Basic Histology: Text and Atlas, 12th edition, www.accessmedicin.com*

As depicted in Figure 8, IHC can be either direct or indirect. In **Articles I and III**, indirect IHC was implemented according to the following protocol: 4µm paraffin-embedded sections were first deparaffinized and rehydrated. Antigen retrieval was carried out by microwave heating in citrate buffer (pH 6) for 20 minutes followed by the blocking of endogenous biotin and hyperoxidase. Then the sections were blocked in 1% BSA before allowing them to incubate with the primary antibody solution overnight at 4°C. Subsequently, a horseradish peroxidase (HRP) labeled secondary antibody was allowed to react with the primary and this specific reaction was visualized by the avidin-biotin complex (ABC) method. Finally, the peroxidase reaction was visualized by incubation in 3,3-diaminobenzidine (DAB). Hematoxylin was used as counterstaining. Indirect IHC might be more laborious as it requires additional steps and the use of two antibodies, but it is also more sensitive than direct IHC.

2.2.2.3. Protein pre-fractionation (Articles II, III, IV)

Proteome fractionation, i.e. the separation of sub-cellular protein fractions, has been recommended during the past few years as a way of enhancing the resolution, the quantity and the quality of information obtained from a protein sample.[54, 75] In **Article II**, we developed and optimized our own pre-fractionation protocol specifically for frozen, archival tumor tissue. In brief, this protocol begins with tumor lysis and isolation of tumor cells by filtration. Fractions enriched for cytosolic and nuclear proteins are then extracted by sequential steps of exposure to different buffers and centrifugation. The success of the fractionation procedure is then verified by Western blot analyses detecting a certain protein characteristic for each fraction (prohibitin for

the cytosolic and lamin A/C for the nuclear fraction). [76] The samples used in **Articles III and IV** were fractionated according to the same protocol.

2.2.2.4. Two-dimensional electrophoresis (2DE) (Articles II, IV)

The basic principles of 2DE have already been presented in section 1.3.2.1. For the experiments included in **Articles II and IV**, cytosolic protein fractions were separated by 2DE following the procedure described by Hellman *et al*. [77] The gels were then scanned on a flatbed scanner and images were analyzed (spot detection, matching, intensity measurements) with PDQuest image analysis software (ver.7.3.0, Bio-Rad, Hercules, CA). A special method of multivariate statistical analysis (see section 2.2.2.5) was implemented to decide which spots should be picked up and identified by MS.

2.2.2.5. Principal Component Analysis (PCA) and Partial Least Squares (PLS) (Articles II, IV)

PCA is virtually a variable-reduction procedure first described by Pearson in 1901 [78] as cited in [79]. The main idea is to transform a data set with a great number of interrelated variables into a new set with fewer, uncorrelated variables, the principal components (PCs), which are ordered in such a way that the first few of them retain most of the variation present in the original variables. [79]

PLS is a recent development of multiple linear regression (MLR). Modeling one or more dependent variables Y (responses) by means of another set of predictor variables X (i.e., the regression problem) is a very common data-analytical problem in both science and technology. PLS is, in this aspect, a very interesting and powerful method as it can analyze data with numerous, correlated, "noisy" X variables and at the same time model several response variables Y. (Information from [80])

2.2.2.6. SELDI-TOF-MS (Articles II, III)

In **Article II** protein fractions were analyzed by using two different SELDI Protein Chip® Arrays with different chemical surfaces (strong anionic exchange and reverse phase). Incubation, washing and matrix application were carried out according to the manufacter's protocols. Spectra collection was done on a SELDI-TOF protein biology system IIC using two separate settings per chip (referring to laser intensity, detector sensitivity and time-lag focusing) allowing for optimal analysis between the regions 3-10 and 10-35 kDa. Data analysis was performed with the Ciphergen Express (CE) software package (Biorad, Hercules, CA).

Apart from using only one type of chemical surface (strong anion exchange), experimental conditions in **Article III** were as mentioned above. Moreover, an

immunocapture experiment was included to exclusively bind protein S100A6 on a chip surface. Briefly, an RS100 Protein Chip® Array was used, which allows for the coupling of an antibody on its surface. After incubating with the monoclonal anti-S100A6 antibody, the array was washed and incubated with protein samples aiming at specifically capturing S100A6.

2.2.2.7. MALDI-TOF-MS (Article IV)

Protein spots excised from 2DE gels were subjected to in-gel digestion to acquire peptides. One or more gel plugs corresponding to the same protein were pooled and destained by Farmer's reagent (50 mM sodium thiosulphate / 15 mM potassium ferricyanide at a 1:1 ratio). Treatment with ammonium bicarbonate (AMBIC) was followed by gel drying in neat acetonitrile (ACN). In-gel digestion began by rehydrating the gel plugs with the trypsine-containing solution and letting them incubate overnight at 37°C. Trifluoroacetic acid (TFA) was applied to terminate digestion and peptide retrieval was enhanced by mechanical vortex and sonication. All peptide samples were desalted and concentrated in micro-Zip Tips and, finally, eluted directly on a MALDI target plate. Alpha-cyano-4-hydroxycinnamic acid (CHCA) in 75% ACN was used as matrix.

Spectrum collection was optimized according to the manufacturer. Four autolytic trypsin peptides (842.51, 1045.56, 2211.10 and 3337.76) were used as internal calibrants for all spectra. For protein identification peptide mass lists were matched against the NCBI non-redundant protein sequence database (2010/02/01).

2.2.2.8. Western Blot (WB) (Articles II, III, IV)

Western Blot or immunoblot is a very common protein detection method. Proteins are separated in a one-dimensional (1D) gel and then transferred on a membrane. This membrane is then incubated with an antibody against the protein of interest (primary antibody). Visualization of the result is made possible by coupling the primary antibody with another antibody (called "secondary") which recognizes a species-specific portion of the primary antibody. The secondary antibody also carries an enzyme (usually horseradish peroxidase) which plays a decisive role in producing luminescence and, thus, visualizing the protein of interest. There is a number of gel running options (e.g. gel density, voltage, duration) which enable the optimization of experimental conditions according to the qualities of the protein one wishes to detect. The use of standard size protein markers, for example, provides information about the size of the detected protein.

In **Articles II, III** and **IV**, 1D-PAGE was used and proteins were separated under denaturing conditions. The objective in **Article II** was to detect prohibitin and lamin A/C

as markers of the fractions enriched for cytosolic and nuclear proteins, respectively. In **Article III** Western blot was used to extract both qualitative and quantitative information about the expression levels of S100A6. Finally, in **Article IV** immunoblotting served as a validation method, confirming the existence of the proteins previously identified by MALDI-TOF-MS.

2.3. Results and Discussion

2.3.1. Article I – Use of MIB-1 index in thyroid FNAB

In this article, the question of using MIB-1 proliferation index as a diagnostic and/or prognostic marker for thyroid tumors was addressed. The study included 504 samples from patients who underwent thyroid FNAB over a period of 18 years (1987-2005), which were analyzed for MIB-1 in routine cytology. MIB-1 was shown to be prominently higher in ATC than in most of the other tumors or benign lesions (except PDTC and atypical Hürthle adenoma). No significant differences were noticed when FTA, FTC and PTC were compared pair-wise (Table 2).

Table 2. Diagnostic Groups and MIB-1 Index from 504 Thyroid Fine Needle Aspiration Biopsies

		MIB-1 index (%)		
Diagnosis*	***n***	***mean***	***median***	***range***
Follicular thyroid adenoma - FTA	53	1.6	1.0	[0 - 6]
Atypical follicular thyroid adenoma - AFTA	16	3.0	2.0	[0.5 - 15]
Follicular thyroid carcinoma - FTC	25	3.5	2.5	[0.5 - 13]
Hürthle cell adenoma - Hü ad	34	1.9	1.0	[0 - 10]
Atypical Hürthle cell adenoma - At Hü ad	5	6.6	2.0	[1 - 25]
Hürthle cell carcinoma - Hü ca	16	3.8	2.75	[0.5 - 10]
Papillary thyroid carcinoma - PTC	185	2.9	2.0	[0 - 40]
Medullary thyroid carcinoma - MTC	21	4.4	2.0	[0.5 - 20]
Poorly differentiated thyroid carcinoma - PDTC	9	13.1	6.0	[0 - 50]
Anaplastic thyroid carcinoma - ATC	24	42.6	42.5	[10 - 100]
Goiter & Adenomatous Hyperplasia †	91	1.6	1.0	[0 - 15]
Thyroiditis ‡	25	4.2	2.0	[0 - 20]
Total	*504*			

** All diagnoses were set on histopathology, unless stated otherwise.*

† 54 diagnosed on cytology. ‡ 23 diagnosed on cytology

Moreover, there was evidence that MIB-1 equal to or greater than 4% was independently associated with increased risk for local recurrence, distant metastasis or disease-related mortality in patients with PTC. Besides that, age at operation also turned out to be of certain significance in connection to distant metastasis and disease-related mortality. The clinicopathological and follow-up information for these PTC patients is summarized in Table 3 (Pages 38-40).

The utility of MIB-1 in clinical routine and its value as a prognostic marker on cytology has been established in breast cancer, lymphomas and soft tissue tumors. [81] It has long been debated, though, whether it can be equally valuable in thyroid oncology and no consensus has been achieved so far. In any case, it is interesting that the majority of studies of MIB-1 in thyroid tumors have been carried out in histopathological material. [82-86]

Our study was to our knowledge the first one to address this issue by using a large and broad-range preoperative thyroid material. The absence of significant difference in MIB-1 between FTA (including atypical FTA) and FTC is in agreement with other immunohistochemical studies [83, 87], although there have been reports providing evidence for the opposite. [82, 84, 85]

PTC is a tumor which can be readily diagnosed on cytology and our institution has achieved a 98% agreement rate between cytology and histopathology. [88] Nevertheless, based on the findings of this study, a high MIB-1 index (where “high” is defined as ≥ 4%) should be considered as a sign of increased aggressiveness and risk for distant metastasis and, therefore, influence the therapeutic scheme. In times when the extent of surgical treatment, as well as that of nodal dissection, is a matter of dispute and varies greatly between different medical centers, our study supports the use of high MIB-1 as an indicator of a PTC which probably calls for a more aggressive and radical therapeutic strategy.

Table 3. Clinical details, MIB-1 index and follow-up information for 183 PTC patients (Continued in pages 39,40)

Patient data				*Histopathology after surgery*					*Local recurrence*	*Distant metastasis*	*Follow up - End event*					
Case no	*Sex M/F*	*Age*	*MIB-1*	*LNM*	*DM*	*ExtG*	*MT*	*Size (cm)*	*Yes/No (years after op)*	*Yes/No (years after op)*	*AWOD*	*AWD*	*DWOD*	*DWD*	*DOD*	*Total follow-up (yrs)*
1	F	87	0.0	No	No	No	No	2	No	No	•					5.7
2	F	39	0.0	No	No	No	No	1.8	No	No	•					5.7
3	M	34	0.0	Yes	No	No	No	3	No	No	•					6.7
4	M	33	0.0	Yes	No	Yes	No	2.3	No	No	•					2.3
5	M	88	0.5	Yes	No	No	No	2	No	No			•			0.1
6	F	77	0.5	Yes	Yes	Yes	No	n/a	No	Yes (0.5)					•	0.5
7	F	76	0.5	No	No	No	No	2	No	No	•					11.5
8	F	75	0.5	No	No	No	Yes	3	No	No	•					4.3
9	F	67	0.5	No	No	No	No	1.2	No	No			•			1.7
10	F	50	0.5	No	No	No	No	1.5	No	No	•					14.9
11	F	65	0.5	No	No	No	No	0.4	No	No	•					1.2
12	F	62	0.5	Yes	No	Yes	No	1.5	No	No	•					3.5
13	F	51	0.5	No	No	No	No	1.7	No	No			•			11.5
14	F	59	0.5	No	No	No	No	1	No	No	•					3.9
15	F	55	0.5	Yes	No	No	No	1.5	No	No	•					6.0
16	F	46	0.5	No	No	No	No	2.5	No	No	•					11.4
17	F	54	0.5	No	No	No	No	2	No	No	•					4.7
18	F	46	0.5	Yes	No	No	No	1.5	No	No	•					10.0
19	M	51	0.5	Yes	No	No	Yes	1.7	No	No	•					4.8
20	M	41	0.5	Yes	No	No	No	3	No	No	•					5.5
21	F	42	0.5	No	No	No	No	2	No	No	•					4.9
22	F	45	0.5	Yes	No	No	No	1.4	No	No	•					2.0
23	F	41	0.5	Yes	No	Yes	No	3	No	No	•					5.4
24	F	34	0.5	Yes	No	Yes	Yes	3	Yes (11.0)	No		•				11.7
25	F	37	0.5	Yes	Yes	Yes	No	1.5	No	Yes (1.3)	•					6.6
26	F	28	0.5	No	No	No	No	1	No	No	•					14.5
27	F	34	0.5	Yes	No	Yes	Yes	1	No	No	•					9.2
28	F	35	0.5	Yes	No	No	No	2.8	No	No	•					6.3
29	F	29	0.5	No	No	No	No	0.8	No	No	•					10.2
30	M	33	0.5	No	No	Yes	Yes	3	No	No	•					4.9
31	F	35	0.5	Yes	No	No	No	0.8	No	No	•					2.5
32	F	30	0.5	Yes	No	No	No	3	No	No	•					4.8
33	F	26	0.5	Yes	No	Yes	Yes	2	No	No	•					7.9
34	F	25	0.5	Yes	No	No	Yes	0.5	No	No	•					8.0
35	F	19	0.5	Yes	No	No	No	2	No	No	•					12.4
36	F	75	1.0	No	No	Yes	Yes	2.5	No	No			•			11.6
37	F	72	1.0	No	No	No	No	1.3	No	No			•			7.8
38	F	76	1.0	No	No	Yes	No	4	No	No	•					8.1
39	F	81	1.0	Yes	No	Yes	No	4	No	No	•					3.1
40	F	76	1.0	No	No	No	No	4	No	No	•					5.5
41	F	75	1.0	Yes	No	Yes	No	3	No	No	•					4.3
42	M	72	1.0	Yes	No	No	No	3	No	No	•					7.0
43	F	71	1.0	No	No	No	No	2	No	No	•					4.9
44	F	72	1.0	No	No	No	No	3	No	No	•					2.2
45	F	68	1.0	No	No	No	No	2.5	No	No	•					4.8
46	F	66	1.0	No	No	No	Yes	2	No	No	•					4.7
47	F	56	1.0	No	Yes	Yes	No	3	No	Yes (0.0)					•	0.4
48	F	53	1.0	No	No	No	No	2	No	No	•					14.3
49	F	58	1.0	No	No	No	No	2	No	No	•					7.9
50	M	60	1.0	No	No	No	No	5	No	No	•					5.6
51	F	57	1.0	No	No	No	No	1.5	No	No	•					8.1
52	M	62	1.0	No	No	No	No	2.3	No	No	•					3.3
53	F	53	1.0	No	No	Yes	No	1.2	No	No			•			10.8
54	F	49	1.0	No	No	No	No	1.2	No	No	•					13.0

LNM = Lymph node metastasis. DM = Distant metastasis. ExtG = Extrathyroidal growth. MT = Multiple tumors. n/a = not available

AWOD = Alive without disease. AWD = Alive with disease. DWOD = Dead without disease. DWD = Dead with disease. DOD = Dead of disease

55	F	48	1.0	No	No	No	No	3.5	Yes (3.7)	No		•	14.2
56	F	58	1.0	Yes	No	No	No	3	No	No	•		3.5
57	M	46	1.0	No	No	No	No	2	No	No	•		3.6
58	F	49	1.0	No	No	No	Yes	4.5	No	No	•		10.4
59	F	53	1.0	Yes	No	Yes	No	2	No	No	•		4.7
60	F	51	1.0	No	No	No	No	3.5	No	No	•		4.8
61	F	53	1.0	No	No	No	No	1.6	No	No	•		2.0
62	F	41	1.0	No	No	No	No	1.2	No	No	•		13.7
63	F	49	1.0	Yes	No	No	No	2.1	No	No	•		5.2
64	M	43	1.0	Yes	No	Yes	No	2.5	Yes (6.2)	No	•		9.9
65	F	35	1.0	No	No	Yes	No	1.2	No	No	•		14.8
66	M	36	1.0	Yes	No	No	Yes	1	No	No	•		13.0
67	M	32	1.0	Yes	No	Yes	No	4	No	No	•		16.3
68	F	35	1.0	No	No	Yes	Yes	2	No	No	•		11.0
69	F	37	1.0	No	No	No	No	2	No	No	•		7.5
70	F	39	1.0	No	No	No	No	3	No	No	•		5.4
71	F	38	1.0	Yes	No	Yes	Yes	1.5	No	No	•		4.6
72	F	34	1.0	No	No	No	No	0.7	No	No	•		8.2
73	M	34	1.0	Yes	No	Yes	No	4	No	No	•		6.1
74	F	35	1.0	No	No	No	No	2	n/a	n/a	•		5.4
75	F	34	1.0	Yes	No	No	No	4.5	No	No	•		5.3
76	F	25	1.0	Yes	No	Yes	Yes	1.5	Yes (13.1)	No		•	13.1
77	F	33	1.0	No	No	No	No	0.3	No	No	•		3.9
78	M	33	1.0	No	No	No	No	4	No	No	•		3.9
79	F	25	1.0	Yes	No	No	No	2	No	No	•		11.1
80	M	30	1.0	Yes	No	Yes	No	2.5	No	No	•		5.0
81	M	23	1.0	Yes	No	No	No	5.5	Yes (7.2)	No	•		12.1
82	M	21	1.0	Yes	No	Yes	No	1.2	No	No	•		10.8
83	F	27	1.0	Yes	No	Yes	No	0.7	No	No	•		5.5
84	M	28	1.0	Yes	Yes	Yes	No	3.5	No	No	•		3.9
85	F	26	1.0	Yes	No	No	No	2	No	No	•		4.8
86	M	25	1.0	Yes	No	No	No	2	No	No	•		4.8
87	F	22	1.0	No	No	No	No	1.8	No	No	•		7.5
88	F	79	1.0	No	No	Yes	Yes	2.5	No	No		•	6.9
89	F	33	1.5	No	No	Yes	No	4	No	No	•		13.6
90	F	36	1.5	Yes	No	No	No	1.1	No	No	•		2.3
91	M	79	2.0	No	No	Yes	No	n/a	No	No		•	3.8
92	M	77	2.0	n/a	n/a	n/a	No	4	n/a	n/a			• 8.5
93	F	80	2.0	No	No	No	No	3.5	Yes (3.0)	Yes (3.0)			• 4.0
94	M	72	2.0	Yes	No	Yes	Yes	12	No	No		•	0.6
95	M	70	2.0	Yes	No	Yes	No	6	Yes (5.0)	No	•		15.6
96	F	80	2.0	No	No	Yes	No	2	No	Yes (1.3)	•		5.8
97	F	69	2.0	No	No	No	No	n/a	No	No		•	12.7
98	F	67	2.0	No	No	No	No	2.4	No	No	•		15.1
99	F	79	2.0	Yes	No	No	No	2.2	No	No		•	1.3
100	F	68	2.0	No	No	No	Yes	2.8	No	No	•		6.7
101	F	76	2.0	No	No	Yes	No	3	No	No	•		5.4
102	F	63	2.0	No	No	No	No	3.5	No	No		•	13.0
103	M	70	2.0	No	No	Yes	Yes	5.5	Yes (5.4)	No	•		7.4
104	F	65	2.0	No	No	No	No	2.2	No	No	•		7.4
105	M	58	2.0	Yes	Yes	Yes	No	1.5	No	No		•	7.0
106	F	59	2.0	No	No	No	No	2	No	No	•		0.5
107	M	53	2.0	No	No	Yes	No	1.8	No	No	•		8.2
108	F	51	2.0	Yes	No	No	No	3.5	No	No	•		9.3
109	F	52	2.0	Yes	No	No	No	n/a	No	No	•		6.9
110	F	54	2.0	No	No	No	No	2	No	No	•		4.3
111	F	53	2.0	No	No	Yes	No	2.5	No	No	•		5.6
112	F	47	2.0	Yes	No	No	Yes	3.5	No	No	•		10.2
113	F	42	2.0	No	No	No	No	1.2	No	No	•		11.9
114	F	37	2.0	No	No	No	No	3.5	No	No	•		5.5
115	M	39	2.0	No	No	No	No	5	No	No	•		12.2
116	F	46	2.0	Yes	No	Yes	Yes	2	No	No	•		4.7
117	F	32	2.0	Yes	No	No	No	2	No	No	•		16.7
118	F	40	2.0	No	No	No	No	1.5	Yes (1.9)	No	•		8.0
119	F	30	2.0	Yes	No	Yes	Yes	5	No	No		•	3.3

120	F	41	2.0	No	No	No	No	2	No	No	•		4.9
121	F	44	2.0	No	No	No	No	1.4	No	No	•		1.9
122	F	41	2.0	No	No	No	No	5	No	No	•		2.3
123	F	24	2.0	Yes	No	Yes	No	5	No	No	•		13.4
124	F	32	2.0	Yes	No	Yes	No	n/a	No	No	•		5.7
125	F	32	2.0	Yes	No	No	No	4.5	No	No	•		2.3
126	F	21	2.0	No	No	No	No	1	No	No	•		11.7
127	F	15	2.0	No	No	No	No	n/a	No	No	•		7.5
128	F	97	2.0	No	No	Yes	No	4.5	No	No		•	0.6
129	F	79	3.0	No	No	No	Yes	2	No	No		•	8.1
130	M	76	3.0	No	No	No	Yes	3	No	Yes (9.5)			• 10.5
131	F	74	3.0	No	No	No	No	3.5	No	No	•		10.0
132	F	57	3.0	No	No	No	No	1.5	No	No	•		9.3
133	F	49	3.0	No	No	No	No	2.5	No	No	•		12.7
134	M	42	3.0	No	No	No	No	0.7	No	No	•		15.1
135	F	46	3.0	Yes	No	No	Yes	1.7	No	No	•		5.6
136	F	42	3.0	Yes	No	No	No	2.5	No	No	•		7.2
137	F	38	3.0	No	No	No	No	2	No	No	•		6.1
138	F	37	3.0	Yes	No	No	No	3	No	No	•		7.0
139	M	30	3.0	No	No	No	No	2	No	No	•		9.3
140	F	27	3.0	No	No	No	No	2	No	No	•		10.7
141	F	30	3.0	Yes	No	No	No	1.5	No	No	•		7.6
142	F	28	3.0	No	No	No	No	2	No	No	•		7.3
143	F	27	3.0	Yes	No	Yes	Yes	2	No	Yes (0.3)		•	7.5
144	M	11	3.0	Yes	Yes	Yes	No	3.5	No	No		•	2.8
145	F	85	4.0	No	No	Yes	No	7	No	No	•		7.5
146	F	81	4.0	Yes	No	No	No	2	No	No		•	0.5
147	F	77	4.0	Yes	No	Yes	No	3	No	No	•		5.7
148	F	68	4.0	Yes	Yes	Yes	No	4	No	Yes (0.0)			• 0.2
149	F	60	4.0	Yes	No	No	Yes	2.5	No	No	•		4.4
150	F	42	4.0	No	No	No	No	1.7	No	No	•		5.6
151	F	19	4.0	Yes	No	No	No	4.7	No	No	•		5.7
152	F	16	4.0	No	No	No	No	5	No	No	•		2.4
153	F	70	5.0	No	No	No	No	3.5	No	No	•		10.5
154	F	68	5.0	No	No	Yes	No	3	No	Yes (1.8)			• 2.2
155	F	67	5.0	No	No	No	No	2	No	No	•		11.5
156	M	70	5.0	Yes	No	Yes	No	5	Yes	Yes (5.0)			• 5.0
157	F	66	5.0	No	No	Yes	No	5.5	No	No	•		4.4
158	M	63	5.0	Yes	No	Yes	No	2.5	No	No	•		6.8
159	F	60	5.0	No	No	No	No	2	No	No	•		5.8
160	F	54	5.0	No	No	No	No	2	No	No	•		11.5
161	F	50	5.0	No	No	No	No	1.8	No	No	•		10.5
162	F	53	5.0	Yes	No	No	No	2.5	No	No	•		5.4
163	F	32	5.0	Yes	No	No	No	n/a	No	No	•		7.1
164	F	32	5.0	No	No	No	No	2	No	No	•		3.5
165	F	23	5.0	No	No	No	No	1.5	No	No	•		7.7
166	M	79	6.0	n/a	n/a	n/a	n/a	6	n/a	n/a			• 0.1
167	F	56	6.0	No	No	Yes	No	2.5	No	No	•		13.7
168	F	40	6.0	Yes	No	Yes	Yes	1.5	No	No	•		16.2
169	F	20	6.0	Yes	Yes	Yes	No	1	No	No	•		10.7
170	F	69	7.0	Yes	No	Yes	No	5	Yes (0.5)	Yes (5.4)			• 5.5
171	F	66	7.0	Yes	No	No	No	4	No	Yes (0.2)			• 0.4
172	F	61	7.0	No	No	No	No	2	No	No	•		10.1
173	M	24	7.0	Yes	No	No	No	1.3	No	No	•		7.9
174	F	74	9.0	No	No	No	No	2.8	No	No	•		5.7
175	M	79	10.0	n/a	n/a	n/a	No	n/a	n/a	n/a			• 0.8
176	F	68	10.0	No	No	No	No	2.5	No	No	•		11.8
177	F	47	10.0	Yes	No	No	No	2	No	No	•		9.4
178	F	30	10.0	No	No	No	No	2	No	No	•		9.3
179	F	17	10.0	No	No	No	No	4	No	No	•		8.4
180	M	83	15.0	No	Yes	No	No	7.5	No	Yes (0.0)		•	1.8
181	M	35	25.0	Yes	Yes	No	Yes	6	Yes	Yes			• 3.6
182	M	62	35.0	Yes	No	No	No	0.9	Yes (0.6)	Yes (1.0)			• 2.3
183	F	75	40.0	Yes	No	No	No	5	Yes (0.5)	Yes (0.7)			• 2.0

2.3.2. Article II – Methodology for protein pre-fractionation

The development of a protocol for protein pre-fractionation and separation of highly enriched protein fractions specifically from frozen tumor tissue was the main objective of our study described in this article. As presented in Figure 9, the quality of the fractions procured was verified by immunoblotting where only one fraction-specific protein per fraction was detected. Fractions 1 and 2 were enriched for cytosolic and nuclear/nuclear membrane proteins, respectively. β-actin analysis, as well as Ponceau

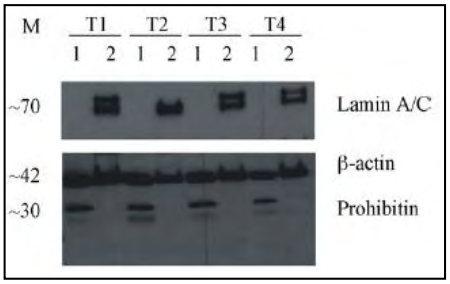

Figure 9. Western Blot analysis showing expression of the fraction-specific proteins in 4 tumor samples (T1-T4). Prohibitin is specific for Fraction 1 (cytosolic proteins enriched), while Lamin A/C is specific for Fraction 2 (nuclear/nuclear membrane proteins enriched.

staining (data not shown), confirmed equal amounts of protein loaded in each lane. SELDI-TOF-MS was then applied to investigate the quality and quantity of information provided by the two fractions in comparison with the total protein extract. The results advocate that the two separate fractions conveyed not only more, but also better information (Figure 10).

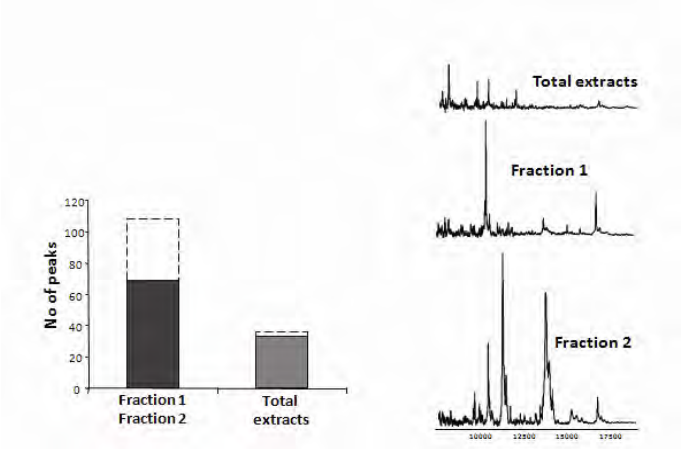

Figure 10. The column-graph (left) presents the total number of peaks detected within the SELDI spectra of Fractions 1 and 2 (black) and that of the total protein extract (grey). Dashed rectangles correspond to the sum of unique peaks detected. A representative area from the SELDI spectra (right) acquired for total protein extract, Fraction 1 and Fraction 2 is revealing how the two fractions give both better (more peaks) and stronger signals (higher peaks) as compared to the total extract.

Moreover, a small subset of our tissue samples was freshly prepared in order to check for possible differences between them and frozen tissue. Our subsequent analysis showed that similar protein fractions were extracted from both sorts of tissue. Finally, the protein fractions produced were also compatible with 2DE giving results of good quality without sample-related defects as far as the gel running was concerned.

Sample fractionation is, for a number of reasons, a challenging issue in modern functional proteomics. First of all, the majority of currently running projects involve complex tissue material which contains a number of different types of cells (e.g., connective tissue or inflammatory cells) whose analysis may not be desirable. Then, the issue of detecting low abundant proteins emerges as none of today's high-end proteomics technologies can cope with the high dynamic range of complex protein solutions such as serum or plasma. Last, but not least, identifying proteins on the sub-cellular level is crucial for fully understanding cellular function. It is widely known that proteins translocate between different cellular compartments depending on certain physiological or other states or that they cycle between the cell surface and intracellular protein pools. Therefore, adjusting our studies in focusing on the subcellular distribution of proteins can be of great value in deciphering underlying mechanisms and expression patterns in various conditions (e.g., disease, tumor, response to treatment). [89, 90]

In developing this pre-fractionation protocol, our goal was to address the issues described above. Having a well-established Biobank of frozen tissue in our hospital, our wish, as well as that of all patients that had entrusted research community with this material, was to make the most out of it. One of the most important components of this protocol is that the samples are not only controlled for tissue representativity, but also filtered following mechanical tissue lysis, in order to obtain a population of cells which is as pure as possible. Furthermore, we have managed to standardize a procedure that ends in acquiring two fractions highly enriched in cytosolic and nuclear/nuclear membrane proteins and of good quality that are also compatible with subsequent analysis, for example, by 2DE.

2.3.3. Article III – Over-expression of S100A6 in PTC

This study is a typical example of a so-called "top-down" proteomics[‡] study. SELDI-TOF-MS spectra from cytosolic protein extracts were collected, peaks were detected

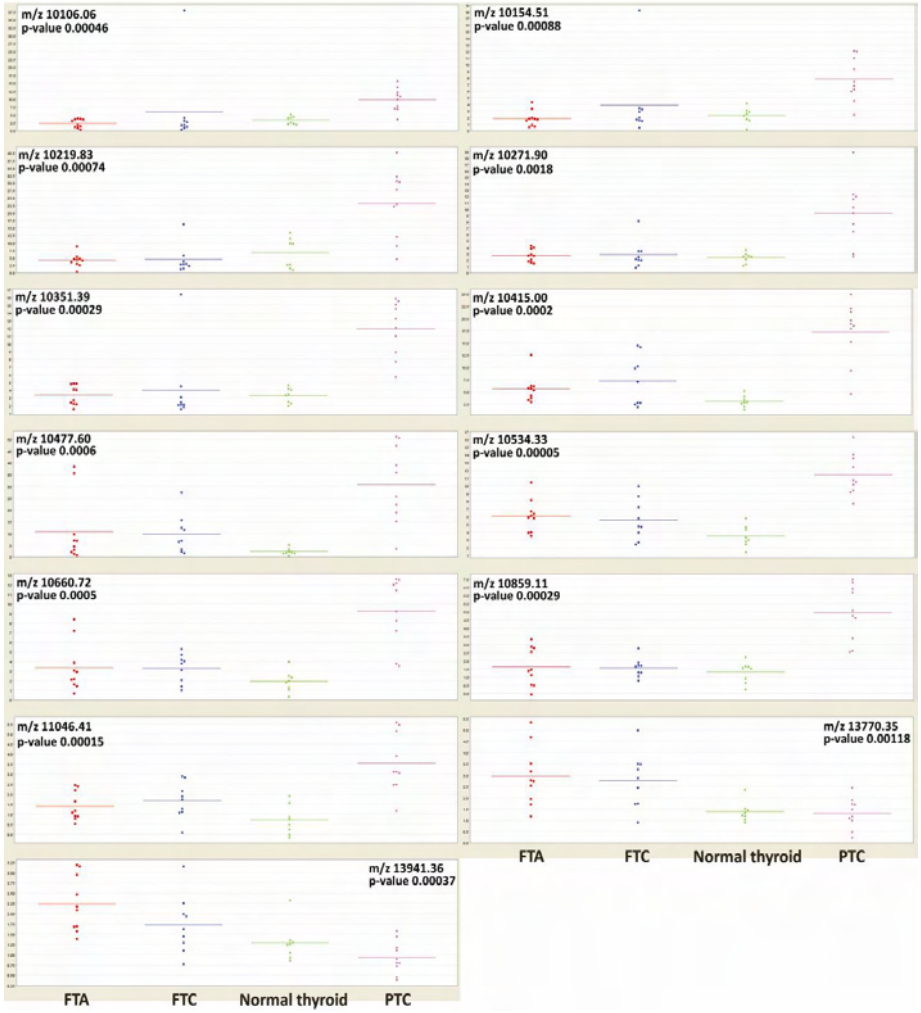

Figure 11. Scatter plots of the 13 peak clusters best distinguishing PTC from follicular tumors. X axis: study groups plotted in red (FTA), blue (FTC), green (normal thyroid) and purple (PTC). Y axis: signal intensity (the horizontal line represents the mean intensity for each group)

[‡] A "top-down" proteomics approach begins by analyzing intact protein molecules, which can subsequently be fragmented into peptides. The opposite approach, i.e. analyzing peptide mixtures derived from enzymatic cleavage of proteins first, is called "bottom-up" proteomics.

and normalized and peak clusters were identified. P-values were calculated (Mann-Whitney non-parametric test) testing the null hypothesis that the medians of the peak intensities of our study groups are equal. Sixty four out of the 155 identified clusters had a significant p-value ($p < 0.05$) and 13 of them were distinguishing between PTC and follicular tumors, as determined after manual inspection following certain criteria (Figure 11).

Based on molecular mass (m/z ratio), the array's binding specificity and previous experience [91] we hypothesized that a certain peak cluster detected at a m/z ratio of about 10,200 Da corresponded to the Ca^{+2}-binding protein S100A6. This hypothesis was confirmed by an immunocapture experiment using an RS100 Protein Chip® Array and validated by Western blot. Moreover, as illustrated in Figure 12, a set of three distinct peaks was visible on the RS100 chip depicting the expected masses of S100A6 (~ 10,100 Da) and two of its modifications by cysteinylation (~ 10,200 Da) and glutathionylation (~ 10,400 Da) as previously described also by Orre *et al*. [91] The quantitative differences of S100A6 observed on the SELDI-TOF-MS profiling were further validated by immunoblotting in a larger series of samples (8 per study group). PTC was shown to express S100A6 in significantly higher levels as compared to normal thyroid tissue ($p = 0.001$), FTA ($p = 0.019$) and FTC ($p = 0.012$). Immunohistochemical analysis on sections from the same cases displayed significantly stronger cytosolic staining and a larger proportion of stained nuclei for S100A6 in PTC than in follicular tumors ($p = 0.004$ and 0.01, respectively).

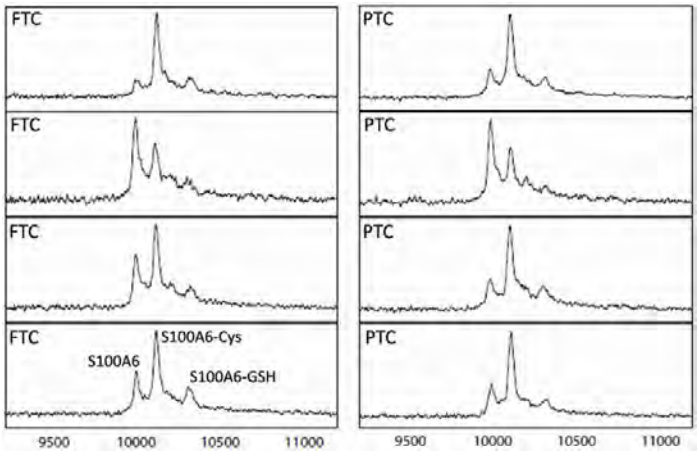

Figure 12. SELDI-TOF-MS spectra from an RS100 Protein Chip® Array treated with a monoclonal anti-S100A6 antibody. Unmodifed S100A6, along with two of its modifications (Cys: Cysteine, GSH: Glutathione) were detected in 4 FTC and 4 PTC samples. All S100A6 forms are depicted on the far lower, left spectrum.

Finally we investigated the possible correlation between S100A6 expression and the V600E mutation on the *BRAF* gene, a mutation which is evidently associated with PTC. The mutation was detected in 50% of the PTC cases, but its presence could not be related to S100A6 levels.

During the past few years numerous studies have provided evidence on the relation between thyroid tumorigenesis and members of the S100 protein family.[92-95] S100A6 in particular has been extensively studied and associated to a wide range of tumors such as colorectal cancer, lung cancer, gastric cancer, malignant melanoma, cutaneous tumors, hepatocellular carcinoma, pancreatic cancer and leukemia. [96-106] As far as the relation between S100A6 and thyroid tumors is concerned, only two studies have been reported so far; one based entirely on IHC and one combining DIGE with IHC-validation. [107, 108] Some of the unique characteristics of our study were that we used protein fractions from normal thyroid tissue as well as from differentiated thyroid tumors and that we combined quantitative methods like SELDI-TOF-MS and WB.

Our findings about S100A6 being significantly over-expressed in PTC as compared to follicular tumors or normal thyroid tissue, are in concordance with previous studies. It still remains to show how these quantitative differences affect its interactions with its molecular partners. Although the molecular functions of S100A6 have not been clarified in their entirety, light has been recently shed over its protein-protein interactions, which provide indications on possible functions. According to Fernandez-Fernandez *et al* and Slomnicki *et al*, S100A6 seems to participate in the regulation of cell growth and apoptosis by interacting with the tumor suppressor p53. [109, 110] In addition, very recent studies elaborate on the interaction between S100A6 and Calcyclin-binding protein (CacyBP) and its involvement in the ERK1/2 pathway. [111, 112]

2.3.4. Article IV – Novel candidate markers for thyroid cancer revealed by 2DE

This study is an example of "bottom-up" proteomics since protein cleavage products (i.e. peptides) are processed in order to obtain the protein's identity. To reduce the complexity of the peptide mixture, there was a protein separation step, which in this case involved 2DE. After gel image analysis (Figure 13), all detected and matched protein spots were subjected to multivariate statistical analysis (PLS-DA) in order to define those that can best distinguish between the different tumor groups. Two PLS-DA models were built; the first one compared FTA to FTC (FTA-FTC model) and the second one compared FTC to PTC (FTC-PTC model).

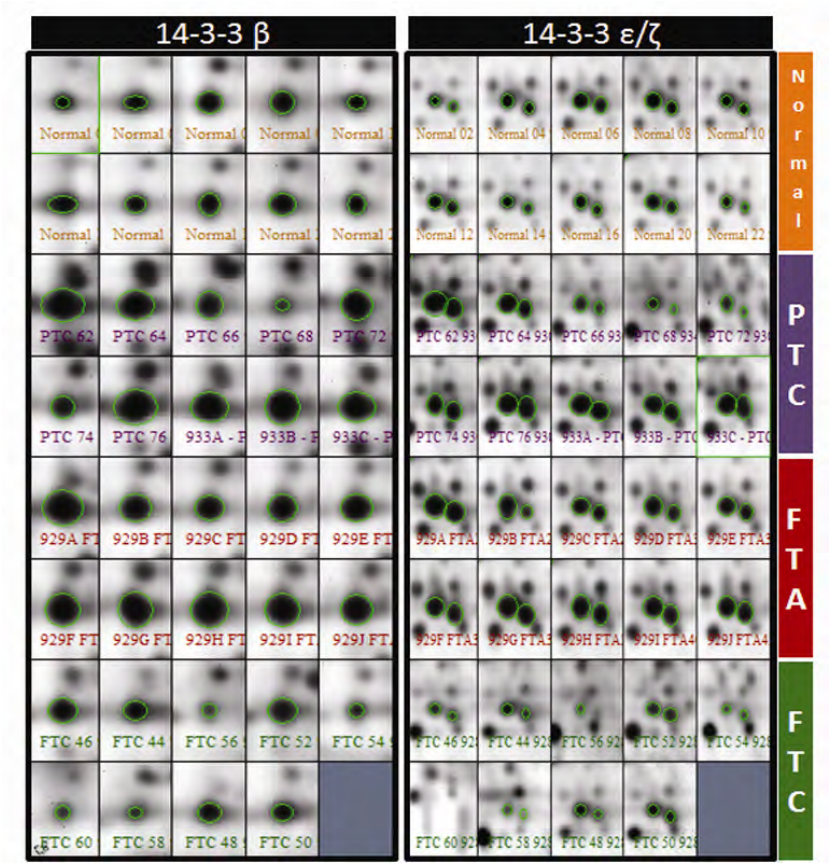

Figure 13. Snapshots from the 2D-gel image analysis software PDQuest (Biorad, Hercules, CA). In green circles the protein spots later identified as protein 14-3-3 isoforms β (left panel), ε and ζ (right panel).

The former model revealed 25 protein spots that could distinguish between follicular tumors. The latter one showed that FTC and PTC could be distinguished by a panel of

19 proteins. After visual inspection, one of these spots was excluded from further analysis (poorly defined, elongated spot situated close to the precipitation line on the acidic edge of the 2DE gel), while the remaining 18 spots were completely overlapping with the 25 spots from the FTA-FTC model. After tryptic digestion of the gel spots and MS analysis of the produced peptides, all proteins were identified as shown in Table 4.

Table 4. List of the identified protein spots belonging to both PLS-DA models

Protein full name (Abbreviation)	Accession no	PLS-DA model	
		FTA-FTC	FTC-PTC
14-3-3 epsilon (14-3-3 ε)	NP006752.1	+	+
Calreticulin	NP004334.1	+	+
14-3-3 zeta (14-3-3 ζ)	NP003397.1	+	+
14-3-3 beta(14-3-3 β)	NP003395.1	+	-
Heat Shock protein gp96 precursor (hsp gp96 prec.)	AAK74072.1	+	+
Heat Shock protein gp96 precursor (hsp gp96 prec.)	AAK74072.1	+	+
Heat Shock protein gp96 precursor (hsp gp96 prec.)	AAK74072.1	+	-
Heat Shock protein gp96 precursor (hsp gp96 prec.)	AAK74072.1	+	+
prolyl 4-hydroxylase beta peptide (P4HB) or Thyroid hormone binding protein precursor	NP000909.2	+	+
Heat shock protein 90 beta, member 1 (hsp90 β1)	NP003290.1	+	+
Heat shock protein 90 beta, member 1 (hsp90 β1)	NP003290.1	+	+
Annexin V (ANXA5)	NP001145.1	+	+
Heat shock protein 90 beta, member 1 (hsp90 β1)	NP003290.1	+	-
α1 antitrypsin (A1AT) or Serine proteinase inhibitor A1 (SERPINA1)	NP000286.3	+	-
Heat shock 70kDa protein 5	NP005338.1	+	-
Heat shock 70kDa protein 5	NP005338.1	+	+
Protein disulfide isomerase A6	NP005733.1	+	+
Tubulin α	NP006073.2	+	+
β-actin (ACTB)	AAH12854.1	+	+
β-actin (ACTB)	AAH12854.1	+	+
Brain creatine kinase	NP001814.2	+	-
Heat shock protein beta-1 (hsp β1)	NP001531.1	+	+
Human serum albumin (HSA)	AAH41789.1	+	-
Selenium-binding protein 1 (SELENBP1)	NP003935.2	+	+
Peroxiredoxin 6 (PRX VI)	NP004896.1	+	+

Nine of these 25 proteins were selected for further validation by Western Blot analysis (overlap with 7 out of 18 proteins from the FTC-PTC model). By using unique antibodies against each protein (except for all 14-3-3 isoforms for which one single antibody was used), we were able to verify their expression in our study groups.

Conclusively, by using biological replicates from normal thyroid tissue and differentiated thyroid tumors and by applying both older and novel proteomics methods, we attempted to address a mind-breaking issue of thyroid cytology. Besides certain shortcomings, we believe that our study can set the foundation for future, prospective studies on the candidate markers we indicate as discriminatory between follicular adenomas and carcinomas.

2.4. Conclusions, general discussion and future perspectives

Despite all advances and breakthroughs, safely diagnosing a thyroid tumor on an FNAB still remains a challenge for cytologists. The most troublesome diagnostic problem is the dilemma between FTA and FTC, where one needs to prove the existence of vascular and/or capsular invasion. Moreover, in the cases of PTC – a malignancy which in many cases develops and remains indolent for a long period of time – one would wish for tangible information concerning the tumor's metastatic potential, behavior or response to treatment, assuming that the diagnosis is a relatively easier step.

The search of molecular markers to address these issues, as well as shedding more light to tumorigenetic mechanisms behind thyroid tumors, was the driving force of this study. Describing our findings in a nutshell:

- ✓ MIB-1 was shown to be a useful prognostic marker for PTC patients. A high MIB-1 value (>4%) was associated with a higher risk of local recurrence, distant metastasis and disease-related mortality.

- ✓ A new protein pre-fractionation protocol was developed intended for use with archival frozen tissue samples. Based on alternate steps of exposure to buffers and centrifugation, this protocol yields high-quality fractions enriched in cytosolic or nuclear/nuclear membrane proteins which are compatible with downstream proteomics applications.

- ✓ The Ca^{+2}-binding protein S100A6 presented as being significantly over-expressed in PTC.

- ✓ We provide evidence for the potential use of a panel of 9 proteins (14-3-3 isoforms β/ε/ζ, annexin V, peroxiredoxin 6, α1-antitrypsin, selenium-binding protein 1, tubulin α and prolyl-4 hydroxylase peptide β) as a tool to distinguish between follicular tumors. A subset of those proteins can also be useful in discerning FTC from PTC.

The management of thyroid nodule has been extensively elaborated on and some of its aspects remain, until today, a matter of scientific dispute. In 2006 two different sets of guidelines and a major consensus statement on this issue were published by the American Thyroid Association (ATA), the American Association of Clinical Endocrinologists/Associazone Medici Endocrinologi (AACE/AME) and the European Thyroid Association (ETA), respectively. [46, 113-115] Despite the overall similarities, there are also numerous disparities probably reflecting differences in patterns of medical practice, regional differences in available resources and variability in interpreting existing data. [116] Some of the most debated questions are presented and discussed here:

i. *The use of pre-operative ^{131}I thyroid scan (scintigraphy):* In cases of multinodular goiter or when the levels of TSH are in the low normal range, ETA recommends the use of thyroid scan to exclude or verify the existence of a hyperfunctioning nodule, which is more likely benign. [46] AACE/AME partly agrees with this recommendation differentiating between iodine-deficient and iodine-rich regions [114] whereas ATA recommends "based on expert opinion" (Recommendation rating: C) the consideration of thyroid scintigraphy in case the cytology report reads "follicular neoplasm", especially with low-normal levels of serum TSH. [115]

ii. *The extent of surgical treatment:* The low incidence and good prognosis of thyroid cancer explains to a great extent the lack of prospective, controlled studies that would compare the two major surgical treatment options, namely thyroid lobectomy and total thyroidectomy. Proponents of total thyroidectomy as the treatment of choice argue on the presence of occult, multifocal PTC which involves the contralateral lobe in 30-85% of cases. Moreover, complete thyroid gland removal facilitates treatment of residual disease with radioactive ablation as well as the patients' follow-up for recurrence by using serum TG measurements. On the other hand, the main argument of those supporting thyroid lobectomy is the lower rate of surgical complications such as hypoparathyroidism and recurrent laryngeal nerve injury. It should be mentioned, though, that this recommendation usually concerns patients with small tumors (<1cm) or patients with problematic access or compliance to thyroid hormone substitution therapy. [117, 118] It is quite characteristic that two recent studies of approximately 5,500 and 52,000 patients respectively, concluded in contradictory results on the impact of the extent of surgical resection on recurrence and survival. [119, 120]

iii. *The extent of nodal dissection:* The widely accepted agreement is that therapeutic, compartment-oriented lymph node dissection should be included in the surgical treatment of differentiated thyroid cancer (DTC) upon nodal disease

confirmed on palpation, biopsy or imaging. The controversy lies on whether DTC patients without pre- or intraoperatively confirmed lymph node metastasis should undergo a prophylactic dissection of various compartment nodes, such as the central compartment. [121, 122] The surgeons in favor of this approach claim that lymph node metastasis carries a negative effect on disease outcome, while the involvement of central neck lymph nodes cannot reliably be confirmed before or during the operation. Moreover, prophylactic central neck dissection can improve staging accuracy, lowers postoperative TG levels and may be responsible for decreased recurrence and mortality rates. [122] In contrast, those opposing this surgical intervention doubt that it can improve disease-free and survival rates and claim that, in the hands of inexperienced surgeons, it can lead to higher incidence of surgical complications (permanent hypoparathyroidism, injury of the recurrent laryngeal nerve). [122] Previous studies in our center suggested that identifying lymph node metastases is important in terms of prognosis and removing them decreases the risk of recurrence and might also improve survival. [123, 124]

To the above debated topics, one could also add the use of neo-adjuvant or adjuvant therapy and the application of radiotherapy or ablation with radioactive iodine. Recently emerged therapeutic approaches include the administration of redifferentiation agents (rosiglitazone) or gene therapy (induction of NIS gene expression which codes for the sodium-iodide symporter), in an attempt to increase radioactive iodine uptake in "radioiodine-negative" differentiated thyroid cancers. [125-127] Finally, targeting signaling pathways (Ras, BRAF, *PAX8-PPARγ*), the vascular endothelial growth factor (VEGF) or inhibiting receptor tyrosine kinases (such as the epidermal growth factor receptor, the VEGF receptor and RET) also pose as promising therapeutic options against thyroid tumor growth. [128-133]

It is discussed and widely accepted that medical research in the 21st century should focus more on so-called "tailor-made" treatments. Each tumor is completely unique and represents a system which has developed under the very certain and specific conditions its host provides. Therefore, the optimal treatment should take into account all those characteristics in order to be as effective as possible. Our hope is that this study will add information to the foundations for unraveling the molecular events behind thyroid tumorigenesis, thus allowing all clinicians involved to reach a secure diagnosis, make safe conclusions on the prognosis and choose the most appropriate therapeutic scheme. Our pursuit towards the direction of the proteins revealed here is to simplify and consolidate diagnostic and treatment algorithms for thyroid cancer in favor of a more efficacious health-care for both the patients and the health-care providers.

3. ΠΕΡΙΛΗΨΗ ΣΤΑ ΕΛΛΗΝΙΚΑ

Ενώ οι καλοήθεις παθήσεις του θυρεοειδούς αδένα είναι αρκετά συχνές, ο καρκίνος του θυρεοειδούς αποτελεί μια σχετικά σπάνια κακοήθεια με επίπτωση που κυμαίνεται περί τα 1,3 (άνδρες) και 3,3 (γυναίκες) κρούσματα ανά 100,000 κατοίκους ανά έτος. Σύμφωνα με την κατηγοριοποίηση του Παγκόσμιου Οργανισμού Υγείας οι κακοήθεις όγκοι του θυρεοειδούς διακρίνονται σε: α. Θηλακιώδη καρκίνο, β. Θηλωματώδη καρκίνο, γ. Μυελοειδή καρκίνο, δ. Καρκίνο χαμηλής διαφοροποίησης και ε. Αδιαφοροποίητο ή αναπλαστικό καρκίνο. Το θηλακιώδες αδένωμα αποτελεί τη μοναδική μορφή καλοήθους όγκου.

Η έγκαιρη διάγνωση, καθώς και η ταυτοποίηση στοιχείων προγνωστικών για την πορεία της νόσου, μπορούν να επηρεάσουν σημαντικά τη θεραπευτική προσέγγιση ενός ασθενούς που παρουσιάζεται με ψηλαφητό μόρφωμα στον θυρεοειδή. Η βιοψία λεπτής βελόνας, με ή χωρίς την καθοδήγηση μέσω υπερήχων, αποτελεί σημαντική διαγνωστική τεχνική η οποία, όμως, σε σημαντικό ποσοστό αποτυγχάνει να διαφοροδιαγνώσει συγκεκριμένους όγκους, π.χ. θηλακιώδες αδένωμα και θηλακιώδη καρκίνο. Το γεγονός αυτό καθιστά επιτακτική την ανάγκη ανεύρεσης διαγνωστικών ή/και προγνωστικών μοριακών καρκινικών δεικτών, εύκολα ανιχνεύσιμων σε κυτταρολογικό υλικό.

Στο Άρθρο Ι μελετήσαμε τον δείκτη MIB-1 και την πιθανή χρησιμότητα του στη διάγνωση και πρόγνωση όγκων του θυρεοειδούς. Συμπερασματικά, παρουσιάσαμε στοιχεία που συνηγορούν υπέρ της χρήσης του MIB-1 μάλλον ως δείκτη κακής πρόγνωσης σε ασθενείς με θηλωματώδη θυρεοειδικό καρκίνο, παρά ως διαγνωστικού δείκτη.

Τα Άρθρα ΙΙ, ΙΙΙ και IV αφορούν αποκλειστικά τη μελέτη πρωτεϊνών («πρωτεωμική» από τον αγγλικό όρο “proteomics”) που δυνητικά θα μπορούσαν να χρησιμοποιηθούν ως θυρεοειδικοί καρκινικοί δείκτες. Ειδικότερα, στο Άρθρο ΙΙ περιγράφεται ο καθορισμός ενός πρωτόκολλου κλασματοποίησης θυρεοειδικών πρωτεϊνών σε δύο κλάσματα, εμπλουτισμένα σε κυτταροπλασματικές και πυρηνικές πρωτεΐνες, αντίστοιχα. Το πρωτόκολλο αυτό απετέλεσε τη βάση προετοιμασίας του πρωτεϊνικού υλικού που χρησιμοποιήθηκε στα Άρθρα ΙΙΙ και IV. Στα άρθρα αυτά ανιχνεύθηκε και ταυτοποιήθηκε ένας αριθμός πρωτεϊνών που εκφράζονται σε διαφορετικά επίπεδα μεταξύ των διαφόρων θυρεοειδικών όγκων που εξετάσαμε, γεγονός που καθιστά τα μόρια αυτά δυνητικώς χρήσιμα για διαγνωστικούς σκοπούς. Μεταξύ των πρωτεϊνών αυτών συμπεριλαμβάνονται οι 14-3-3 (ισομορφές β, ε και ζ), Annexin V, α1-antitrypsin, S100A6, SELENBP1 κ.α.

Οι μελέτες που περιγράφονται στην παρούσα διατριβή δίνουν μια νέα προοπτική στην προσπάθεια ανεύρεσης καινοφανών θυρεοειδικών καρκινικών δεικτών. Πεποίθησή μας είναι ότι η διενέργεια προοπτικών μελετών επικεντρωμένων στις πρωτεΐνες που περιγράφονται σε αυτήν τη διατριβή, θα μπορούσε μελλοντικά να διευκολύνει τη διαφορική διάγνωση όγκων του θυρεοειδούς χάριν μιας πιο άμεσης και πιο αποτελεσματικής θεραπευτικής προσέγγισης των ασθενών. Είναι, όμως, εξίσου σημαντικό να μελετηθούν και να αποκαλυφθούν λεπτομερώς οι μοριακοί μηχανισμοί που ευθύνονται για τη γένεση και ανάπτυξη των θυρεοειδικών νεοπλασμάτων.

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"As you set out for Ithaca,
wish your road is a long one,
full of adventure, full of knowledge..."
(by Konstantinos Kavafis, "Ithaca" 1911)

Now that I have reached my Ithaca (well…before setting out for a new one!), I feel the need of thanking all those that, in one way or another, have contributed in this long journey.

First and foremost (pardon me, if I am breaking the protocol here…), I would like to thank, from the bottom of my heart, my two elementary school teachers, **Mr. Kostas Kotoulas** and his lovely wife **Mrs. Voula Kotoula**. For I believe that it is at that age that we set the foundations upon which we build our future as human beings. And I could not have been luckier than to have these two gifted and devoted teachers educating me and instilling me the virtues of seeking knowledge and being not just a good professional, but most importantly a good man. Σας ευχαριστώ θερμά!

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