# SUMMARY

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# ABSTRACT

**Background**: High mitotic index, high nuclear grade and reticulin distruption are part of representative hallmarks of adrenocortical cancer (ACC). A characteristic neutrophil/T-lymphocytes infiltrate ratio has been often implicated in carcinogenesis, progression and clinical outcome of several cancer types. However, its role in adrenal cortical tumors is unclear. Histology-based diagnosis may also suffer of a moment of subjectivity due to inter- and intra-observer variations. Proteomic studies of malignant tumors represent the future both for possible diagnostic and prognostic implication; whole proteome analysis of adrenocortical tumors from fresh tissues may represent the lacking piece of puzzling management of this tumor.

**Aim**: to assess by computerized morphometry morphological features, vascular, inflammatory, reticulin and proliferative index pattern in adrenocortical adenomas (ACAs) and carcinomas and to assess proteomic profiles from adrenocortical tumors fresh tissues.

**Methods**: A single Institution series of 11 ACAs and 18 ACCs samples was analyzed using a Kontron-Zeiss KS400 image analyzer. Four consecutive sections 4 μm thick were obtained with a total of 250–300 HPF examined for each case. Immunohistochemistry for Ki67, reticulin and CD8/CD15 was obtained. To minimize subjectivity, particularly relevant when quantitative results are expected, we generated a morphometric model based on analysis of volume fractions occupied by Ki67 positive and negative cells (nuclei, cytoplasm) and inflammatory compartments (CD15+ granulocytes, CD8+ lymphocytes) and reticulin framework surface. Lastly, the assessment of Ki-67 by computerized morphometry was compared with pathologist's evaluation.

After sample preparation protocol of 7 ACCs, 5 ACAs and 5 normal adrenal tissue samples, difference In Gel Electrophoresis (DIGE) and following the protein spots individuation and isolation, Mass Spectrometry, were performed to allow protein identification.

**Results**: The volume fraction of Ki-67 positive cells was highest in ACC. The volume fraction of nuclei in unit volume and the nuclear/cytoplasmic ratio in both Ki-67 negative cells and Ki-67 positive cells were prominent in ACC. The surface fraction of reticulin was considerably lower in ACC. Moreover, when comparing morphometric analysis of Ki67+ cells to pathologist's scores, the data of the point grid analysis revealed significantly lower values compared to conventional histopathology. These values, once statistically analyzed, demonstrated that our morphometric model could improve the sensitivity and specificity of Ki-67 evaluation in ACCs and ACAs (reaching 94% of sensitivity, 100% of specificity) and also that it cold contribute to a better prognosis definition.

Proteomics individuated 62% overexpressed proteins in ACCs with respect to ACAs: among them vimentin and vitamin D-binding protein resulted the most varied (3.2 and 3-fold more expressed in ACCs than in ACAs respectively). On the other hand the remaining 38% of proteins resulted under-expressed in ACCs with regard to ACAs, being cathepsin D and aldose reductase both 3-fold less expressed in ACCs than in ACAs. The protein profile of ACCs versus normal adrenal tissue was similar (although with slight differences in terms of fold variation) to that of ACCs versus ACAs; nonetheless a varied new protein (lactate dehydrogenase, 1.8 fold increase in ACCs) with a possible role in tumorigenesis and tumor progression, was detected.

**Conclusions**: Our computerized morphometric model is simple, lacks observer or subjective bias and can be used to supplement objective methods to achieve precise and reader-independent quantification of morphological characteristics and histological biomarkers of adrenocortical tumors. We speculate that the assessment of inflammatory infiltrate may found a place in the diagnostic algorithm of adrenal benign and malignant tumors. The promising preliminary results obtained by the proteomic study of ACCs and ACAs could contribute to the identification of new histological biomarkers. These data, once integrated into a complex algorithm including histological assessment, morphometric analysis and clinical data evaluation would easily contribute to create a prognostic stratification of ACCs with clear advantages for the clinical management of the disease.

# INTRODUCTION

## Benign and malignant adrenocortical tumors

The cortical component of the adrenal gland consists of three different areas called zona glomerulosa, fasciculata and reticularis, each with specific hormonal secretion characteristics: the zona glomerulosa secretes glucocorticoids, the zona fasciculata mineralocorticoid, the reticularis mainly androgens. On the basis of autopsy findings adrenal neoplasms could be considered among the most common malignancies, with a prevalence average of 2.3% (range 1-8.7%, probably due to the small size of many lesions).[1] Neoplasms of the adrenal cortex are mostly benign (adenomas) and are often found incidentally (the so-called *adrenal incidentalomas*) during diagnostic investigations performed for non-adrenal diseases (3-5% of radiological examinations); the definition of incidentaloma does not refer to adrenal nodules found in patients undergoing investigations for diagnostic workup of a known neoplastic disease.[2,3]

The prevalence of adrenocortical adenomas (ACAs) increases with age, being approximately 0.2% in the young while it is above 7% in patients with more than 70 years.[4] The increase of the average with the age of the general population and the improvement of the resolution of imaging techniques (as well as their increased use) will further contribute to the rise of its prevalence with a consequent impact on clinical management and healthcare costs.[5] In clinical practice, the radiological findings of an adrenal incidentaloma can be considered a diagnostic "dilemma", particularly for small lesions.[6] Although the majority of the adrenal lesions turn out to be benign at the end of the

diagnostic workup, fifteen to 20% of adrenal incidentalomas are characterized by hormonal hypersecretion syndromes (usually hypercortisolism, in particular in the subclinical form; much more rarely occur the neoplasms of the medullary component called pheochromocytomas, that are not part of this topic). If not treated, these syndromes are associated with high morbidity and mortality.[7,8] Although the radiological parameters considered as distinctive for a likely benign adrenal lesion (Hounsfield Unit, HU, for radiodensity less than 10 at baseline CT scan and rapid washout of contrast medium) are relatively characteristic of an adrenal adenoma, not infrequently they are also present in malignant lesions, as demonstrated by a study of the Cleveland Clinic in a wide series.[9] A careful assessment as to whether these lesions with atypical or suspicious radiological features are already *hyperfunctioning* at the diagnosis is therefore crucial. Recognizing any potentially evolving lesion (progression from a non-secreting to hypersecreting subclinical or clinical form) and predicting the growth rate or the possible progression to malignancy are the major endpoints for the clinician.

Concerning the clinical management the most recent guidelines recommend, after the diagnosis of an adrenal incidentaloma, a radiological control at 6, 12 and 24 months, and even more frequently in cases suspicious for malignancy. This impacts undoubtedly the patient's quality of life (stress, exposure to ionizing radiation, hospital access both inpatient and Day Hospital care) and also the subsequent increase in costs for the National Health System. Moreover in consideration of the complexity of the disease management, the diagnostic and curative workup of adrenal lesions necessitates the involvement of a multidisciplinary team of expert endocrinologists, radiologists, surgeons and pathologists.

In 2002, the NIH *Consensus Conference of the clinical unapparent adrenal mass* strongly recommended that adrenal masses larger than 6 cm should be surgically removed, suggesting instead a conservative approach for adrenal tumors with less than 4 cm size; for the approach to intermediate size lesions the indication was to consider additional diagnostic and clinical criteria.[10] Actually these additional criteria are not sufficiently standardized nor widely established. Moreover in view of the increased frequency of the diagnosis of ACCs of small size, where surgery would be potentially curative, the above described scenario becomes even more complex, as recently documented by a review of the National Cancer Data Base.[11] This study concluded that not even the size of an adrenocortical carcinoma (ACC), previously considered as a significant prognostic parameter, correlates with long-term survival of patients. On the other hand the presence of regional and distant metastases, the nearby surrounding organs invasion and the grading of the tumor appeared to be related to worst prognosis. Nonetheless the size of the adrenal mass at CT or MRI remains currently one of the best indicators of malignancy available. In fact, more than 60% of incidentalomas with a diameter less than or equal to 4 cm consists of benign adenomas, while less than 2% are carcinomas. On the contrary, the risk of ACC increases up to 25% in case of lesions larger than 6 cm, while adenomas are less than 15% and finally nearly 6% of the nodules between 4 and 6 cm size turns out to be malignant.[12,10]

The histological diagnosis of the majority of operated adrenal incidentalomas is represented by ACAs; more uncommon are their oncocytic variant (presenting a clinical-biological attitude slightly more aggressive than conventional adenomas). About 5% of the surgically removed incidentalomas is represented by pheochromocytomas, while about 2% are adrenal metastases

from other undetected cancers. Other categories such as myelolipomas, lipomas, cysts, hemangiomas, angiosarcomas and lymphomas are pretty rare.[13,14,15,16]

The ACC (with all its histological variants) represents the remaining 5% of these lesions (both incidentally and non-incidentally discovered); this rare malignancy has a heterogeneous and only partially known pathogenesis and it is associated to a very poor prognosis. Its prevalence is 4-12 cases per million adults and an incidence of 1-2 new cases per million per year. Women seem to be more affected than men (ratio 1.5), but this difference could be partly explained by the fact that women tend to undergo diagnostic tests more frequently than men. The ACC is characterized by a bimodal distribution, with a first frequency peak in children aged less than five years and a second peak in adults in the fourth and fifth decade of life; in children, the incidence is 10 times lower, except in some areas of southern Brazil where it has been described a high incidence of a germline mutation (in the gene encoding p53) implicated in its pathogenesis.[12]

Due to the advanced stage of disease at diagnosis, the survival rate of patients at 5 years is quite low, ranging from 20% to 45% (with a median survival of 18 months); approximately 2/3 of the patients experience a recurrence within two years and about 85% of them develop a local recurrence or distant metastasis. Moreover about half of patients with ACC has already distant metastases at diagnosis: this precludes a surgical approach as a possible curative choice.[17] Proper staging is therefore essential to distinguish subgroups of patients with potentially resectable disease rather than eligible to chemotherapy. The most recent revision of the previous staging system has been proposed (and nowadays widely applied) by the European Network for the Study of Adrenal Tumours (ENS@T)[19]:

**Stage I**: Disease limited to the adrenal gland with no evidence of local invasion or distant metastases, with maximum size of the tumor ≤5 cm (T1N0M0)

**Stage II**: disease limited to the adrenal gland with no evidence of local invasion or distant metastases (like stage I) with maximum size of the tumor >5 cm (T2N0M0)

**Stage III**: tumor of any size, with at least one of the following elements: tumor infiltration into the surrounding tissues (T3), involvement of adjacent organs or presence of neoplastic thrombus at the level of the inferior vena cava or renal vein (T4), the presence of lymph node metastases (N1) without distant metastasis (M0)

**Stage IV**: the presence of distant metastases (M1) regardless of the tumor size and local extent (T1-T4) and/or of the involvement lymph nodes (N0-N1)

Lesions limited to the adrenal gland (about 30% of adrenal tumors), especially when small, still represent a diagnostic problem that often cannot be dealt with radiological image alone nor with additional procedures such as fine needle biopsy and cytology. In fact fine needle biopsy of an adrenal lesion it is no longer indicated as rarely useful due to the ambiguity of the cytological results and to the potential risk of tumor cells spreading.[20] Currently this procedure is only used during an oncological workup of a primary tumor in case of an adrenal lesion suspicious for metastasis.[21]

A careful histologic examination is therefore considered crucial in the diagnostic workup of a surgically removed adrenal lesion, in order to make appropriate choices for therapy and follow up of an adrenocortical

tumor.[22,23,24] Since middle of 80s some authors have tried to identify surgical and histological parameters capable to predict the clinical behavior of an ACC. In 1984 Weiss and coworkers, through a comparative study of 43 cases of ACC, identified nine descriptive histological criteria (named "*Weisse score*", see Table 1) able to differentiate with reasonable sensitivity adrenal adenomas from carcinomas.[25] Unfortunately none of the 9 criteria of the Weiss score unequivocally represents *per se* an absolute feature of malignancy. Moreover, due to the heterogeneity of this tumor, Weiss score modifications have been proposed accordingly to the different tumor variants (classic, oncocytic, myxoid and pediatric), where the classic Weiss criteria has been demonstrated not applicable.[26]

**Morphology and histology of benign and malignant adrenal tumors:**

The diagnosis of ACC can be very difficult. First, it is essential to make a macroscopic evaluation: in fact the majority of ACCs has a weight greater than 100 grams and a diameter greater than 6.5 cm, with presence of hemorrhagic areas and/or necrosis and calcifications. These tumors commonly have a lobulated aspect, characterized by extensive fibrous septa that separate areas of parenchyma, and in some cases involvement of adjacent organs may be evident; occasionally a macroscopic angioinvasion could be described.[27]

In the past the majority of ACCs possessed all the macroscopic features described above, including the large size, therefore the diagnosis appeared easier already at the first macroscopic evaluation. Serious diagnostic problems arose from the increasing number of small adrenal nodules operated that do not show the typical macroscopic characteristics of malignancy. The

differential diagnosis between one of these "small" ACCs and an adenoma can actually be challenging for the pathologists' routine evaluation.

Overall the tissue architecture in ACCs is less organized than in adenomas, and may remain constant or also vary markedly between different areas of the tumor. Normally the organization is in trabeculae, with large strings separated by capillary cells, but it is often possible to observe a pattern of widespread growth; compact cells are more prevalent in ACCs than in adenomas. Nuclear hyperchromatism and pleomorphism is often present in ACCs cells, sometimes in presence of multinucleated giant cells. Many of the cancerous cells are also characterized by vesicular nuclei with prominent nucleoli and nuclear membranes; mitoses are frequent and not rarely atypical.[28]

**Table 1. Weiss Score**

| | | | |
|---|---|---|---|
| 1. | High nuclear grade (Fuhrman III or IV) | 2. | Mitotic index >5/50 High Power Fields |
| 3. | Atypical mitosis | 4. | Clear cells≤ 25% of the histological slide |
| 5. | Diffuse architecture >1/3 histological slide | 6. | Presence of necrosis |
| 7. | Venous invasion | 8. | Sinusoidal invasion |
| 9. | Tumor capsule invasion | | |

More in detail, mitotic rate is performed with x40 objective by counting 10 random high power fields (HPF) in the area of the greatest numbers of mitotic figures on the five slides with greatest number of mitoses. Atypical mitotic figures are regarded as atypical when they definitely show an abnormal distribution of chromosomes or an excessive number of mitotic spindles.

The presence of clear cells is defined as less than or equal to 25% of clear or vacuolated cells resembling the normal zona fasciculata. Diffuse architecture is observed if greater than one-third of the tumor formed patternless sheets of cells: trabecular, columnar, alveolar or nesting organizations are regarded as non-diffuse patterns.

Necrosis is regarded as present when occurring in at least confluent nests of cells.

Concerning vascular invasion (venous or sinusoidal) Weiss defined a vein as an "endothelial-lined vessel with smooth muscle as a component of the wall and a sinusoid as endothelial-lined vessel in the adrenal gland with little supportive tissues". In case of sinusoids only those located within the tumor are considered.

Invasion of tumor capsule is accepted as present when nests or cords of tumor are extended into or through the capsule, with a corresponding stromal reaction.

The Weiss score system as described above has become in the following years, till today, the standard method for the evaluation and classification of adrenocortical neoplasms also because it does not require clinical data (often incomplete or inaccessible for the pathologist) to be performed; furthermore, this system allows to obtain information about the biological behavior of the lesion, providing important information for the therapeutic approach and also contributing to a prognosis assessment. The application of the Weiss score however requires experienced pathologists and, in consideration of the relative rarity of the disease, several authors agree that the interpretation and recognition of some of the histological criteria proposed is difficult and operator dependent. Several revisions of this score have been

proposed trying to simplify the method and to improve its sensitivity (Van Slooten, Hough system), but an adequate standardization is still far to be attained. In 2002 Aubert et al. proposed a simplification of Weiss system, eliminating the parameters difficult to be assessed and keeping only the most reliable and repeatable (Table 2):[29]

**Table 2. Weiss revisited index, WRI**

| |
|---|
| 1. mitotic Index> 5/50 fields at high resolution (HPF) |
| 2. Presence of atypical mitosis |
| 3. Percentage of clear cell ≤ 25% |
| 4. Presence of necrosis |
| 5. Presence of capsular invasion |

This new system was well correlated with the one proposed by Weiss and seemed to be easier to apply in clinical practice. A recent study not only confirmed the value of this new system in the diagnosis of the ACC, but also showed how it is significantly correlated with the prognosis and in particular with patient survival.[30]

Following this attempt of Weiss score renovation other authors proposed further modifications of the diagnostic algorithm of ACCs: in 2009 Papotti and coworkers analyzing a large series of ACCs and adenomas found that the disruption of the reticular network (defined histologically with reticulin staining) was present in all the malignant cases. Based on this finding the authors proposed a new easy-to-perform algorithm where the reticulin disruption together with at least one additional parameter (mitotic index>5/50 high-power fields (HPF), presence of necrosis and presence of

vascular invasion) showed 100% sensitivity and specificity in recognizing malignant tumors, also according to the Weiss system, but with more practical applicability: this study has been also recently validated in a multicentric study in a larger series, showing also a high interobserver reproducibility. Immunohistochemistry may also provide important additional information to conventional histology in adrenocortical tumor diagnostic workup. Especially, the evaluation of proliferative tumor markers (e.g. ki-67) proved to be useful in the differential diagnosis between benign and malignant adrenocortical neoplasms.[31,32,33]

The expression of the protein Ki-67 is closely related to cell proliferation, as it is present within the nuclei of cells during all stages of the cell cycle (G1, S, G2 and mitosis), but is absent in resting cells (G0);[32] the protein Ki-67 is considered to be an excellent marker for the assessment of the growth fraction of any human cell population (both normal and neoplastic), so that the percentage of labeled cells (Ki-67 Labeling Index) is commonly used for the diagnosis and evaluation of the "biological behavior" of different neoplasms.[34,35] As for adrenocortical tumors many studies have highlighted the utility of the assessment of Ki-67 expression in clinical practice, the labeling index resulting significantly higher in carcinomas than adenomas.[36,37] Additionally some authors have observed that in patients affected by ACC, the Ki-67 Labeling Index (from here on, only Ki-67) is inversely related to overall survival. These results suggest that this marker may be helpful in identifying patients with more severe prognosis, suggesting also a possible role in indicating different therapeutic approaches. A Ki-67 greater than 5-7% is considered an important clue of malignancy for adrenal tumors, although a well-defined cutoff for Ki-67 able to correlate unambiguously with the morphology and prognosis of these tumors is not

available so far, especially because of a relevant interobserver variability. Ki-67 is thoroughly studied in breast cancer and Ki-67 immunostaining has been shown to be accurately evaluable by computerized methods. Previous studies already found that semi-automated analysis of Ki-67 staining with image analysis could be used for prognostic assessment of patients with breast cancer.[38,39,40]

## Computerized morphometry and stereology: state of the art

During the past 30 years, the number of laboratory tests available to be used by physicians has grown exponentially. The number and quality of tests used in anatomic pathology with the goal of improving the patient care have also increased. These technologies range from special histological stains to molecular assays at the DNA, RNA, and protein levels. However, histopathology remains the gold standard for most diagnostic and therapeutic decisions in surgical pathology. Pathologists are increasingly involved in clinical practice as part of the team responsible for the management of a particular patient and disease. The significant technical improvement of former, and the advent of new methodologies gave pathologists also a decisional role, especially in oncology. The pathologist judgment is extremely important with regard to biomarkers and their cutoff values, presence/absence of a particular connotative mutation, infiltrative disease and morphological features. In this scenario computerized image analysis and morphometric/stereological models could become very helpful. In fact there is an expectation in Medicine that the critical part of the operation of an instrument should be fully automatic. Indeed, many users express mistrust against manual operations, often regarded as subjective and irreproducible. Low reproducibility reduces the clinical value of morphologic grading of malignant tumors, and the replacement of subjective classification with objective quantification has been suggested.

The interpretation of histologic sections is an inherently subjective process based primarily on morphologic features. With the aim of making

pathologic examinations less subjective, the extensive use of immunohistochemistry (IHC) assisted pathologists in making diagnoses, adding to or complementing morphologic information with molecular information. Despite the fact that it often lacks reproducibility and standardization, immunohistochemistry has become a standard assay in surgical pathology. Sources of variability in immunohistochemistry are numerous and include fixation conditions, specimen pre-treatment, reagents, detection methods, and interpretation of result. With regards to this last point the use of the pathologists' subjective scoring method is less accurate and reproducible; Cohen's kappa coefficient, frequently applied as a statistical measure of inter-observer agreement for qualitative (categorical) items, still shows some pitfalls. Therefore, being impossible the standardization of all the potential variables in IHC, a standardizing approach to IHC results through quantitative methods has been proposed. More recently, IHC has also been used to predict response to targeted therapy. This new role further underlines the need of reproducibility and accuracy for this technology.[41]

A considerable number of published scientific studies have addressed computer vision to quantification of protein expression as determined by immunohistochemistry. [42,43,44] Very few studies have compared human visual interpretation and computer vision of a biomarker immunostaining with regard to clinically important endpoints, such as disease outcome. [45,46]

While tissue sample processing and IHC staining methods are increasingly automated, the evaluation of staining results is still predominantly performed by visual assessment. A human interpreter has excellent image comprehension and pattern recognition capabilities, but is prone to substantial variability in quantification tasks. Computer vision methods are capable of processing images consistently and generally they

perform well in repetitive processes. Virtual microscopy combined with computer vision techniques can aid the human observer by analysis of large tissue areas at a high magnification. The digital sample (i.e. the virtual slide) can be an entire section of a single cancerous tumor or an array of 100-200 tumor tissue samples assembled by the use of tissue microarray technology.[47]

The stereology challenge is to understand the structural inner three-dimensional arrangement based on the analysis of the structure slices only showing two-dimensional information. Through the correct stereology use, a quantitative study with little effort could be performed: efficiency in stereology means a minimum slices sample counting (little work), low cost (slices preparation), but good accuracy. Recently, morphologists have benefited of the use of genetic and molecular techniques to help improving biological and biomedical researches. However, questions concerning quantitative alterations of tissues, cells or cellular organelles (which frequently appear in adaptation, evolution or pathology of an organism), as well as a better correlation between morphology and function, need a quantitative approach to become well understood.[48,49] Despite the argument about nomenclature morphometry and stereology could denominate the same method.[50] Most authors consider morphometry as a two-dimensional quantitative method that uses a caliper (mainly a caliper micrometer, a gauge with a calibrated micrometer screw for the measurement of thin objects under microscopic observation). On the contrary, stereology does not use a caliper (and does not perform a direct measurement), but it uses a test-system usually composed by test-points, test-lines over a known frame (or test-area). Major differences between morphometry and stereology are summarized in the table here below (modified from Mandarim-de-Lacerda 2003).[51]

**Table 3. Major differences between morphometry and stereology**

| Characteristics | Morphometry | Stereology |
|---|---|---|
| macro, meso, micro levels | yes | yes |
| measurement in the plane | yes | no |
| 3-D information | no | yes |
| caliper | yes | no |
| test-system | no | yes |
| unit | mm | $mm/mm^3$ |
| slices of the structure | not necessarily | yes |
| formulas | simple | complex |
| probabilistic statistics | no | yes |
| image analysis facilities | usually yes | usually not |
| statistics | parametric | non parametric |

Morphometry determines lengths, perimeters, areas, and benefits of image analysis software's facilities. Stereology estimates densities; the most interesting are the densities per volume: volume density, length density, surface density and numerical density. The accuracy of calculations is based on the statistics principles (sample size, randomization, and isotropy). The aim of the sampling design for stereology is to obtain the maximal amount of quantitative structural information at a given total cost or effort. In general, the variation between different individuals (the biological variation) is the major determinant of overall efficiency, whereas the variation between single microscopic features is less important. However, spending time and/or money in order to increase the precision of the single measurements is describe as irrational in almost all studies. This could be summarized by the sentence ''do more less well''.[52]

Low reproducibility reduces the clinical value of morphologic evaluation of malignant tumors: simple mitosis counting has been employed for objective malignancy grading most frequently and has proved its

prognostic significance in sarcomas and carcinomas of the breast and ovary. These and other measurements of morphometry are, however, obtained in two dimensions only, introducing bias due to ignorance of the fact that biologic structures are three-dimensional. Stereological estimators are well suited, because they enable the assessment of spatial structure from sections. It has been shown that many stereological evaluations are applicable to ordinary histologic sections processed under routine conditions. If a systematic random scheme of sampling is employed then the efficiency of estimation is usually high and therefore reproducible, accurate and representative results are ensured.

The main problem with instrumental image analysis is that it critically depends on image segmentation (detection of the nuclear boundary) as a first step, an operation easily resolved in other fields of engineering but that represents a formidable obstacle in biology. The prejudice against interactive procedures, which so easily would resolve many problems, also appears unwarranted in view of the extensive stereological literature proving most manual procedures to be efficient. Additionally, all morphometric point counting procedures have been consistently proved to be more reliable and reproducible than computerized approaches. The unfulfilled promise of image analysis in all fields of pathology is that computers would potentially provide a dependable, reproducible, and objective instrument of making diagnoses. The problem is to find a proper reference system (consider, for instance, the stroma, blood vessels, the variable portion of tissue occupied by tumor, even the non-stained parts of the tumor cells). Some manufacturers propose to quantitate simply by averaging the results over the number of malignant cells. This is a conceptually simple approach that belies the difficulty of properly segmenting and, therefore, counting nuclei. If not limited by the

unfriendliness of the community toward manual and semi-quantitative methodologies, a solution would be to limit counts to areas present within an adjustable test frame of known area (rather than the entire video screen) and to cells identified by a manual approach (clicking on them). Despite this interest in morphometric diagnosis, it is evident that, nowadays, the majority of diagnoses in pathology are achieved by subjective and visual evaluation.[53]

This is generally easy for a trained pathologist, but there are numerous cases where an objective quantitative way of resolving a difficult differential diagnosis would be welcome, and adrenal tumors are part of this setting. Recently virtual microscopy has been proposed to improve histological analysis of adrenocortical tumors. In one of these studies twelve pathologists, all from reference Centers, analyzed 50 selected adrenocortical tumors using a web-based virtual microscopy approach in a blind design and allowed determining the intra-observer and inter-observer reproducibility of the Weiss system. Interestingly the method was able to very simply grade the intra-observer reproducibility of every single item: necrosis being the most reproducible criteria, whereas sinusoidal invasion resulted almost irreproducible. On the other hand nuclear grade and sinusoidal invasion that were ruled out of the modified Weiss score proposed by Aubert and coworkers because of their low intra-observer reproducibility performed poorly also with virtual microscopy. Moreover the two other Weiss criteria that were most improved with virtual microscopy were the number of mitotic figures and the presence of atypical mitoses: these criteria are among the most useful for determining the malignancy and prognosis of ACCs.[54]

Overall we could argue that the image analysis–based approach, more likely to give rise to a new anatomic subspecialty than to replace pathologist evaluation, could only be conceived as one of the ancillary technique at

disposal of pathologists. Analysis of the diagnostic approach followed by expert pathologists reveals critical reliance on a number of features that can be quantified by computerized image analysis, like nuclear size, nuclear pleomorphism or chromatin texture. Each of these descriptors could be substituted with a quantifiable number, giving the opportunity of creating new classification systems and diagnostic algorithms. Morphometric diagnostic methods can be applied to both cytology and histology, however cytological applications are easier and convincing. In cytology in fact the entire nucleus is visible and not just sections of it, nonetheless processing morphometric data obtained on histologic sections of paraffin-embedded tissues and achieving a diagnosis on them is possible (but it requires considerable adaptation).[55]

The procedure that consents a correct histological sample examination is segmentation, which designs the delimitation of boundaries between two compartments. Once the nucleus has been properly segmented, the following computerized measurements inside the nuclear outline (enclosed area, largest diameter, intercepts with test lines, even shape factors) by the methods of image analysis are easy, straightforward and dependable. Many multipurpose graphics programs that can be easily adapted to cytology are commercially available. In other words, the quality of the measurements and, therefore, the reliability of the diagnosis depend mainly on the nuclear segmentation, which is the real Achilles' heel of diagnostic morphometry.[56]

Operator independent and reproducible malignancy grading has interest for the clinical pathologist and oncologist, because replacement of subjective scoring by quantification offers the potential for improving the accuracy of the biologic information provided. Numerous studies have employed 2D techniques, and it is known that several morphometric variables may possess prognostic impact.[57] It seems that especially mitotic counts have strong

predictive capacity, in particular in breast and ovary,[58,59] and in sarcomas.[60] Objective tumor grading by stereological estimation of the mean nuclear volume was first introduced by Nielsen and co-workers in 1986 in a study of bladder cancer patients.[61] Reviewed by Serrensen six years later this variable has been successfully applied to predict the prognosis in a large number of tumor types, including malignant melanoma, carcinomas of the uterine cervix, oral mucosa,[62] and more recently lung carcinoma and carcinoma of the ampulla of Vater.[63,64] Moreover in breast cancer (and central nervous system malignancies as well) evidence is accumulating regarding the value of stereological mean nuclear size estimation as a prognostic marker, and also stereological application to IHC (e.g. Ki-67) has already emerged as a powerful tool able to increase diagnostic and prognostic accuracy in several malignancies.[65] Many promising studies have been performed also on prostate cancer. Ananthanarayanan and coworkers developed an automated method for subcellular scoring of p27 without individual segmentation of the cells: this method consented to identify a strong relationship between p27 expression and prostate cancer recurrence, independent of tumor grade, stage, and prostate-specific antigen and regardless of subcellular location. Interestingly this relationship was not observed with manual scoring demonstrating an improvement of the histological analysis in this cancer thanks to the application of an automated model.[66]

As for endocrine oncology, several interesting results have been obtained with the application of morphometric/stereological models to thyroid cytology and histology. In fact due to the difficult identification of malignant follicular cells or adjacent normal follicular cells on conventional cytology, morphometric parameters could help in pre-surgical diagnosis and or simplifying the histological study.[67] Some authors found a very strong

statistical difference between the morphometric high and low values for area, perimeter and large/small nuclear diameter ratio of follicular adenomas and carcinomas in agreement with literature data that indicate that the differential diagnosis between the two thyroid neoplasms could be based on nuclear characteristics, especially nuclear size and perimeter.[68,69]

One of the first studies on adrenal disease, particularly on benign and malignant pheochromocytomas, demonstrated distribution plots of the maximum nuclear cord length and of the nuclear area showing a narrow peak for the malignant cases, whereas benign cases showed a right-shifted curve with a wider peak: this evidence underlined that nuclear morphometric analysis could yield two distinctive patterns and may be of utility in the assessment of the diagnosis of the biological behavior of pheochromocytomas.[70]

Very recently some authors showed how stereology applied to in vitro studies of pituitary adenomas is able to give information on basal and stimulated/inhibited cell morphology.[71]

At present, few data with regard to computerized morphometry application on adrenal lesions are available. Some evidences have been obtained on age-dependent adrenocortical cell migration and only preliminary data on nuclear differences between ACAs and ACCs have been reported. Particularly Scarpelli and coworkers several years ago performed a quantitative analysis of adrenocortical adenomas and carcinomas: nuclei in ACCs resulted larger than in ACAs, total optical density had a near-diploid distribution in the adenomas while it was clearly aneuploid in the carcinomas and also differences in nuclear chromatin texture were found between the two groups. Multivariate analysis showed good discrimination between

carcinomas and adenomas and between different histological types of adenomas. However, based on Bayesian decision boundaries, 20-25% of carcinoma nuclei could be expected to be in the range of adenoma and about 12% of Cushing's adenomas nuclei and 15% of Conn's adenoma nuclei would be classified as carcinoma. This is one of the most crucial results, pointing out that the morphological/stereological study *per se* could be able only partly to distinguish between benign and malignant adrenocortical neoplasms, suggesting that other tools may be useful, as integrated diagnostic approach, to give significant differential morphological information.[72] A year later, the same group of authors extended the evaluation also to incidentalomas and normal adrenal tissue, concluding that computer-assisted analysis of nuclear characteristics proved to be useful in identifying and describing differences between groups of tumors arising in the adrenal cortex and highlighted the similarity between incidentalomas and adjacent normal-appearing cortex and between Cushing's adenoma and adrenal carcinoma. Other evidences supporting the need of an integrated method emerged also more recently: Shirata and coll. evaluated with an image analyzer the chromatin texture of pediatric and adulthood ACCs. Although the authors could not show a statistical significant correlation of the chromatin texture parameters, they were able to demonstrate a marginally significant correlation of DNA-ploidy with clinical behavior in children.[73]

The precise quantification of morphometric estimators and IHC markers is even more important when these parameters are included in classification and diagnostic criteria or in prognostic algorithms, as for neuroendocrine tumors.[74] Precisely for this particular type of endocrine tumors a panel of experts indicated the computerized morphometry with

digital image analysis as the most reproducible method for the quantification of Ki-67 positive cells, although it is not routinely available anywhere.[75,76]

The computerized morphometric analysis is not only able to provide specific characteristics of cancerous cells, but it could also differentiate between the tumor cell compartment and the structures in the surrounding microenvironment, especially lymphatic and blood vessels (e.g. interesting for the tumor-dependent angiogenesis).[77]

Similarly also the intra- and peritumoral inflammatory infiltrate could be studied through a morphometric-immunohistochemical approach obtaining a more detailed pattern of inflammatory infiltration, already described by several authors to be involved with the degree of invasiveness and aggression of a particular tumor.[78]

## Proteomic expression analysis: state of the art

Proteomics is a methodology extremely vast and complex that, through a combination of different technologies, allows identifying, quantifying and characterizing all the proteins of a cell, tissue or organism, including those still unknown. Proteome analysis has gained considerable attention when the human genome has been discovered consisting of far fewer genes than expected, disagreeing with the dogma of biology "one gene, one protein". It is estimated that approximately 100,000 human genes with mRNA splicing and post-translational modifications could generate in humans a proteome of nearly 1,000,000 of proteins. The possible applications of proteomics are wide and range from the study of the molecular processes that occur in course of a disease, such as post-translational modifications of proteins or their cellular redistribution, to the exploration of new protein biomarkers specific to certain conditions or modified proteins (i.e. phosphorylated, glycosylated,

etc.). It is evident in fact that the sole genomic information is not sufficient to explain all the biological events and to allow identifying new drug targets. The comparison between the protein expression profiles of normal versus neoplastic (adenoma, carcinoma) tissue may in fact show a large number of hypo- or hyper-expressed proteins, lack or presence of a particular protein, and also post-translational modifications. Usually the samples are prepared with highly complex protein mixtures such as cell lysates, tissue extracts and other biological materials. But it is only with the identification of all the proteins in the sample, together with their function, that it would be possible a better understanding of the complex mechanism of onset and progression of a disease. A large number of studies have reported that there is no single protein (so called *biomarker*) able to discriminate a disease: therefore it is suggested to assume that only a combination of biomarkers can better identify a particular disease. The ideal marker should be as specific as possible and sensitive; it should have clinical utility higher than the one of markers already in use, it should be present in biological samples obtained easily through non-invasive procedures. The liquid biological samples are certainly the most suitable for the detection of prognostic or diagnostic biomarkers, thanks to their easy accessibility and to the non-invasiveness.

The sample preparation, prior to its analysis, is one of the most critical points of the proteomic study, as it can affect the final results. Some phases of proteomic expression analysis cannot be automated, such as sample preparation and two-dimensional electrophoresis gel, therefore hiding an operator dependent process.[79]

The cornerstone of proteomics is the two-dimensional polyacrylamide gel electrophoresis (2D-PAGE), used for the separation of proteins according to their isoelectric point in the first dimension and to their molecular weight in

the second dimension. A 2D-PAGE is able to individuate thousands of proteins simultaneously in a single procedure leading theoretically to the separation of 10,000 proteins in a single gel. The matching of two electrophoretic techniques gives the two-Dimension Electrophoresis (2DE) a high-resolution power, making it currently the most powerful separation technique for the study of the proteome. This technique obtains information on the isoelectric points and molecular weights of proteins, their total amount, the protein isoforms and some post-translational modifications. This procedure is indicated for the separation of proteins with molecular weights between 10 kDa and 100 kDa and isoelectric points between pH 3 and pH 10. The 2 DE unfortunately presents some limits, because it is not possible expecting for all the proteins contained in a cell to be displayed in a 2-D gel. Some proteins with poor solubility will not remain in solution, proteins of molecular weight greater than 150 kDa will not enter the first or the second dimension, and some of them will be present in quantities too low to be visualized. The major limitation is related to the technical difficulties: multiple operations are required to achieve a two-dimensional map, therefore it is necessary a highly specialized personnel to be in charge of these important steps, in order to ensure reproducibility. Some of these problems have been eliminated by the introduction of very sophisticated equipment for the separation in the first and second dimensions that allow to separate many samples simultaneously and to perform fine control on the maintenance of conditions of temperature, and current voltage during separations. The sample preparation influences the effectiveness of electrophoretic separation: in fact a proteome is not a stable mixture, but is constantly changing. The methods of sampling and sample preparation are therefore of critical importance to preserve the protein composition of the tissue from the time of the extraction throughout the rest of the procedure.[80]

A process of preparation of the sample includes four phases:

- Cell lysate;

- Sample solubilization;

- Protease inhibition, to protect the proteins by the action of proteolytic enzymes that would lead to their degradation;

- Impurity removal usually using ultracentrifugation;

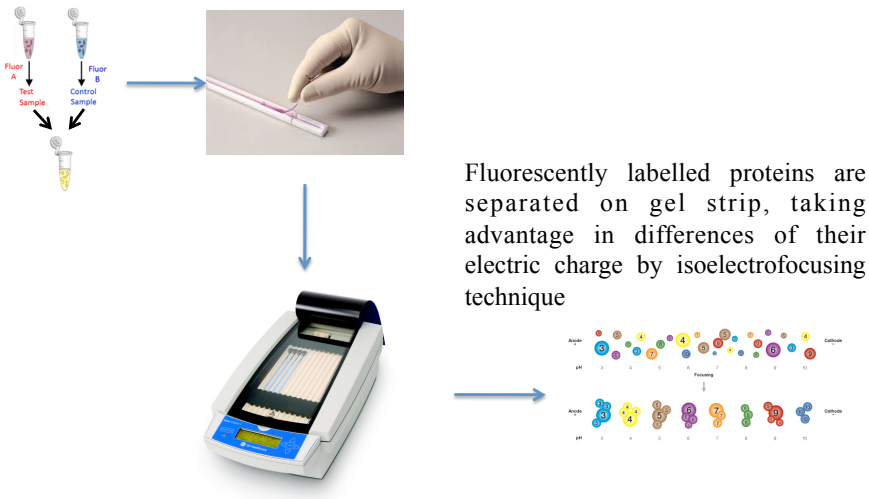
The solubilization procedure must be compatible with the methods of analysis used, which can be based on charge differences of the proteins or on the mass difference. The proteins are treated with a lyses buffer, consisting of a solution containing a mixture of 7M urea and 2M thiourea; this combination greatly increases the degree of denaturation and solubilization of proteins. Crucial importance is the inclusion of non-ionic detergents, such as CHAPS 4% and 1% TRITON X100, solubilization in the solution, to completely remove the hydrophobic interactions of the protein and to allow the solubilization of the greater number of membrane proteins. In fact another critical point of the solubilization process is represented by the difficult solubilization of hydrophobic membrane proteins that tend to congregate in aqueous medium.

The first dimension separation is obtained through an electrophoretic run able to differentiate between the isoelectric points of every single protein; it is a high-resolution method capable of separating proteins with isoelectric points that differ by 0.01 pH units. The basic requirement for a highly reproducible separation is the presence of a stable pH gradient and regular and

constant conductivity. Under the effect of an electric field, a protein migrates along the gradient toward the point at which its net charge is zero. The use of immobilized pH gradient (IPG) strip of thin polyacrylamide, linked to a plastic support, has improved the performance and reproducibility of the separation. The separation is then obtained by applying a potential difference at the ends of a gel containing a pH gradient.[81]

With the introduction of Immobiline DryStrips (GE Healthcare), the methodology and the manipulation have been made much easier and it consents the load of a quantity of proteins up to several milligrams. The Immobiline Dry Strips contain a bar code for easy identification of samples. The conditions of the electrophoretic run must be precisely controlled. The most important parameters are the temperature that must be maintained constant at 20°C and the program of applied voltage, which is dependent on the length and the pH gradient of the IPG strip. During the run the strips are coated with oil to avoid contact with the air and maintain sample under the surface. Through a separation platform (e.g. Ettan IPGphor, GE Healthcare) 12 strips in parallel run are feasible. After separation in the first dimension, the strips may be stored in a freezer at -80°C (Figure 1).

**Figure 1. First dimension Isoelectrofocusing**

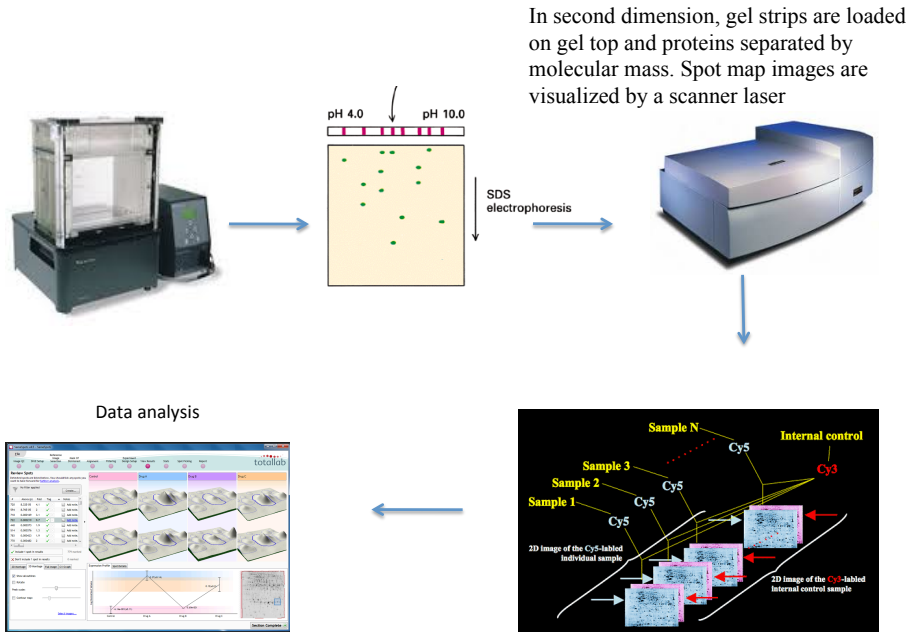



The second dimension separation (SDS-PAGE) is performed according to the protein size. Sodium dodecyl sulfate (SDS) is an ionic detergent. The SDS is added to protein samples leading to their degradation and denaturation from the original three-dimensional state. All the proteins of the mixture are open in a filamentary structure (a molecule of SDS binds every two amino acid residues). The sample buffer also contains a tracer (dye) ionizable, usually the bromophenol blue, that allows following the trend of the electrophoretic run. The SDS-protein complexes, negatively charged, move towards the anode under the applied electric field. The smaller the protein, the easier is the passage through the pores of the gel, while the larger proteins are delayed due to the frictional resistance. The dye bromophenol blue, being a small molecule, is absolutely not delayed representing the migration front. When the dye reaches the bottom of the gel, current is removed; the gel is removed from the two glass plates, kept under stirring for some hours in a dye solution suitable for leaving the proteins visible on a transparent background.

The techniques commonly used for visualization of proteins fractionated on gels are based on the use of dyes that bind indefinitely to any protein.[82]

The introduction of fluorescent reagents with sensitivity similar to previous reagents as silver staining, but with a linear dynamic range of three orders of magnitude, particularly improved the visualization of the proteins. A major step forward in terms of sensitivity has been done with the introduction of the DIGE (Difference In Gel Electrophoresis), a separation procedure described for the first time in 1997 by Unlu and coworkers. This technique uses a two-dimensional electrophoresis as an experimental model, but unlike the classical 2-D the separation is done within the same gel, with two protein extracts labeled with different fluorophores. The fluorophores used as markers are structurally similar and do not affect the mobility and electrical charge of the proteins (fluorescent cyanine dies: Cy2, Cy3 and Cy5). The DIGE is characterized by a degree of sensitivity of 125 pg per single spot of protein, presenting a linear response up to five orders of magnitude. The reproducibility is guaranteed by the presence of an internal standard that represents the protein pattern of all samples that are part of the experiment, and which is separated in the same gel with the sample. The internal standard increases the precision of matching between the gel and consequently the accuracy of the statistical analysis (Figure 2).

**Figure 2. Second dimension SDS-PAGE - DIGE**




The polyacrylamide gel used in this technique are prepared inside of glass sheets with low emission of fluorescence, the concentration of acrylamide being variable from 3% to 20% compared to the total volume of the gel. A concentration of 12% (also used in the present study) offers a good resolution of proteins between 14 and 100 kD. The scanning of the gel at two different wavelengths, which excite the two dye molecules, reveals if each individual spot is associated with a single or double dye. Most of the spots will have fluorescence emission at both wavelengths, but if a spot is associated with a single dye molecule it indicates that the protein is present in only one of the biological samples.[83]

The modern era of proteomics as a method of molecular "fingerprinting" of human tumors was made possible by several technological advances:

- Genomic sequencing (ie, completion of the first phase of the Human Genome Project) and the availability of publicly available databases that catalog known protein and nucleic acid sequences

- Highly sensitive quantitative techniques to detect small amounts of protein in biologic samples

- Laser capture micro-dissection (LCM) technology, which permits the isolation of selected cells out of a tissue section

- Technical advances in mass spectrometry (MS), allowing highly sensitive and specific protein identification both in the solubilized (liquid) state and in the solid state (ie, directly from tissue samples or sections)

Despite these advances, 2-Dimension Electrophoresis (2-DE) remains the foundation of most proteomic studies (particularly investigations of biomarkers in serum), because of its ability to separate relatively large proteins from biologic materials. With this simple technique and subsequent analysis of gel images with specialized software, differences in protein expression (eg, presence versus absence, or intensity of different proteins or isoforms) between healthy and diseased tissues can be revealed.[84] 2-DE fails to resolve most proteins that are larger than approximately 100 kDa or smaller than 5 kDa. Furthermore, 2-DE cannot routinely resolve complex protein mixtures into more than 1000 spots.

Afterwards the proteins of interest can then be identified, typically by Mass Spectrometry-based strategies.

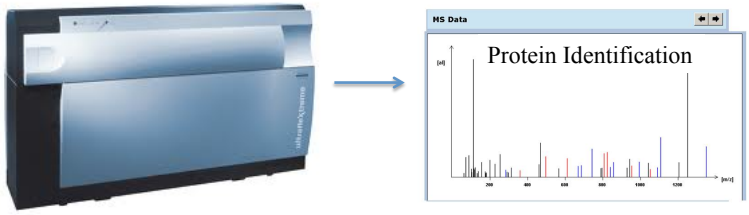
After the 2D-DIGE gels analysis the process passes to the identification of all proteins or only those of specific interest; in most cases, this is done by means of peptide mass fingerprint.

The main aim of a proteomic study is to characterize all the proteins expression and the protein identification is the essential endpoint. Traditionally the protein patterns in separate 2D gels were identified by comparison with protein maps available in reference databases or through recognition by specific antibodies. Important progresses in the identification of the proteins were made thanks to the development of the techniques of MS. A mass spectrometer is a highly accurate instrument that can separate individual proteins of very similar molecular weight or mass. MS requires a smaller amount of material and has a higher throughput than other sequencing methods for protein identification. The goal of applying MS in clinical proteomics is to generate protein profiles (mass:charge [m/z] ratio versus signal intensity) from body fluids (eg, serum, saliva, urine) or tissue samples to detect quantitative and qualitative changes in protein expression levels that correlate with the disease states.

MS operates in the gas phase, and peptides thus must first be ionized; the development of newer, gentler ionization methods has led to breakthroughs in the application of MS to biotechnology and clinical proteomics. Ionization can be accomplished by electrospray ionization for solubilized (liquid state) samples, or by laser desorption techniques for samples in the solid state. Therefore a “time of flight” analyzer measures the ions mass. In simple terms, the MS instrument accelerates the ionized proteins across a fixed voltage potential in a high vacuum, measuring the amount of time (the "time of flight" or TOF) required for the ion to hit a detector (Figure 3). This allows the molecular weight or mass of a single positively charged

molecule to be accurately measured and displayed according to their mass: charge ratio (m/z).[85]

**Figure 3. MALDI-Tof mass spectrometer**

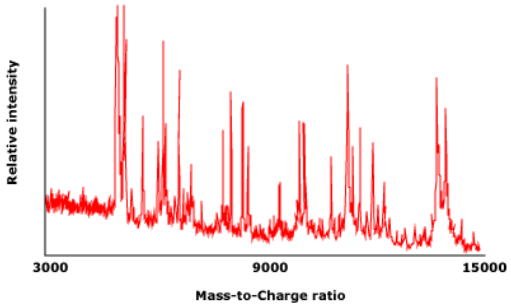



Cutting individual spots from the gels can identify proteins that are separated on 2-DE gels. The excised protein spot is digested with trypsin and separated into specific amino acid sequences and broke down into a mixture of peptides. The peptide fragments produced by trypsin digestion of any protein have a unique mass that can be measured accurately with MS to produce a peptide mass "fingerprint". This protein signature or fingerprint is then compared with the peptide masses predicted from theoretical digestion of protein sequences contained within published databases such as SWISS-PROT,[86] thus allowing identification of the protein from which the fragments originated.[87] Its high sensitivity and the possibility of analysis of complex mixtures have made this technique a method in common use in the characterization of peptides isolated by electrophoresis or chromatography. Mass spectrometry has proved to be effective not only in the identification of proteins but also in the determination of post-translational modifications, and more recently also in the context of relative quantification via labeling of proteins.

Complementary technologies have been developed able to apply the high sensitivity and specificity of MS to rapid and direct mapping and imaging of biomolecules that are present in body fluids or tissue sections. The available techniques, SELDI-TOF MS (surface-enhanced laser desorption ionization time of flight mass spectrometry) and MALDI-TOF MS (matrix-assisted laser desorption ionization time of flight mass spectrometry), use a laser to desorb and ionize proteins from a solid surface and thus are called "laser desorption" techniques. MALDI-TOF MS does not require solubilization or pre-separation of tissue samples. Using this technique, high resolution mass spectrograms can be obtained directly from solid phase samples (e.g., in tissue sections).[88,89] In a typical procedure, a 12-micron frozen tissue section is freeze-dried onto a conductive MALDI plate, and coated with a solution of a matrix, usually a low molecular weight crystalline compound such as a cinnamic acid analog. When dry, the sample is introduced into the vacuum inlet of a mass spectrometer, and then irradiated with a finely focused laser beam from a 377 nm N2 laser. The beam, which is approximately circular with a diameter of 25 microns, is directed at regions of interest in the tumor, which are selected by examining adjacent tissue sections that are processed for conventional light microscopy (hematoxylin and eosin, HE). Each consecutive spot produces a mass spectrum that reflects the molecules that are present within the irradiated area. For most tissue sections, over 200 protein and peptide peaks are found in the mass spectrum from each spot ablated by the laser. Application of the laser, acquisition of data, download to a computer, data processing, and repositioning of the sample stage are all automated, and the procedure is rapid. Samples can be obtained from well-defined regions (e.g., normal tissue, malignant tissue, necrotic areas, the leading edge of a tumor), many of which have unique subsets of proteins and peptides. Images can then be created of different patterns of

protein expression by integrating the peak areas and plotting the relative values using a color scale (Figure 4).

**Figure 4. Representative matrix-assisted laser desorption ionization time-of flight mass spectrography (MALDI-TOF MS) protein spectrum obtained directly from human lung tumor tissue**




MALDI-TOF MS is used to generate protein profiles in both linear and reflector modes, with subsequent MALDI-TOF MS to identify low molecular weight serum peptides in the protein profile with high spectral resolution.[90] On the other hand, due to the presence of matrix ions, one of the limits of this procedure is the loss of sensitivity in the higher region of the protein size.

Mainly for the conventional proteomic procedures the following step is often represented by the analysis through a so called “validation technique”, which aims to verify the real over- or under-expression of a particular protein through universally accepted methodologies like western blotting, immunohistochemistry and/or tissue microarray immunohistochemistry. Nonetheless very recently, direct quantification techniques has been successfully proposed.[91]

Concerning the possible application of molecular technologies to clinical oncology, it has to be considered that despite significant progress in the field of DNA microarrays and gene expression profile, there are some limitations in using a genomic (nucleic acid) approach to molecular tumor

classification. This is the reason of the growing interest on proteomics. In fact proteins rather than nucleic acids represent the functional output of the cell and RNA expression is often not tightly correlated with protein expression.[92]

Moreover genomic analysis does not detect posttranslational changes such as proteolytic processing, phosphorylation or glycosylation, which can alter the activity of a protein. These posttranslational modifications are often critical in various signaling pathways involved in tumorigenesis.[93]

Compared to the study of DNA or RNA expression patterns, analysis of protein expression may provide a more accurate understanding of the molecular complexities of human tumors.[94]

Preliminary reports of the analysis of protein expression profiles in oncologic patients using 2-DE could describe only differences in patterns of protein expression rather than specific proteins.[95] Separated proteins could only be identified by co-migration, specific antibody labeling, or chemical microsequencing, all of which were time-consuming and technically demanding. Because there are no simple methods for protein amplification, highly sensitive methodologies needed to be developed to identify low-abundance proteins that are important in the malignant process. Although 2-DE is a powerful tool for the study of differences in protein expression that characterize cell types or tissues, it is difficult to apply this technique to large numbers of clinical samples.[96] The number of unique proteins contained in a tumor is higher than the number of spots detectable with 2-DE. Moreover the study of lysates from cell lines and human tissues with the aim of identifying "tumor-specific" changes in protein expression is limited by the heterogeneity of human tumors, the inclusion of stromal, normal cells, and premalignant lesions along with tumor in many samples.

The result of a proteomic analysis is therefore the correlation of

changes in protein expression with a given phenotype. Once a protein (or biomarker) "signature profile" has been identified, results must be validated in independent, large study sets. This requires high throughput technology, consenting the analysis of hundreds of cancer tissue samples at a time. The identified proteins may represent important biomarkers for early diagnosis, yield valuable information about the disease process and represent targets for novel therapeutic intervention, or even provide surrogate markers for therapeutic efficacy or treatment-related toxicity. Preliminary data suggest that MALDI-TOF MS analysis of protein expression profiles from frozen sections of human surgically resected lung tumors (non-small cell lung cancers, NSCLC) allows the separation of tumors according to histologic type, providing potentially useful prognostic information.[97,98]

In one of the preliminary reports of proteomics' application to clinical oncology, protein spectra were obtained from 79 lung tumors and 14 normal lung tissues. A model was established using 42 lung tumors and eight normal samples, which was then validated on a blinded test cohort of 37 lung tumors and six normal lung samples. Using this approach over 1000 distinct protein species could be analyzed from less than one nanogram of each tissue sample. The protein spectra of lung cancers and normal lung resulted different with peaks in the tumor samples higher than in normal lung, and clearly distinguishable from background noise. A discriminatory pattern of protein expression was also demonstrated when lung adenocarcinoma and squamous cell carcinoma were compared and when lung primary tumors were compared to pulmonary metastasis. These proteomics results were shown having prognostic value as well generating 15 distinct MS peaks that could distinguish between patients with good or poor prognoses. This association was present even after adjustment for stage and histologic grade.[97]

A commercially available serum proteomic MALDI-TOF based classifier (VeriStrat) was adopted for stratifying and predicting survival after treatment with erlotinib in a blinded fashion to pretreatment sera from recurrent advanced NSCLC patients. VeriStrat was found to be able to classify these patients into two significantly different outcome groups that had "better" and "worse" outcomes.[99]

Proteomics demonstrated a good clinical-scientific impact also in urologic oncology. In fact the well demonstrated lack of specificity of serum PSA for discriminating between prostate cancer and benign prostatic hyperplasia (BPH) limits its utility for early diagnosis. Several reports already suggested that serum proteomics might provide a better biomarker for early detection of prostate cancer.[100]

One of the first studies that used a serum SELDI MS to analyze serum proteins and differentiate prostate cancer from generated a serum protein profiles that allowed developing a decision tree classification algorithm, which correctly classified 96% of the samples. In a subsequent blinded validation set, this approach still showed 83% sensitivity and 97% of specificity.[101]

In hematology a study from peripheral blood samples of 57 adults with a confirmed diagnosis of acute lymphoblastic leukemia could successfully provide a decision tree based on proteomics providing a predictive value of 84 to 92 percent. Interestingly recurrence prediction was independent of cytogenetics, marrow blast count, and serum level of lactate dehydrogenase, beta-2 microglobulin or surface marker studies.[102]

Potential biomarkers have been identified by proteomics in many other cancers so far: Olsen and coll. recently demonstrated that antigens expression

profile of breast cancers could represent potentially a high specific biomarker;[103] in ovarian cancers MALDI imaging mass spectrometry identified a protein (*PA28* or *Reg-alpha*) whose specific antibody could be used to discriminate the borderline tumor cases.[104]

As for endocrine tumors and disease, the application of 2D-DIGE has been successfully demonstrated in several studies on thyroid tumors. Despite the tremendous effort that have been made in deciphering alterations in gene and protein expression pattern of cold thyroid nodules, the ultimate link to the clonal molecular offspring of a cold thyroid nodule is far from being understood. Nonetheless some different expression between papillary thyroid carcinomas (PTC) and normal thyroid tissue has been already found. Of all overexpressed proteins in PTC, the protein S100A6 displayed the highest volume ratio between PTC and normal thyroid (6.5-fold). This protein was also used for validation of the proteomic approach by immunohistochemistry on independent samples of benign and malignant tumors reaching 85% sensitivity and 69% specificity for the discrimination between benign and malignant thyroid neoplasms.[105]

Interesting data have been obtained also by proteomic analysis of pituitary adenomas: Ribeiro de Oliveira and coll. studied a series of 20 secreting and non-secreting pituitary adenomas comparing their protein profile with normal pituitary tissue. Between the differently expressed proteins (many of them never disclosed before), particular attention was given to proteins possibly involved in the pathogenesis of the pituitary adenomas (HSP110, B2 bradykinin receptor, CSK and annexin II): all the data but one (CSK) were confirmed by western blotting being HSP110 significantly overexpressed in prolactinomas and non functioning pituitary adenomas, B2 bradykinin receptor significantly overexpressed in prolactinomas and annexin

II significantly under-expressed in somatotrophinomas.[106]

As for adrenal adenomas and carcinomas, although many molecular studies addressed to describe genome and transcriptome profiling of these tumors have already been published with noteworthy results, few data are available at present on adrenocortical tumors proteomic profile.[107,108,109] Very recently Kjellin and coll. compared the microsomal protein composition of eight malignant and six benign adrenocortical tumors with proteomic methods. The IGF2 increased level in ACCs confirmed previous microarray studies. On the other hand some enzyme and proteins belonging to mitochondrial respiration chain and involved in tumor metabolism, showed different expression between malignant and benign/normal adrenal tissue. This may indicate a shift in energy metabolism where glycolysis may be favored (Warburg effect).[110] One of these proteins (GRIM-19, Tumor Suppressive Protein Gene Associated with Retinoid-Interferon-Induced Mortality), under expressed in ACCs, has already been identified in other malignancies and seems to be able to interact with STAT3 with regulatory role, as suggested by Li and coll.[111] Moreover other authors have also reviewed the role of GRIM-19 in cancer development: some of these studies could demonstrate an involvement of GRIM-19 also in endocrine tumors (e.g., Hurtle cells thyroid adenomas).[112,113]

Although performed on cell lines lysate and not on fresh human tissues, another piece of the puzzling proteomic profile of adrenal tumors have been found by Stigliano and coll.: the authors showed that mitotane (the unique adjuvant therapy in adrenal cancer available to date) could affect proteome in an adrenal malignant tumor cell line. The data allowed identifying some classes of proteins involved in energetic metabolism, stress reaction, cytoskeleton structure and tumorigenesis.[114]

# AIM OF THE STUDY

The main purpose of this study is to improve the diagnostic process of malignant and benign adrenal tumors trying to provide the pathologist with a morphometric valuable and reliable model that could be easily integrated into the present diagnostic algorithm of the disease. We decided to develop a computer vision method for IHC analysis and to compare the method to visual interpretation of IHC staining. A highly studied biomarker (Ki-67) with known prognostic value in many cancers (also adrenal cancer) was chosen as target. In this study, a tool for semi-automated quantitative assessment of Ki-67 expression is presented. The IHC quantification method was evaluated by comparing the results with visual assessment of Ki-67 expression in a comprehensive series of adrenocortical adenoma and carcinoma specimens. Moreover the same morphometric model was applied also for the quantification of reticulin staining and inflammatory infiltration.

The secondary aim of this study was the individuation of sensitive biomarkers with a possible diagnostic and prognostic role: immunohistochemistry and proteomics evaluation should consent the individuation of different subtypes of benign and malignant lesions contributing to a better characterization of pathogenic mechanisms and disease progression. The identification of novel biological markers suggestive of malignancy would afford an earlier diagnosis of ACCs and the development of potential target therapies would lead to increased likelihood of long-term survival for the patients affected. A better definition of peculiar subtypes of adrenal lesions would also allow an earlier identification of silent benign tumors with secreting attitude before any irreversible metabolic

damage would be established, with a significant impact also on healthcare costs.

Eventually data obtained by morphometry and proteomic signature of ACCs and ACAs could be integrated in future with clinical-surgical-radiological and histological data trying to explain the inconsistencies often found among the histological data and the clinical course of the disease and possibly providing an accurate prognostic stratification of these heterogeneous tumors.

# METHODS

## Morphometric Model

In this study, we examined retrospectively 11 ACA (median size= 6.2 cm, range 5-16 cm) and 18 ACC (median size= 11 cm, range 4-19 cm). Specimens were obtained from the archival files of 29 patients (9M/20F) submitted to adrenal surgery at the Division of Minimally Invasive and Oncological Surgery, Niguarda Hospital in Milan (Table 4). All patients were followed at the Department of Endocrinology at the same hospital. For all samples (for morphometric model and also for proteomic study, see below) informed consent of the patient was obtained after the approval of the Hospital Ethical Committee.

**Table 4**. Characteristics of patients.

| Lesion | Dimensions (cm) | Age | M/F |
|---|---|---|---|
| **ACA** | 4-11 | 28-59 | 4/7 |
| **ACC** | 5-19 | 25-68 | 5/13 |

ACAs and ACCs were defined grossly and microscopically following the criteria and the nomenclature system of pathologic features proposed by Weiss et al. (see above). All primary malignant tumors reviewed as part of this study demonstrated three or more of the histologic criteria needed for the diagnosis of ACC as defined by Weiss score.

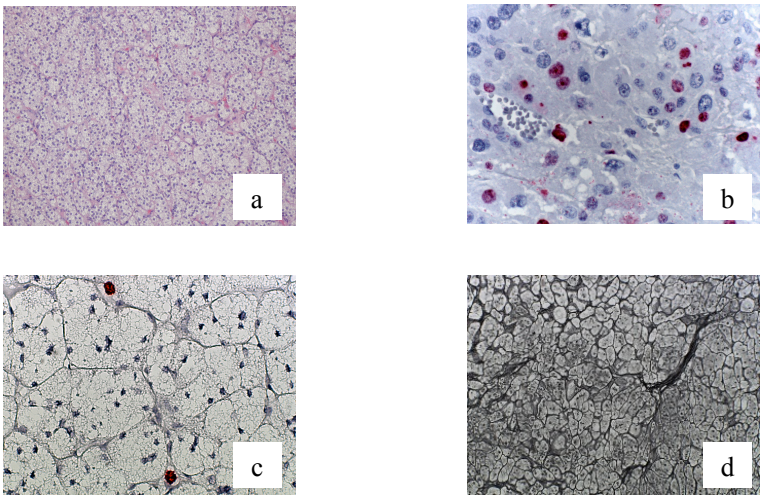
Two experienced pathologists without the pathologist's knowledge of the patients' clinical characteristics or outcomes reviewed all specimens.

In all cases investigated, 2-cm3 blocks were obtained, then immediately formalin fixed soon after the receipt of the adrenal specimen and embedded in paraffin. For each case, three consecutive sections 4 μm thick were obtained from the corresponding block.

Each section series was stained with different methods (see Figure 5):

- Hematoxylin-Eosin (HE) to confirm the diagnosis of adrenal nodular lesions.
- Monoclonal antibodies against Ki67 (Rabbit anti-human ki-67 monoclonal Ab Clone SP6, 1:400 - Thermo Fisher Scientific, CA - USA) to assess volume fractions of KI67 negative and positive cells (nuclei and cytoplasm), nucleus\cytoplasm (N\C) ratio both for marked/unmarked cells
- Monoclonal antibodies against CD8 (mouse monoclonal anti-CD8 1:100, Abcam-USA), a two chain glycoprotein that is expressed on the surface of circulating T-cells.
- Monoclonal antibodies against CD15 (Mouse monoclonal anti-CD15 clone G10F5, BD Pharmingen™-USA), a glycosyl-phosphatidyl-inositol linked protein expressed on granulocytes.[115]
- Silver impregnation for reticulin fibers using the Gordon and Sweets method to assess the cell plate architecture.[116]
- Blood and lymphatic vessels were also identified.

**Figure 5. Material and Methods: Hematoxylin-Eosin (HE, a, 10x), immunostaining for Ki-67 (b, 40x), CD15 (c, 40x), reticulin (d, 10x)**




The variables assessed by morphometry are listed in Table 5 and include cellular compartment, fibrous stromal, and vascular supply in both groups of lesions.

**Table 5**. Morphometric model.

| |
|---|
| **Ki-67 (40x magnification)**: **Volume fractions referred to the entire tissue occupied by** |
| nuclei of Ki-67 negative cells (Vvnucneg) |
| cytoplasm of Ki-67 negative cells (Vvcytneg) |
| nuclei of Ki-67 positive cells (Vvnucpos) |
| cytoplasm of Ki-67 positive cells (Vvcytpos) |
| ratio nuclei/cytoplasm of Ki-67 negative cells (N/Cneg) |
| ratio nuclei/cytoplasm of Ki-67 positive cells (N/Cpos) |
| other structure (vessels and inflammatory infiltrate) (Vvother) |
| **Inflammatory infiltration (40x): Volume fractions referred to the entire tissue occupied by** |
| T lymphocytes (VvCD8) |
| Granulocytes (VvCD15) |
| **Reticulin (10x): surface fraction (surface in unit volume) of reticulin** |
| Reticulin (Svret) |

During the analysis of the data we decided also to create a secondary category of cell volume fraction (see results): Vvcellpos, meaning the Volume fraction of Ki-67 cells positive referred only to cell tumor compartment (vessels and blood cells excluded).

The morphometric analysis was performed at two magnification levels using an interactive approach with a high-resolution computerized image analyzer (Kontron-Zeiss KS 400) that included a color video camera (JVC TK-C1381EG) attached to a light microscope equipped with a motorized stage with 10X and 40 X objectives and auto-focusing software. The software

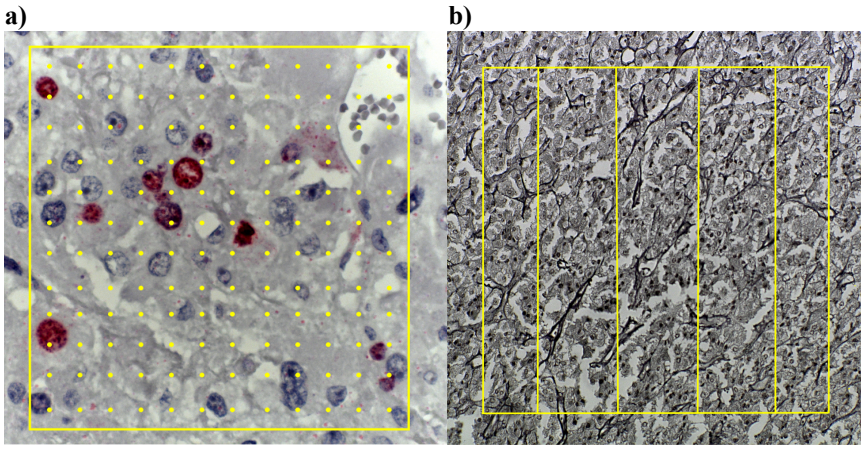
system, tailored on the research needs of our team, consisted of different programs to control interactively the scanning stage and autofocus functions.

The analysis works as follows: images are acquired by the video camera and displayed on the monitor. The analyzer automatically superimposes to each microscopic field different grids of points and lines, included in a test area 504 x 504 pixels, allowing an evaluation of the stereological variables.[117]

The observer can interactively apply techniques of enhancement for a better definition of the different structures. It is also possible to exclude fields in which the tissue section may not be suitable for analysis due to technical artifact. An algorithm automatically controls the scanning stage operation in order to avoid duplicate measurements of the same structures.

Two different grids have been used: a 144-points square lattice to evaluate the volume fractions of the components investigated and a 4-lines grid to evaluate reticulin surface (Figure 6a and 6b). To examine the microscopic fields, two objectives 10X and 40 X have been used.

**Figure 6. a) 144-points square lattice to evaluate the volume fractions of the cellular compartment of the tumors, b) a 4-lines grid to evaluate reticulin surface in the tumors.**



On stained sections at 10X magnification surface area of reticulin was evaluated by superimposing on each microscopic field displayed on the monitor a grid of lines: surface densities were calculated by differential intersection counting. The grid was then rotated by 45°, 90°, and 135° and counts repeated each time. On stained slices at 40X, volumetric analysis of Ki-67 negative and Ki-67 positive cells was performed by differential point counting[118] and more than 250 microscopic fields systematically selected were examined. During point counting procedure, a single experienced operator blinded to pathologist's diagnosis or clinical history identified the different structures.

Ki-67 assessment by morphometry was compared with the histological semi-quantitative scoring by regression analysis.

Lastly, to assess the reproducibility of our morphometric model, 10 specimens were randomly selected from the entire pool of specimens, reassessed as above by another blinded operator and compared to the initial counting.

Statistical Analysis: SPSS 21.0 software were used for statistical analysis. For each parameter, a comparison between the two groups of lesions has been performed by variance analysis. Statistical significance was established at the $p<0.05$. Furthermore, regression analysis was used to test reproducibility of the morphometric analysis, according to the results obtained by two blinded operators.

## Proteomic Expression Analysis

The 2D fluorescence differential gel electrophoresis (DIGE) technique represents to date the most sophisticated and straightforward approach to discovering protein patterns that distinguish disease and disease-free states with high sensitivity and specificity. In a DIGE system, experimental and control samples and an internal standard (consisting of equal protein amounts of all samples) are labeled with spectrally resolvable fluorescent dyes before isoelectric focusing. Subsequently, the pre-labeled samples are resolved within the same analytical 2D gel run. The multiplexing of different samples allows the direct quantitative measurement of experimental and control samples co-migrating within one 2D gel. In addition, the normalization of all 2D images to the common 2D image of the internal standard permits inter- and intragel matching as well as the normalization of spot volumes across different gels. Thus, each spot has its own standard, which allows the direct quantitative measurement of the differentially labeled experimental and control sample and the equivalent protein signal from the standard. The sensitivity of commercially available fluorescent dyes is comparable to that of silver stain. Furthermore, because of its high linearity and wide dynamic range, the use of CyDyes is a valuable tool for quantitative proteomics.[118,119] More in detail:

### Sample preparation

Frozen tissue samples from 7 ACCs, 5 ACAs and 5 normal adrenal glands (surgically removed for other non influencing-contaminating disease) with were grounded by mortar and pestle in dry ice, and an aliquot of each sample powder was solubilized in lyses buffer containing: 30 mM Tris-HCl

pH 8.5, 7 M Urea, 2 M Thiourea, 4% CHAPS , 0.1% Triton X100 and protease inhibitor. In order to remove contaminants, lipids, salts, detergents and other non IEF compatible reagent, sample extracts were treated by 2D clean-up kit (GE Healthcare). The proteins were than re-suspended in lyses buffer and protein concentrations were determined using 2D Quant Kit (GE Healthcare).

**2D-DIGE**

First of all we have to consider that Two Dimensional Gel Electrophoresis (2D-DIGE) is a modification of 2-DE technique in which protein samples, labeled with different fluorescent dyes, are separated in the first dimension according to their isoelectric point and in the second dimension by their molecular mass.

The gels obtained were scanned with excitation wavelength of each dye and detected using fluorescent imaging scanner. Protein minimal labeling reactions were performed: 50 μg of protein extract from each sample, were labeled with 400 pmol of Cy5 dye (CyDye, GE Healthcare), while an internal standard, generated by pooling an aliquot of all sample protein extracts, was labeled with Cy3 dye according to manufacture's recommendations. For each 2D-DIGE gel, 50 μg of a Cy5-labled sample and 50 μg of a Cy3-labled pool were mixed and the final volume brought up to 250 μL with rehydration buffer (7 M urea/2 M thiourea/4% CHAPS/40 mM Tris- HCl, pH 8.8) containing 1% IPGphor ampholytes, pI range 3-10 (GE Healthcare), 30 mM dithiothreitol (DTT) and a trace of bromophenol blue. 24 cm IPG DryStrip pH 3-10 non linear gradient (GE Healthcare) were rehydrated and focused on an Ettan IPGphor II (GE Healthcare) using the following protocol: 6h at 30 V, 6h at 60V, 2h at 200V, 2h at 500V, 2h at 1000 V, 1.5h at 2000V, 1.5h at 3000V,

4h gradient 3000-8000V, 5h at 8000V. The next day, DryStrips were placed at −80 °C.

DryStrips were thawed at RT for 15 min, equilibrated in SDS-sample buffer (6 M urea, 75 mM Tris-HCL, pH 8.8, 20% glycerol, 2% SDS, 65 mM DTT) for 15 min and then for 8 min in SDS-sample buffer containing freshly added iodoacetamide (135 mM). Each strip was then placed on top of a 12% constant polyacrylamide SDS-PAGE gel. Second-dimension separation for all gels was carried out in Ettan Dalt separation unit (GE Healthcare) overnight at 20°C, until bromophenol blue reached the bottom of the gel.

After electrophoretic separation, CyDy labeled gels were scanned on a Typhoon imager 9200 (GE Healthcare) using the specific laser band-pass filters for each dye's excitation and emission wavelengths, generating overlaid multi-channel images for each gel. Each gel was scanned individually and Photo Multiplier Tube (PMT) voltages were adjusted for maximum image quality with minimal signal saturation and clipping. Images were cropped and exported as 16-bit GEL files using ImageQuant TL (GE Healthcare) and imported into SameSpot Progenesis (Non Linear Dynamics).

**Images and statistical analysis**

All images are loaded in the analysis software, which automatically detects and matches spots and normalizes signals using the info contained on the internal standard, which is identical on every gels prepared. The internal standard is a balanced mix of all samples included in the experiment and it allows the representation of all spots present in samples also in the reference. Only proteins with spot volumes consistently different in all replicates will be considered differentially expressed. Statistical analysis was performed using traditional paired or non-paired T-test or Anova's to calculate p-Value

associated with distribution of spots signal across groups of samples to be compared. Other type of supervised and non-supervised classification methods can be applied with PCA, hierarchical clustering, and single decision tree.

**Protein identification by Mass Spectrometry**

Protein spots, selected by statistical analysis, were automatically picked from preparative gels by Ettan Spot Picker (GE Healthcare) and transferred to 96-well plates. Spots were destained in 50% methanol/50 mM ammonium bicarbonate and incubated with 30 mL of 4 ng/mL trypsin (Promega) dissolved in 10 mM ammonium bicarbonate for 16 h at 371C. Released peptides were subjected to reverse-phase chromatography (Zip-Tip C18 micro, Millipore), eluted with 50% ACN/1% formic acid. Briefly, 1 mL of peptides mixture was spotted onto the sample plate of an Ultraflex III MALDI-ToF/ToF (Bruker Daltonics) mass spectrometer; an equal volume of 10 mg/ mL CHCA matrix dissolved in 70% ACN/30% 50 mM citric acid was applied and spots were air dried at room temperature. MS proceeded at an accelerating voltage of 25 kV and spectra were externally calibrated using Peptide Calibration Standard mixture (Bruker Daltonics); 1000 laser shots were taken per spectrum. Proteins were identified by comparing the digest peaks with a computer-generated database of tryptic peptides from known proteins using MASCOT, which utilizes a robust probabilistic scoring algorithm. Search was carried out by correlation of uninterpreted spectra to Homo sapiens entries in NCBI Data-base. With regard to MASCOT parameters, one missed cleavage per peptide was allowed and carbamidomethylation, as fixed modification, and methionine oxidation, as variable modification, were set. Peptide mass tolerance was set at 30ppm. Proteins were identified by searches of a mammalian subset of the SWISS-

PROT and TrEMBL databases.

# RESULTS

## Morphometric model:

Results of the morphometric study are shown in Tables 6-9 and in Figures 7–10.

On Ki-67 stained sections, the volume fraction of nuclei in unit volume showed highest values in ACC both in Ki-67 negative cells (ACC .12046, ACA .06637) and in Ki-67 positive cells (ACC .01227, ACA .00104).

The volume fraction of Ki-67 negative cells was highest in ACA (ACC .71031, ACA .78537); on the contrary, the volume fraction of Ki-67 positive cells was highest in ACC (ACC .02988, ACA .00503).

When considering N/C ratio, ACC showed the highest values in both Ki-67 negative cells (ACC .20535, ACA .09260) and Ki-67 positive cells (ACC .68022, ACA .27281).

The volume fractions of the other compartments of the lesion (vascular structures, inflammatory infiltrate) were higher in ACC (ACC .25969, ACA .20963).

**Table 6. Morphometric variables evaulated by Ki-67 immunostaining for the different lesions (mean±standard deviation; degrees of freedom 1; 27)**

| Variables | Dimension | ACA | ACC | p | F |
|---|---|---|---|---|---|
| Vvnucneg | mm3/mm3 | .06637±.0153 | .12046±.0226 | <.0001 | 45.718 |
| Vvcytneg | mm3/mm3 | .71900±.0295 | .58985±.0571 | <.0001 | 52.973 |
| Vvnucpos | mm3/mm3 | .00104±.0004 | .01227±.0088 | <.0001 | 20.656 |
| Vvcytpos | mm3/mm3 | .00399±.0011 | .01771±.0094 | <.0001 | 26.409 |
| Vvcelneg | mm3/mm3 | .78537±.0304 | .71031±.0656 | <.001 | 15.209 |
| Vvcelpos | mm3/mm3 | .00503±.0013 | .02988±.0186 | <.0001 | 24.304 |
| Vvother | mm3/mm3 | .20963±.0299 | .25969±.0155 | <.05 | 6.366 |
| Vvcellpos* | mm3/mm3 | .00639±.0017 | .04039±.0245 | <.0001 | 20.82 |
| N/C neg | | .09260±.0228 | .20535±.0396 | <.0001 | 69.151 |
| N/C pos | | .27281±.1449 | .68022±.1693 | <.0001 | 44.493 |

Volume fractions referred to the entire lesion in the test area = 504x504 pixels occupied by nuclei and cytoplasm respectively in Ki-67 cells negative ($V_v$nucneg and $V_v$cytneg) and Ki-67 cells positive ($V_v$nucpos and $V_v$cytpos), vascular and inflammatory infiltrate ($V_v$other), Ki-67 cells negative and positive ($V_v$celneg and $V_v$celpos); N/C represents the nuclei/cytoplasm ratio in both type of cells.

*$V_v$cellpos: Volume fraction of Ki-67 cells positive referred to cellular compartment.

The volume fractions of inflammatory infiltration were higher in ACCs than in ACAs (CD15: ACC .00312, ACA .00098; CD8: ACC .01070, ACA .00356). The surface fraction of reticulin was significantly lower in ACC (SvRet: ACC 10.43, ACA 37.24).

**Table 7. Morphometric variables evaluated by CD15/CD18 and reticulin immunostaining for the different lesions (mean±standard deviation; degrees of freedom 1; 27)**

| Marker | Dimensions | ACA | ACC | p | F |
|---|---|---|---|---|---|
| $V_v$CD15 | $mm^3/mm^3$ | .00098±.0004 | .00312±.0007 | <.0001 | 60.231 |
| $V_v$CD8 | $mm^3/mm^3$ | .00356±.0012 | .01070±.0094 | .068 | 3.802 |
| $S_v$ret | $mm^2/mm^3$ | 37.24±9.29 | 10.43±5.89 | <.0001 | 60.019 |

Volume fractions occupied by CD15 granulocytes ($V_v$CD15), CD8 T-lymphocytes ($V_v$CD8), and surface fraction occupied by reticulin ($S_v$Ret).

**Figure 7. Volume fractions of nuclei and cytoplasm respectively in Ki-67 negative cells (Panel A) and Ki-67 positive cells (Panel B), in both lesions.**

**Panel A** **Panel B**

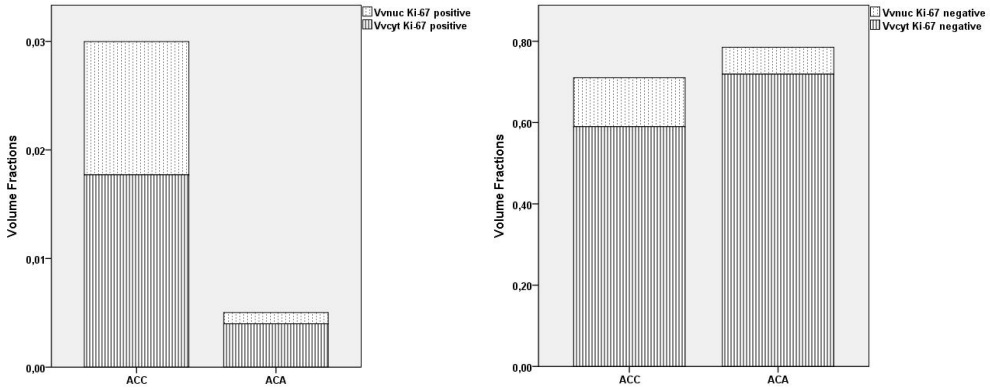



**Figure 8. Linear regression of pathologist assessment of the percentage of Ki-67 positive cells on the morphometric evaluation. When comparing morphometric analysis to pathologist's scores, the data of the point grid analysis revealed significantly lower values with respect to conventional histopathology, as shown by the regression line.**

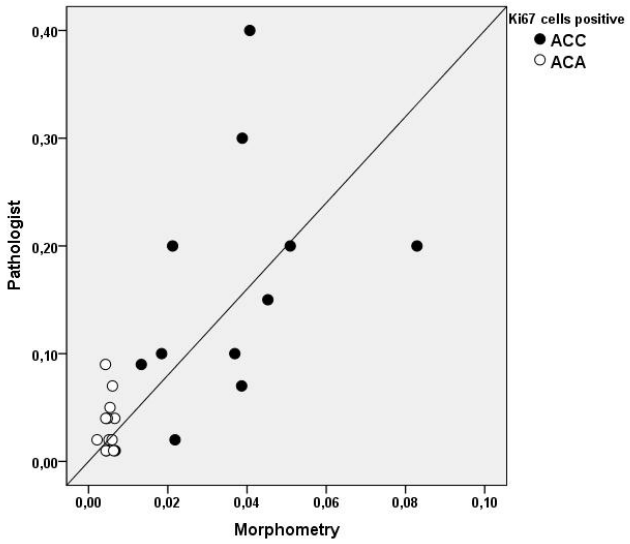


**Figure 9. Surface fraction of reticulin for each lesion investigated**

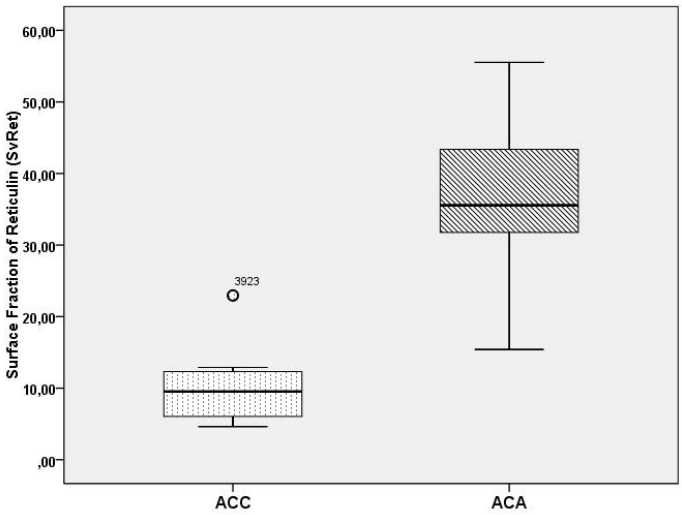



**Figure 10. Volume fractions of CD15 and CD8 positive cells for both lesions**

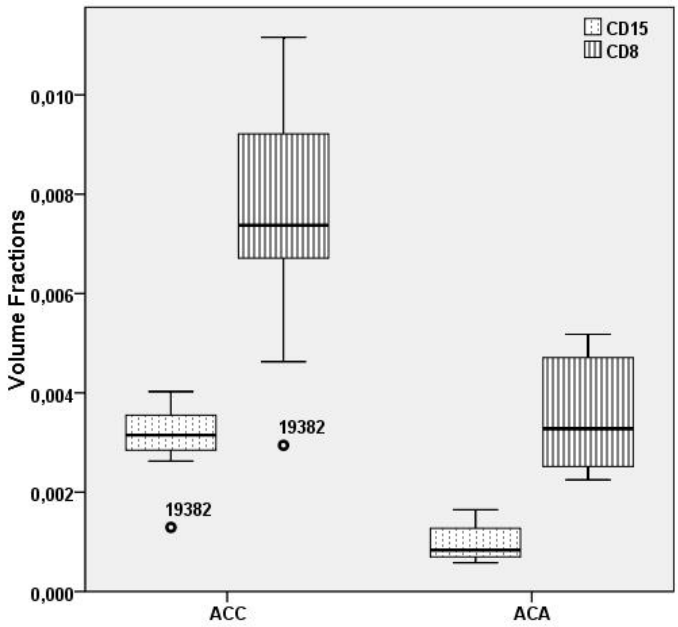


**Table 8. Statistical analysis of inter-observer agreement on morphometric analysis: 10 randomly selected specimens are compared with initial point counting and with differential intersection counting. F represents the variance ratio related to the source of error for each evaluation by the two operators.**

| | $V_v$nuc neg | $V_v$cyt neg | $V_v$nuc pos | $V_v$cyt pos | $V_v$oth | CD15 | CD8 | $S_v$ret |
|---|---|---|---|---|---|---|---|---|
| F | 0.654 | 0.587 | 0.102 | 0.447 | 0.091 | 0.895 | 0.065 | 0.754 |
| *p* | 0.321 | 0.654 | 0.729 | 0.573 | 0.528 | 0.367 | 0.619 | 0.301 |

Finally we also evaluated the prognostic value of new ENSAT staging classification in the same series of ACCs comparing our findings to tumor Weiss score and ki-67 expression (both from pathologist visual evaluation and morphometric approach), with regard to the disease-free and disease-specific survival. Clinical and pathological data of the 18 pts affected by ACCs (14F, 4M, median age 52 yrs, range 25-78) surgically treated, were retrospectively reviewed; the follow up period was 26 months (median, range 6-192). ACC staging was performed accordingly to ENSAT classification; tumor malignancy was assessed accordingly to Weiss system. A multivariate analysis of disease-free and overall survival in ACC according to Weiss score and ki-67 was performed.

The 70% of tumors were hypersecreting; mean size was 10,7cm (range 50-2500), mean weight was 512g (range 50-2500). In our series the visual evaluation of the pathologist led to consider a Ki-67 greater than 7% (sensitivity 85,7%, specificity 62,5%, ROC analysis) and Weiss score>4 (sensitivity 100%, specificity 50%) were the best cut-off values suggestive for a poor prognosis (Kaplan-Meyer, p=0,06 and p=0,04 respectively). Taken together the two risk factors showed a significant effect on survival probability (Cox-regression, p= 0,04). Ki67>7% and Weiss score>4 showed a

slight correlation also with disease-free survival (Kaplan-Meyer, p=0,06 and p=0,04 respectively); when both risk factors were concomitantly present, the correlation with probability of disease recurrence was higher (Cox-regression, p=0,02).

When we applied our morphometric model, the Ki-67 cutoff value decreased to 1,2% with a sensitivity of 94% and a specificity of 100% maintaining the same significant correlation with the disease outcome.

**Proteomics expression study:**

After adequate selection of the protein spots obtained by DIGE, taking into account also some technical pitfalls (e.g. some of the spots was not removable from the gel, the total amount of some proteins was not enough to perform the following step, the MS) we could detect 30 spots with significant differential expression in ACCs versus ACAs and 45 protein spots differently expressed between ACCs and normal adrenal tissue. Many of these proteins have been also identified by mass spectrometry; some of them are still on study. As for the interpretation of the results, in the analysis of this database we decided arbitrarily to select only the proteins with relevant possible role involved in tumorigenesis, tumor metabolism and/or aggressiveness to proceed with the validation step (now ongoing). We focused our attention on the proteins with the higher fold variation.

In ACCs the 62% of differentially expressed proteins resulted overexpressed with respect to ACAs: among them vimentin (accession nr P08670) and vitamin D-binding protein (DBP, accession nr P04276) resulted the most varied (3.2 and 3-fold more expressed in ACCs than in ACAs respectively). On the other hand the remaining 38% of proteins resulted under-expressed in ACCs with regard to ACAs, being cathepsin D (CD,

accession nr P07339) and aldose reductase (AR, accession nr P15121) both 3-fold less expressed in ACCs than in ACAs.

We could find similar differences once we compared normal adrenal tissue to ACC protein expression profile (62% of the 35 differential protein spots resulted overexpressed in ACCs), also considering the same proteins described above, although with slight modifications in terms of fold variation (vimentin showed 2.4 fold increase and DBP 1.6 fold increase, CD showed 1.8 fold decrease and AR 2.4 fold decrease, in ACCs with regard to normal adrenal tissue). Considering the purpose of the study, the more remarkable additional protein we could demonstrate to be overexpressed in ACCs versus normal adrenal tissue (1.8 fold increase) and not present in ACAs vs ACCS protein expression study, was lactate dehydrogenase (LDH, chain A accession nr P00338).

Table 9 and Table 10 summarize the proteins detected so far, and their fold variation.

**Table 9. Protein expression profiles of ACAs versus ACCs: list of the most relevant proteins detected so far and reciprocal fold variation (fold). Anova (p): statistical evaluation results. ANV: Average of normalized volumes. PPM: parts per million. In gray the average of normalized volumes of overexpressed proteins.**

| # | Anova (p) | Fold | ANV | ANV | Accession Number | Protein | MW (Mascot) | Matches | ppm | Score |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | ACA | ACC | | | | | | |
| 884 | 0,002 | 3,2 | 0,513 | 1,648 | P08670 | Vimentin | 53676 | 21/61 | 90 | 355 |
| 888 | 6,34E-04 | 3 | 2,188 | 0,741 | P07339 | Cathepsin D | 45037 | 11/37 | 41 | 126 |
| 519 | 0,003 | 3 | 1,698 | 0,575 | P15121 | Aldose reductase | 36230 | 9/18 | 65 | 129 |
| 864 | 0,04 | 2,9 | 2,099 | 0,715 | | | | | | |
| 594 | 0,025 | 2,6 | 1,773 | 0,68 | | | | | | |
| 880 | 0,015 | 2,5 | 2,444 | 0,987 | | | | | | |
| 54 | 0,023 | 2,5 | 0,526 | 1,342 | | | | | | |
| 104 | 4,77E-05 | 2,4 | 0,553 | 1,31 | | | | | | |
| 875 | 0,003 | 2,2 | 0,658 | 1,462 | Q6LDC6 | Vitamin D-binding protein | 52964 | 30/58 | 70 | 285 |
| 50 | 0,005 | 2,2 | 0,59 | 1,268 | | | | | | |
| 545 | 0,028 | 1,9 | 0,712 | 1,327 | Q06520 | Bile salt sulfotransferase | 33929 | 9/31 | 20 | 114 |
| 108 | 1,95E-04 | 1,8 | 0,696 | 1,258 | | | | | | |
| 624 | 0,004 | 1,6 | 1,298 | 0,793 | | | | | | |
| 637 | 0,006 | 1,6 | 0,976 | 1,568 | | | | | | |
| 708 | 0,04 | 1,6 | 1,463 | 0,913 | LRC29 | Leucine-rich repeat-containing protein 29 | | | 110 | 62,70 |
| 893 | 0,044 | 1,6 | 1,202 | 0,752 | P60709 | Actin | 42052 | 28/51 | 50 | 193 |
| 112 | 0,009 | 1,5 | 0,759 | 1,152 | | | | | | |
| 401 | 0,021 | 1,5 | 0,881 | 1,313 | P22570 | NADPH:adrenodoxin oxidoreductase, mitochondrial | 54259 | 21/26 | 70 | 308 |
| 820 | 0,023 | 1,5 | 1,736 | 1,122 | | | | | | |
| 526 | 0,046 | 1,5 | 0,972 | 1,446 | | | | | | |
| 787 | 0,028 | 1,4 | 0,974 | 1,377 | | | | | | |
| 92 | 0,032 | 1,3 | 0,759 | 1,019 | | | | | | |
| 105 | 0,035 | 1,3 | 0,92 | 1,182 | P45974 | Ubiquitin carboxyl-terminal hydrolase 5 | 95768 | | 20 | 60 |
| 96 | 0,038 | 1,3 | 0,858 | 1,137 | P14625 | Endoplasmin | 92696 | 29/62 | 80 | 261 |

**Table 10. Protein expression profiles of normal adrenal tissue versus ACCs: list of the most relevant proteins detected so far and reciprocal fold variation (fold). Anova (p): statistical evaluation results. ANV: Average of normalized volumes. PPM: parts per million. In gray the average of normalized volumes of overexpressed proteins.**

| # | Anova (p) | Fold | ANV | ANV | Accession Number | Proteina | MW (Mascot) | Matches | ppm | Score |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | NA | ACC | | | | | | |
| 874 | 0,008 | 2,4 | 0,701 | 1,68 | P08670 | Vimentin | 53676 | 31/65 | 150 | 334 |
| 519 | 0,002 | 2,3 | 1,297 | 0,575 | P15121 | Aldose reductase | 36230 | 9/18 | 65 | 129 |
| 545 | 0,017 | 2,2 | 0,611 | 1,327 | Q06520 | Bile salt sulfotransferase | 33929 | 9/31 | 20 | 114 |
| 531 | 0,012 | 2,1 | 1,205 | 0,57 | | | | | | |
| 893 | 0,002 | 2 | 1,512 | 0,752 | P60709 | Actin | 42052 | 28/51 | 50 | 193 |
| 594 | 0,002 | 1,9 | 1,299 | 0,68 | | | | | | |
| 864 | 0,015 | 1,9 | 1,379 | 0,715 | | | | | | |
| 595 | 0,002 | 1,8 | 1,246 | 0,7 | P07339 | Cathepsin D | 45037 | 11/37 | 41 | 126 |
| 544 | 0,008 | 1,8 | 0,746 | 1,334 | P00338 | L-lactate dehydrogenase A chain | 36950 | 11/40 | 50 | 109 |
| 678 | 3,02E-04 | 1,7 | 1,675 | 0,984 | | | | | | |
| 890 | 4,63E-04 | 1,7 | 2,037 | 1,217 | Q99497 | Protein DJ-1 | | | 25 | 82 |
| 526 | 0,01 | 1,7 | 0,87 | 1,446 | | | | | | |
| 50 | 0,014 | 1,7 | 0,731 | 1,268 | | | | | | |
| 838 | 0,015 | 1,7 | 0,803 | 1,326 | P62937 | Peptidyl-prolyl cis-trans isomerase A | 18229 | 9/28 | 40 | 146 |
| 111 | 9,20E-04 | 1,6 | 0,734 | 1,192 | | | | | | |
| 112 | 0,001 | 1,6 | 0,742 | 1,152 | | | | | | |
| 197 | 0,004 | 1,6 | 1,277 | 0,783 | P02768 | Serum albumin | 71317 | 37/68 | 50 | 448 |
| 785 | 0,005 | 1,6 | 0,943 | 1,48 | | | | | | |
| 439 | 0,009 | 1,6 | 0,837 | 1,336 | | | | | | |
| 875 | 0,037 | 1,6 | 0,888 | 1,462 | Q6LDC6 | Vitamin D-binding protein | 52964 | 30/58 | 70 | 285 |
| 525 | 0,041 | 1,6 | 1,092 | 0,697 | | | | | | |
| 891 | 0,051 | 1,6 | 1,266 | 0,805 | Q06830 | Peroxiredoxin-1 | 22324 | 8/22 | 90 | 112 |
| 106 | 0,004 | 1,5 | 0,833 | 1,287 | | | | | | |
| 787 | 0,004 | 1,5 | 0,898 | 1,377 | | | | | | |
| 104 | 0,008 | 1,5 | 0,862 | 1,31 | | | | | | |
| 507 | 0,008 | 1,5 | 0,784 | 1,213 | P07195 | L-lactate dehydrogenase B chain | 36900 | 15/38 | 150 | 173 |
| 832 | 0,012 | 1,5 | 0,865 | 1,314 | P30086 | Phosphatidylethanolamine-binding protein 1 | 21158 | 11/40 | 40 | 158 |
| 108 | 0,002 | 1,4 | 0,88 | 1,258 | | | | | | |
| 209 | 0,037 | 1,4 | 0,781 | 1,089 | P11142 | Heat shock cognate 71 kDa protein | 71082 | 16/51 | 35 | 185 |
| 399 | 0,042 | 1,4 | 0,863 | 1,203 | P06733 | Alpha-enolase | 47481 | 18/43 | 90 | 223 |
| 427 | 0,044 | 1,4 | 1,242 | 0,874 | | | | | | |
| 849 | 0,016 | 1,3 | 1 | 1,316 | P62937 | Peptidyl-prolyl cis-trans isomerase A | 18229 | 7/24 | 60 | 109 |
| 769 | 0,02 | 1,3 | 0,963 | 1,285 | | | | | | |
| 878 | 0,047 | 1,3 | 1,209 | 0,921 | P02787 | Serotransferrin | 79294 | 11/58 | 140 | 64 |
| 822 | 0,034 | 1,2 | 1,003 | 1,228 | | | | | | |

# DISCUSSION

Several histologic features have been considered in the differential diagnosis between malignant and benign adrenocortical lesion.[38,40,121,122] Adrenocortical cancers are mainly characterized by high mitotic index, high nuclear grade and disruption of the normal adrenal architecture. However, no single histological criterion can certainly define the malignancy of the adrenocortical lesion. Therefore, several diagnostic algorithms and scoring systems have been proposed, including different items of histological alteration. However, these scoring systems retain some limitations and the morphological differentiation still remains controversial especially in small size and purely localized lesions. Moreover, histology-based diagnosis may also suffer of the subjectivity of the individual morphological observations. In this more "technical" part of the study, we used a computerized morphometric approach to evaluate and quantify the morphological features in ACC and ACA. On Ki-67 stained sections, the most intriguing results were related to both cellular populations, i.e. Ki-67 negative and positive cells, cells not stained and stained with Ki-67 marker respectively.

ACC showed a significantly higher volume fraction of nuclei in unit volume both in Ki-67 negative cells (Vvnucneg) and in Ki-67 positive cells (Vvnucpos). The volume fraction of cytoplasm in Ki-67 positive cells was highest in ACC (Vvnucpos), while ACA showed the highest volume fraction of cytoplasm in Ki-67 negative cells (Vvnucneg). These data reflect the different trend of nuclear/cytoplasmic ratio (N/C) between the two groups: ACC showed the highest values of N/C in both Ki-67 negative cells and Ki-67 positive cells, when compared to the corresponding cellular population in

ACA. The volume fraction of Ki-67 negative cells (Vvcelneg) was highest in ACA; conversely, the volume fraction of Ki-67 positive cells (Vvcelpos) was highest in ACC. The volume fractions of the other compartments (vascular structures, inflammatory infiltrate) were highest in ACC.

According to these results, it could be resumed that ACC are characterized by increased nuclear and cellular density, which appear as "nuclear clouding" in histological specimens.

Ki-67 has been studied with a morphometric model in several types of cancer, some of which are breast, bladder, brain, colon and prostate cancers and it is expressed during all phases of the cell cycle, independently of the duration of the cell cycle.[123,124] Different studies have explored the use of Ki-67 as cell proliferation marker in the diagnosis of adrenocortical neoplasm. Wachenfeld et al. found that a Ki-67 staining index over 5% could be considered a good marker for malignancy in adrenocortical neoplasms.[121] Other Authors found a higher Ki-67 index in ACC compared with ACA, but 6 of 15 ACC examined had a Ki-67 labeling index within the range of adenomas.[37] These differences could be related to technical pitfalls but could also underline that the quantification of Ki-67 in histologic samples represents a critical point. Sampling, individual morphological observations subjectivity and mainly inter-observer variability could be sources of error in the interpretation of some lesions therefore limiting a semi-quantitative grading.

In this setting, when comparing morphometric analysis with the quantification of Ki-67 by pathologist, our results were significantly lower with respect to conventional visual histopathology, as shown by the regression line in figure 8.

How can we explain these data?

In assessing the amount of Ki-67 positive cells in histological samples, pathologists usually try to estimate the proportion of these cells, so measuring a cell count fraction instead of a volume fraction. This estimation is not the same as measuring by morphometry the total area occupied by Ki-67 positive cells, where the results are often given as percentage of histologic sample area. Moreover, it must be underlined that pathologists usually count Ki-67 positive cells in 50 high-power fields (HPF) selecting conventionally the areas of greatest concentrations of positively stained cells (named "*hot spot areas"*).

In our analysis, we have examined more than 250 microscopic high power fields at 40X magnification and, to estimate the "total" amount of Ki-67 positive cells, we have referred volume fractions of Ki-67 positive cells to the cellular compartment, i.e. the reference volume (Vvcellpos), as shown in Table 6.

Accordingly to our results, all ACAs showed Ki-67 values < 1% when compared to ACCs. We recognize that our method is not completely comparable to the pathologist evaluation, certainly for the automated approach but also for the rational and the different criteria of selection and evaluation of the samples' areas. On the other hand this method could be helpful, for example, for those cases in which a high Ki-67 labeling observed in hot spot areas is associated with an otherwise inexplicable favorable outcome. There was no overlapping between the two groups apart for one case. One ACC case (Weiss score 2, ENS@T III stage due to vein cava thrombus) in fact showed low Ki-67 value on both computerized morphometric analysis and pathologist's evaluation (1%) fitting in the range of ACAs group. To explain this case we examined the clinical history of the patient: she had a favorable outcome despite the staging of the disease (stage

III ENS@T, with invasion of the vena cava). Although this is not a clinical study, this data could suggest that a low Ki-67 index could be related to a less aggressive clinical course of an adrenocortical carcinoma, authorizing to think to a possible "intermediate-low aggressive" category of ACC. To better investigate this suggestion further larger studies are needed.

Lastly, the surface fraction of reticulin (Svret) was markedly reduced in ACC when compared to ACA (Figure 9). Different Authors have underlined that the disruption of reticulin framework could be considered a highly sensitive pathologic feature of ACC.[32,33] In our series, ACC group showed a significant negative relationship between reticulin and volume fraction of Ki-67 positive cells (preliminary data, not shown): therefore we could argue that a tumor that scores high for Ki-67 is made of cells that are rapidly dividing and growing and it is associated to a progressive decrease and/or fragmentation of the reticulin framework.

Certainly, adding a morphometric semi-automated tool to the classical diagnostic algorithm of ACCs means introducing numerical values and not only semi-quantitative categories that, in future studies, may actually help in identifying subpopulations of adrenocortical tumors depending on their morphometric characteristics. As a matter of fact, figure 9 shows that one ACC had a surface fraction of reticulin well away, i.e. more than 3 times the 75-25 interquartile distance from the rest of the tumors. This, in our opinion, proves that morphometry can indeed identify also outliers, otherwise covert, within a certain cell population.

To evaluate the reproducibility of our method, 10 randomly selected specimens were analyzed by another operator and compared with the results of the initial counting. We found (Table 8) a high inter-observer agreement

between the two independent morphometric evaluations. This is an important endpoint of our study: in fact many authors have recently tried to deal with one of the most relevant obstacle in the histological analysis of adrenocortical neoplasms: the variability due to the observer subjectivity and experience.[54] In other malignancies the data arising from computerized approach to Ki-67 evaluation and tumor morphologic histological features have already been demonstrated accurate, reproducible and effective.[76,123,125] Further validation studies are requested to demonstrate that the system we used is able to produce acceptable digital slides and to give correct diagnostic interpretation. This would permit in the future the clinical use of this technology in a manner that does not compromise patient care.

The outcome of patients with ACC is strictly related to the stage of the disease, being new ENSAT proposal a valuable tool to improve the power of the stage-related prognostic value. Ki67 expression and Weiss score combined may play a role of interest; the computerized morphometric approach was able to demonstrate an improvement in diagnostic sensitivity and specificity, maintaining also a good correlation with the outcome of the disease. Nevertheless, Ki-67 evaluation remains only one of the possible useful parameters needed for a better prognostic stratification of these rare and fatal tumors, being the finding of even more specific biomarkers the next endpoint of future studies.

We performed a proteomic study to identify a series of proteins that are differentially expressed ACCs when compared to ACAs and normal adrenal tissue. Following the analysis of protein data, we explored the expression of some proteins of interest for tumor pathogenesis and/or aggressiveness. The proteomic profile has been only recently completed and therefore the data about immunohistochemistry and western blotting are not available at the

moment of the presentation of this manuscript. This is the reason because we could only argue, so far, about hypothesis and interpretation of the data.

High-resolution two-dimensional gel electrophoresis (2-DE) has been the standard tool for expression proteomics since its introduction more than 30 years ago. Despite various improvements of the 2-DE technique, one of the main inherent limitations of this method is the fact that many proteins that are expressed at low levels may escape detection. Bioinformatics software made 2-DE gel analysis much easier, however several challenges remain in order to achieve a non-biased, accurate, and reproducible result. Differences in the spot positions between gels are major challenging issue in image processing because they impede accurate spot matching: we performed a gel image alignment and then the software (DeCyder BVA module V. 6.5) aligned other spots based on these landmarks. Moreover the relationship between the original protein quantity in the sample and the measured spot intensity is affected by various intervening factors such as losses during preliminary proteomic procedure steps. It is expected that some proteins would have a nonlinear relation between concentration and intensity. The quantification of proteins has now become very accurate with the introduction of highly sensitive fluorescent stains with a wide dynamic range, technology that has been very effectively employed in DIGE (differential gel electrophoresis). This avoids ambiguities of spot matching as the samples are separated on the same gel, and inter-gel comparisons are greatly improved by the common internal standard. In addition, the sensitivity is comparable with silver staining, but in contrast with it, fluorescent stains are fully MS compatible. In our study the DIGE technology easily consented the individuation of protein spots differently expressed between ACCs and ACAs and between ACCs and normal adrenal tissue: we could detect 30 and 45 protein spots with

significant differential expression in ACCs versus ACAs and ACCs versus normal adrenal tissue respectively.

As from the previous disclosure (see results) we studied more deeply only part of the proteins resulted differentially expressed in ACCs. Among them we selected vimentin, vitamin D-binding protein, cathepsin D, aldose reductase and lactate dehydrogenase because of their possible implication in tumorigenesis and tumor progression.

Vimentin is a major constituent of the intermediate filament (IF) family of proteins, is ubiquitously expressed in normal mesenchymal cells and is known to maintain cellular integrity and provide resistance against stress. Increased vimentin expression has been reported in various epithelial cancers including prostate cancer, gastrointestinal tumors, CNS tumors, breast cancer, malignant melanoma, lung cancer and other types of cancers. Although the over-expression of this protein in cancer has been shown to correlate with increased tumor growth, invasion and poor prognosis, the role of vimentin in cancer progression remains obscure.[127]

The tumor microenvironment is a dynamic system, composed by various cells including tumor vascular cells, inflammatory cells and fibroblasts. As a major and important component in tumor stroma, increasing evidence has shown that spindle-shaped cancer-associated fibro-blasts (CAFs) are a significant modifier of cancer evolution promoting tumorigenesis, tumor invasion and metastasis by stimulating angiogenesis, malignant cell survival, epithelial-mesenchymal transition (EMT) and proliferation via direct cell-to-cell contact or secretion of soluble factors in most digestive solid tumors.[128] The evidence arose by our proteomic study that in ACCs vimentin is overexpressed may be a clue that CAFs could have a role also in this

malignant endocrine tumor: to our knowledge this is the first time that CAFs is designated as a possible player in adrenocortical cancer tumor processes. Moreover as we have demonstrated by our morphometric model through semi-quantitative assessment of CD15/CD8 immuhistochemical staining, neutrophils infiltration seems to be much more present in ACCs than in ACAs: this could represent a new starting point to address further studies to better describe the ACC microenvironment.

There is evidence that vitamin D is capable of modulating several features of cancer: anti-carcinogenic properties of this hormone include the inhibition of cell proliferation, invasion, metastasis and angiogenesis, and the induction of apoptosis and differentiation. The main action of vitamin D is led through its nuclear receptor. Nonetheless several evidences of vitamin D effects not dependent on gene transcription have already been reported. Presumably, these effects are mediated by cell surface membrane receptors, such as Vitamin D-binding protein. This protein is a multifunctional protein found in plasma, ascitic fluid, cerebrospinal fluid, and urine and on the surface of many cell types. In plasma, it carries the vitamin D sterols and prevents polymerization of actin by binding its monomers. DBP associates with membrane-bound immunoglobulin on the surface of B-lymphocytes and with IgG Fc receptor on the membranes of T-lymphocytes. DBP has been found overexpressed in a proteomic analysis of nipple aspirates fluid of women with breast cancer fine: interestingly in the same series Western blot analysis of pooled samples of from healthy volunteers versus samples from women with breast cancer confirmed the overexpression of vitamin D-binding protein in tumor-bearing breasts.[129] Furthermore, regarding breast cancer, DBP overexpression has been reported in 80% of breast cancers[130] and significantly at higher concentrations in breast cancers than in normal breast

tissue.[131] Moreover vitamin D-binding protein is a Gc-globulin (Group-specific component) and it is known to be the precursor for the principal macrophage activating factor (MAF). During inflammation via modification of serum Gc protein by stimulated lymphocytes as compared with de novo synthesis of lymphokines seems to be an emergency shunt for the host defense response in infectious and inflammatory diseases and appears to be the major inflammation-primed macrophage activation cascade. This possible action of DBP leads one more times to the tumor microenvironment and the possible role of inflammatory infiltration in ACCs. Considering that, when de-glycosylated, DBP lacks its pro-inflammatory action, it would be interesting if further studies could better define the DBP status in neoplastic patients tissues and plasma.[132]

Unlike the previous proteins, Cathepsin D has been found under-expressed in ACCs with respect to ACAs. This protein is a member of the peptidase A1 family, it has specificity similar to but narrower than that of pepsin A. Cathepsin D is the product of a gene whose mutations are involved in the pathogenesis of several diseases, including breast cancer and possibly Alzheimer disease. In the last decade an increasing number of studies demonstrated that enzymatic function of CD is not restricted solely to acidic milieu of lysosomes, with important consequences in regulation of apoptosis: some models of apoptosis appear to be dependent either on cathepsins or caspases, whereas others rely on both caspases and cathepsins for their initiation and execution. In addition to CD enzymatic activity, it has been shown that apoptosis is also regulated by catalytically inactive mutants of CD which suggests that CD interacts with other important molecules influencing cell signaling. While some studies reported that CD could directly induce apoptosis, some others suggest that CD promotes apoptosis induced by

cytotoxic and stress agents. Moreover, procathepsin D (pCD), secreted from cancer cells, might acts as a mitogen on both cancer and stromal cells and stimulates their pro-invasive and pro-metastatic properties. Numerous studies found that pCD/CD level represents an independent prognostic factor in a variety of cancers and is therefore considered to be a potential target of anti-cancer therapy. One of the possible pitfalls of the analysis of cathepsin D expression is due to the fact that there are several simultaneous forms of CD in a cell (pCD, intermediate enzymatically active CD and mature heavy and light chain CD).[133]

The cathepsin D expression evaluation in cancers has been already described with particular attention to its possible role as marker of cancer status and prognosis, its functional role in cancer progression including mitogenic functions, influence on invasion, metastasis, angiogenesis, apoptosis and consequently also as a target of cancer therapy.[134,135,136] Although confirmatory data about cathepsin D (and pro-cathepsin D) as mitogenic autocrine factors secreted by cancer cells and its involvement in angiogenesis and metastatic attitude have already been reported so far, the lack of standardization in measurement techniques of pCD/CD, limits their current clinical usefulness in oncology.[137]

Overall we could conclude that the despite the role of cathepsin D in cancer metastasis is actually poorly understood,[138] this enzyme is a useful marker for identifying breast carcinomas with increased risk of recurrent disease and metastasis.[139] Cathepsin D is recognized as an independent prognostic factor in breast carcinoma because of its impact on the incidence of clinical metastasis; its mechanism of action may involve direct digestion of the basement membrane, promotion of cell growth, and a decreased contact

inhibition.[140] This role in cancer metastasis has been studied also in melanoma, prostatic carcinoma, and ovarian tumors.

In our proteomic study cathepsin D resulted three fold less expressed in adrenal carcinomas than in adenomas. This data seems to be contradictory with respect to the general believing of the literature, which usually describes a direct proportional relationship between cathepsin D expression and tumor grade. Interestingly in some immunohistochemical studies performed on central nervous system (particularly in astrocytomas with higher grade of malignancy) and also urological neoplasms cathepsin D has been found under-expressed, implying a possible down-regulation of the protein in highly malignant tumors with loss of differentiation.[141,142]

Moreover in ovarian cancers cathepsin D showed a negative relation to p53 expression: this could be another source of explanation of the low levels of cathepsin D in our series. In fact it is well known that p53 is generally overexpressed in ACCs and moreover the transcription of the cathepsin D gene is initiated from several sites, including one that is a start site for an estrogen-regulated transcript and both ovarian and adrenocortical cancers may secrete estrogens.[135,143]

Aldose reductase, a member of aldo-keto reductase superfamily of proteins, is the mediator of inflammatory signals induced by growth factors, cytokines, chemokines, carcinogens etc. It reduces glucose to sorbitol glucose to sorbitol in the presence of NADPH during hyperglycemic conditions and it is supposed to play a protective role against toxic aldehydes and free radicals derived from lipid peroxidation and steroidogenesis that could affect cell growth/differentiation when accumulated. More than 250 publications result from searching the literature by using keywords “aldose reductase” and

"cancer". Nonetheless very few of these publications are actually referred to AR and various forms of cancer. Aldose reductase is found more commonly over-expressed in cancers such as lung, breast, prostate, cervix, ovarian and colon; some authors showed that the specific activity of AR was higher in tumor areas than in non-tumor regions of tissues.[144,145,146]

Inversely in our study this enzyme resulted under-expressed in ACCs with regard to ACAs. Adrenal gland is a major site of AR expression; therefore we could expect some variations in its expression in adrenal disease. In fact this is not the first time that AR under-expression has been found in ACCs: Lefrançois and coll. in fact could demonstrate in a series of 37 adrenocortical neoplasms (benign, malignant, functioning and non-functioning) that the majority of ACCs (7/8) exhibited low or hardly detectable amounts of AKR1B1 protein: quantification of AR signals showed significantly lower concentrations of the enzyme in malignant tumors than in normal tissue, adenomas, or Cushing's hyperplasia (data confirmed also by mRNA study). We agree with these authors who highlighted two possible reasons for low AR expression in carcinomas: first, chromosomal alterations, including mutations or rearrangements, could reduce expression of the gene. Unfortunately this is probably only a theoretical suggestion, because no chromosomal abnormalities have been described in adrenal tumorigenesis so far. Alternatively, inhibition of AR gene expression, in carcinomas, may be due to alteration of the mechanisms underlying the control of gene expression: one of them could be cAMP action. Interestingly, the transcription factor cAMP-responsive element-binding protein (CREB) resulted strongly decreased at the protein level in ACCs. Changes in aldose reductase mRNA levels in response to cAMP, in parallel with steroidogenic genes, suggest that

AR could be considered a marker of adrenocortical cell differentiation.[147,148,149]

On the contrary, overexpression of aldose reductase and/or aldose reductase-like proteins in other cancers such as hepatocellular carcinomas has been interpreted as a defense reaction against harmful metabolites produced by the growing cancer cells. Overall the authors concluded that it is far to be elucidate whether the decrease in AR expression in malignant tumors is merely a consequence of a general dedifferentiation of the tumor or whether if it contributes to the pathogenesis of the malignancy by promoting, as a consequence of lipid peroxidation products accumulation, alterations in cell-cell communication through progressive loss of gap junctions. Several evidences pointed out that AR expression could be chosen as a good candidate marker for further studies in ACCs, first of all because 88% of the carcinomas exhibit AR protein concentrations below the lowest value measured in normal tissues and benign neoplasms: this is reflecting the same result we have obtained by proteomic profile. Second, AR is a very stable soluble protein whose detection is easy to be performed simply using a Western blot analysis, although more sensitive quantitative analysis could be easily improved by development of an AR-based RIA or ELISA. Moreover it would be worth investigating whether maintaining a high capacity of AR-dependent detoxication could impair or delay malignant transformation process in adrenal cortex.[147]

Aldose reductase reduced expression levels have also been reported in endometrial cancer, tumors characterized by cell proliferation due to high concentrations of estrogens, and decreased cell differentiation due to low levels of progesterone and retinoic acid. These tumors are also associated with aberrant inflammatory responses and concomitant increased production of

prostaglandins. A significantly lower mRNA and protein level of AR in endometrial cancerous tissues compared to adjacent endometrium has been detected. Immunohistochemistry revealed that the enzyme was present in all of the samples (located in epithelial cells of cancerous and control endometrial glands). Elevated levels in adjacent non-cancerous tissues could be interpreted as the role of AR in the initiation of endometrial cancer more than in its progression.[149]

Lactate dehydrogenase A catalyzes the inter-conversion of pyruvate and L-lactate with concomitant inter-conversion of NADH and NAD+. LDHA is found in most somatic tissues, predominantly in muscle tissue and tumors, and belongs to the lactate dehydrogenase family. Very recently Liu and coll. demonstrated that LDHA is overexpressed in gastric cancers and notably targeting this enzyme with its inhibitor (oxamate) they observed the decrease of lactate production and, in presence of glucose, the inhibition of cell proliferation in a dose-dependent manner. Moreover the pro-apoptotic effect of oxamate was further highlighted by flow cytometry.[150]

From the clinical point of view it is even more important to know that LDHA may modulate (18)F-FDG uptake (e.g. in lung adenocarcinomas) via the AKT-GLUT1 pathway and may increase (18)F-FDG accumulation into non-small cell lung cancer, possibly by upregulation of GLUT1 expression. Based on LDHA expression and on a retrospective radiological clinical study, Zhou and coll. could demonstrate that (18)F-FDG PET/CT may predict LDHA expression levels and response to anti-LDHA therapy in lung adenocarcinoma.[151]

As previously mentioned, cancer cells exhibit altered glucose metabolism, known as Warburg effect. This effect consists by the increase

uptake of glucose and the conversion of glucose to lactate in cancer cells under adequate oxygen tension. Recent genetic and metabolic analyses have provided insights about the molecular mechanisms of genes that are involved in the Warburg effect and tumorigenesis, being LDHA one of these genes products. With regard to tumors of the gastrointestinal tract an association between overexpression of transcriptional factors, metabolite transporters and glycolytic enzymes and both poor prognosis and chemotherapy resistance, has been described.[152]

Furthermore, also in endocrine oncology, a proteomic approach allowed obtaining the same interesting data regarding LDHA overexpression in pheochromocytomas and paragangliomas.[153]

# CONCLUSIONS

In conclusion, our computerized morphometric model showed to be an efficient and simple method of quantification of the morphologic characteristics of adrenocortical lesions. It is reproducible and lacks of observer or subjective bias. Therefore, it could represent an adjunctive tool to complement conventional histological analysis to conceivably enhance the reliability and uniformity of the diagnostic evaluation of adrenocortical nodular lesions. Moreover, data obtained could be integrated and coupled in a dedicated software with classical microscopic pathology assessment to supply further information to conventional histological analysis of the adrenocortical lesions.

It is known that the comparisons between adenomatous tissues generally consisting of a monoclonal, homogeneous tumor cell population and normal adrenal or carcinomas, represented by various proportions of adrenal cell types (ACC often showed a polyclonal cells proliferation pattern), may also carry some bias. Nonetheless we believe that the morphological analysis of adrenocortical neoplasms, possibly driving in the future to a nuclear size distribution evaluation, could represent a step forward for a better understanding of this heterogeneous disease.

Although Ki-67 proliferative activity assessed by the automated system was in reasonably good agreement with visual assessment as for diagnostic phase, its prognostic value with respect to recurrence-free and cancer-specific survival need further investigation and validation studies to be considered accurate and effective.

Visual methods are used widely in the clinical assessment of Ki-67 proliferative activity. We recently participated to an international multicentric study on classical visual Ki-67 assessment in adrenal cancers under the supervision of ENS@T association: centralization of laboratory services and increasing workloads may increase the interest also in computerized image analysis for the assessment of Ki-67 labeling index.[154] Moreover we could argue that a morphometric model applied to an IHC study (particularly with the assessment of proliferation markers such as Ki-67) and assisted by an image analyzer could offer significant advantages, including greater rapidity, high reproducibility as well as the possibility of making a digital images archive to be shared between different Institutions.

Although some authors underlined that a human observer may be better at recognizing non-tumor or stromal areas in the sample than an image analysis algorithm,[47] we hypothesize that with a correct mathematical algorithm together with an expert operator (who could work also as a tutor for other operators) working with a semi-automated system, this obstacle could be easily overtaken.

The genome of a given cell or organism is relatively static, subject to epigenetic modulation, whereas the transcriptome and proteome are more dynamic, and change with time and conditions reflecting the different state of the disease. Indeed, the proteome is much more complex than the transcriptome due to post-translational modifications, translocation, protein–protein interactions and regulation, but otherwise may provide more direct information when compared with the transcriptome in relation to oncogenesis. An important aim of proteomics is to understand the cellular function at the protein level by the dynamic proteome, clarifying the functions of proteins and disclosing new biomarkers for disease states.

In conclusion, stereology is of great value for quantifying tumor elements. For objective malignancy grading especially assessment of the three-dimensional mean nuclear size seems useful. All of these systems and methods have a common goal: the standardization of IHC interpretation. Standardized, controlled assays, in combination with a device that provides quantitative and objective output, can dramatically improve the quality of the data obtained from IHC studies. They allow increased sensitivity in scoring and provide a more reliable and reproducible analysis of protein expression in situ. With the advent of these new systems and a controlled approach, the goal of standardizing IHC, from the fixation of tissues to the analysis of IHC results, is achievable.

The retrospective review of the pre-surgical radiological data in light of the biomolecular evidences could be used to better characterize the sensitivity and specificity of radiological imaging techniques.

On the basis of recent advantages obtained with the revision of the staging of adrenal cortical carcinoma proposed by ENSAT we believe that a reconsideration of the histopathological features of these tumors and the identification of parameters that can clearly identify moderately malignant neoplasms from more aggressive histotypes is absolutely needed: an uncertain biological behavior is directly related to a long follow-up, with the consequent clinical relapses, economic and psychosocial.

The revaluation of the Weiss criteria through the use of new technologies (such as mathematical modeling and image analysis) and the identification of new histological parameters to be used in the analysis of fresh tissues might help to create a prognostic stratification of ACCs with clear advantages for the clinical management and a new decision tree for this

disease (choice of targeted adjuvant chemotherapy, radiotherapy, properly "timing" the follow-up, etc.). Furthermore, although the translation of results from research to clinical practice may not be immediate, we are confident that a further proteomic characterization of ACC may allow the design of new therapies directed to target molecules (possibly also radiolabelled) that can provide an additional therapeutic or diagnostic option for these patients.

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