Summary of the PhD Thesis:

**HISTOPATHOLOGICAL AND EXTENSIVE IMMUNOHISTOCHEMICAL STUDY OF THE TESTICULAR GERM CELL TUMOURS**

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This thesis is structured in two parts: a general part and personal contributions.

- **In the general part** I have briefly presented a simplified embryology of the testis relevant to the germ cell tumours histopathology which is followed by a concise histology of the human normal testis. Later on, some of the most important events in the history of germ cell tumours of the testis are revisited together with their consecutive histogenesis, while the most recent classifications are also mentioned. Epidemiology and risk factors are summarised as some treatment considerations based on the latest publications and consensus of the European Association of Urology are stated. The classic histopathology of germ cell tumours is also reviewed based on the latest WHO classification and recommendations, highlighting their main microscopic features that will represent the “gold standard” in their diagnosis. Finally, the antibodies traditionally used in the diagnosis of testicular germ cell tumour are mentioned and some of the state-of-the-art new ones will be analysed in the immunoprofile section.

- **In the first chapter of my personal contributions** I underline the aims and the objectives of this study. Since testicular germ cell tumours incidences is continuously increasing worldwide and have such an architectural variability prone to misdiagnosis, the immunohistochemical evaluation is mandatory. The classical antibodies used in their diagnostic like PLAP, CD30 and AFP have proven their lack of sensitivity and specificity and in consequence, new antibodies have been recently tested.

- The main objective of this study was to establish whether the use of the new antibodies represents an improvement in the diagnosis of TGCTs when compared with classic markers and optic microscopy. Secondly, I was interested to determine the true specificity of each antibody, its usefulness in distinguishing various tumour patterns and if they would shed some light on their histogenesis and developmental differentiation.

- In the second and third chapters of the personal contributions, materials and methods and then the results are presented. In the fourth chapter I discuss some of the available clinical data, the histology of the tumours and their possible differential diagnosis. Each antibody is later discussed based on its positivity in various architectural patterns and in concordance with their specificity and sensitivity for each type of tumour.

- In this retrospective study were included a number of 5 new antibodies (Glypican 3, D2-40, OCT3/4, SOX2 and SALL4) that were evaluated together with the classical ones. This extensive immunohistochemical study was never been performed before on such a large number of testicular germ cell tumours.

A total number of 152 testicular tumours from the archives of Pathology Departments of both University Hospitals of Tg Mures, Romania and San Cecilio, Granada, Spain were microscopically reviewed, of which only 93 TGCTs matched the inclusion criteria and a representative sample of each of them was further analysed. For each slide an evaluation chart was assigned and their histology, architectural patterns and a semiquantitative interpretation of their immunoprofile were recorded.

- Based on their histology, they corresponded to 40 seminomas, 51 non-seminomas and 2 spermatocytic seminomas while intratubular neoplasia was identified in 73 cases. There were 62 slides presenting a single type of tumour, 2 spermatocytic seminomas while the rest of 29 had a mixed histology. The undifferentiated intratubular neoplasia was constantly positive while intratubular seminoma or embryonal carcinomas were present in only 4 cases each. The characteristic solid architecture was present in the majority of the seminomas while papillary areas were the main architectural feature of embryonal carcinomas. The yolk sac tumours were characterised by the mixture of patterns, the microcystic still being the most common. Teratomas were a mixture of mature and immature somatic elements while choriocarcinomas were always part of the mixed tumours and presented their classic morphology.

- The automated or manual immunohistochemical techniques were performed in the Department of Pathology of San Cecilio University Hospital and the Immunohistochemistry Lab of the Tumour Bank of Andalusia
(RBTA), Granada using monoclonal and polyclonal antibodies. Appropriate positive controls were evaluated before sampling the cases of the study while the negative controls consisted of slides run without the primary antibody. Positive reactions were considered based on the general accepted patterns for each antibody while lack of staining was considered a negative result. The obtained data were included within an individual study chart, coded and statistically analyzed.

The atypical cells of the IGCNU were positive in 100% of the cases for PLAP, D2-40, OCT3/4 and SALL4. Except for a reduced percent of seminomas that did not stain for PLAP, the rest of the cases presented the same immunoprofile.

Embryonal carcinomas stained for the majority of the antibodies and AFP was the only one constantly negative. PLAP, CD30, D2-40, OCT3/4, SOX2 and SALL4 were almost all positive in 100% of the cases. D2-40 had a peculiar apical positivity while OCT3/4, SOX2 and SALL4 had constantly a strong nuclear stain. Glypican 3 stained moderately a reduced number of embryonal carcinoma papillary areas.

Yolk sac tumours immunoprofile was characterised by their distinguishing weak to moderate granular AFP positivity, general background stain and negativity in 12% of the cases. The newly introduced Glypican 3 was constantly positive and had a moderate but mainly strong stain in 80% of the cases. The rest of the cases presented weak positivity and as an advantage, it highlighted overlooked areas of yolk sac tumour and did not presented the background stain of AFP. SALL4 stained all the epithelial areas highlighting a higher percentage of cells and presenting a more intense positivity than both AFP and Glypican 3. Both OCT3/4 and SOX2 were negative and helped in the differential diagnostic of solid, endodermal sinus and hepatic patterns.

Cytotrophoblasts were negative for all the antibodies while the syncytiotrophoblasts were constantly stained by Glypican 3 that was paralleled by PLAP in only one case.

Teratomas had an inconstant, variable immunoprofile that expressed their diverse mixture of somatic mature and immature tissues. Important to be mentioned is the Glypican 3 cytoplasmic and membranous positivity in the neuroepithelium paralleled by the intense nuclear stain of SOX2. SALL4 weak positivity in the immature glandular areas might be used in the differential diagnostic with the areas of glandular differentiation of yolk sac tumours.

Spermatocytic seminomas presented moderate positivity for glypican 3 in one case while SALL4 marked the majority of small and medium cells of both cases.

Based on our results, it is concluded that:

- The current revision is so far the largest comparative study evaluating the expression of classical (PLAP, CD30, AFP), new (Glypican 3, D2-40) and stem cell (OCT3/4, SOX2, SALL4) antibodies in testicular germ cell tumours and performing a correlation with their presence in embryonal tissues.
- **Histologically**, this study has provided the following relevant data:
  - Preinvasive IGCNU lesions were present in all cases with remaining testicular parenchyma and had an almost equal distribution between seminomas and non-seminomas. However, spermatocytic seminomas originated from normal testicular parenchyma.
  - Pagetoid extension of atypical germ cells in the rete testis was characteristic of seminomas, while the direct infiltration of the rete was present in non-seminomatous tumours.
  - Unusual histological patterns of seminoma (intertubular, tubular-trabecular, microcystic and pleomorphic) were always associated with areas of classical seminoma.
  - Unusual histological patterns of yolk sac tumour (solid, glandular, macrocystic, Schiller-Duval bodies, papillary and hepatic) always occurred with areas of classical microcystic yolk sac tumour.
  - The rare hepatic pattern of yolk sac tumour should always be kept in mind when a solid area of a testicular germ cell tumour is evaluated.
  - Papillary areas of some embryonal carcinomas can mimic the Schiller-Duval bodies of yolk sac tumour.
  - In a simultaneous review article, we propose the new nomenclature of PRIMITIVE ENDODERMAL TUMOURS for the yolk sac tumour group.
  - Mixed germ cell tumors represented 38.5% of the cases and in 94.4% (34/36) they contained embryonal carcinoma as the main component. The three most frequent associations were embryonal carcinoma admixed with yolk sac tumour (7 cases), with seminoma (7 cases) or with yolk sac tumour, choriocarcinoma and teratoma (7 cases).
- **Immunohistochemical** study revealed the following:
a. Classical markers have their diagnostic value but are complemented by the new, state-of-the-art markers, adding higher specificity and sensitivity to the diagnosis.

b. OCT3/4, SOX2 and SALL4 are stem cells markers and represent the immunohistochemical expression of intrinsic factors that regulate self-renewal and pluripotency of human embryonic stem/germ cells. Their expression in testicular germ cell tumours confirms the pluripotent capacity of these neoplasms.

c. SOX2 is a highly characteristic marker of embryonal carcinoma while OCT3/4 behaves as a marker of the pluripotent undifferentiated germ cell tumours, proving that seminoma is not an end-type differentiation but a precursor of other germ cell tumours.

d. SALL4 can be considered a broad general marker of germ cell tumours; however, it may be present in rare non-germ cell tumours with a stem cell population.

e. Stem cell markers negativity in choriocarcinomas identify this tumour as a terminal-type differentiation

f. Simultaneous expression of SALL4, SOX2 and OCT7/4 confirms embryonal carcinoma as the pluripotent tumour stem cell tumour (Pierce).

From a **practical**, diagnostic viewpoint

a. Basic immunophenotype for IGCNU should include one or two of the following antibodies: PLAP, D2-40, OCT3/4 or SALL4. This also helps to identify pagetoid invasion.

b. Seminoma diagnosis benefits from the same panel of antibodies as above. However, PLAP, a classic seminoma marker, may be negative in 5% of cases, proving that new stem cell markers may supersede classical ones.

c. The majority of embryonal carcinomas are diagnosed by a combination of CD30 and SOX2 while OCT3/4 could be another useful marker to confirm doubtful cases of embryonal carcinomas with a papillary pattern.

d. D2-40 is helpful to differentiate seminoma from embryonal carcinoma and has the additional advantage to identify lymph vessel endothelia and consequently, the presence of tumour emboli.

e. AFP positive stain is diagnostic for yolk sac tumours but in cases not secreting this protein, re-evaluation with positive Glypican 3 and SALL4 and negative OCT3/4, should be performed.

f. Glypican 3 has a higher sensitivity than AFP in identifying YST areas of pure neoplasms and in mixed testicular germ cell tumours, since it stains more tissue and its clean background facilitates interpretation. It even highlights poorly differentiated foci of YST that can pass otherwise undetected.

g. Glypican 3 in YSTs does not have the high specificity of AFP since it can stain true embryonal carcinoma areas, teratoid glands, syncytiotrophoblast and neuroepithelium

h. A minimum panel of antibodies that will cover the most frequent types of intratubular and invasive germ cell tumours should include D2-40, OCT3/4, SALL4 and GLP3