UNIVERSITY OF MEDICINE AND PHARMACY
"CAROL DAVILA", BUCHAREST
DOCTORAL SCHOOL
FIELD OF MEDICINE

PHD THESIS SUMMARY

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PhD student:
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HISTOTYPIC VARIABILITY AND IMMUNOPHENOTYPIC AND MOLECULAR CONSTELLATION IN GASTRIC ADENOCARCINOMA

ABSTRACT OF DOCTORAL THESIS

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I. Fundamental issue

Gastric cancer (GC) is an aggressive disease, many patients are diagnosed at advanced stages and some are inoperable [1,2]. It consists of several subtypes; their relative incidence is influenced by genetic and environmental factors, so that prevalence of each subtype may vary significantly in different populations [3]. Conventional treatment is of limited success [4–8]. Recent advances in personalized treatment improve outcomes but, for this to be effective, distinct subtypes need to be recognised. Different morphological and molecular subtypes have been highlighted by numerous classifications, but no unifying classification is currently in use.

Classification of GC into actionable diagnostic categories would result in more effective treatment [9]. NGS-based molecular classifications such as The Cancer Genome Atlas Program (TCGA) [10] and the Asian Cancer Research Group (ACRG) [11,12] have identified a number of important subtypes of GC characterized by different molecular signatures, including a hypermutated phenotype associated with mismatch repair deficiency (dMMR), an ultramutated phenotype with chromosomal instability (CIN) and a genomically stable (GS) phenotype. In addition, it is apparent that there are a small proportion of cases that have integrated EBV.

It is recognised that while there is some overlap with TCGA, a major difference is that the ACRG lacks a category that relies solely on EBV status, whilst the TCGA does not have a category reliant solely on p53 status. Nevertheless, these multiomics classifications are difficult to implement in the diagnostic routine and several groups have tried to identify these subtypes using a small number of on-slide tests.

The Boston group (Setia *et al*.) was the first to propose a hierarchical classification using a small number of on-slide tests that would stratify these patients into molecular-based and clinically-actionable categories [13]. More recently, others have used the same portfolio of on-slide tests and the same subclassification. For example, Ramos and colleagues demonstrated that such an approach is viable in a prospective study [14]. Importantly, they highlighted the potential difficulty in classifying tumours when expression of these four markers is heterogeneous. They raised the issue of sampling bias, recognizing the importance of correctly interpreting mixed profiles. Ahn and colleagues tested a retrospective cohort of GC patients using tissue microarrays (TMAs) and showed similar correlation with prognosis [15]. Zhao and colleagues used retrospective tissue in TMAs stained by IHC for mismatch repair proteins (PMS2, MLH1,

MSH2 and MSH6), E- cadherin and p21 to classify GC into four subtypes, which correlate with different prognoses [16].

All this work demonstrates the strength of the hierarchical approach and shows a significant degree of concordance in subtypes and biomarkers used but no single widely adopted classification has emerged. More importantly, there has been little attention paid to the distinction between diffuse and intestinal subtypes identified by Laurén that remains a cornerstone of treatment decision making [17].

Nowadays, there are a number of GC classifications based on different tests. Some of the diagnostic categories have considerable overlap but this is not always clear, since they use different terminology and also, sometimes, different markers.

The present study has a first *general part* that contains the present classifications based on morphology proposed by Laurén as well as the WHO classification, followed by the presentation of the most known intrinsic/molecular classifications developed by TCGA, ACRG and Singapore-Duke group and the surrogate ones proposed by Setia *et al.*, Ramos and colleagues, Ahn *et al.* and Zhao *et al.*

The second part is dedicated to the *special section* where we defined our hypothesis, the objectives of the research, materials and methods used, followed by the obtained results and discussion. The final part of the study contains the *final conclusions and personal contributions* which highlight the importance of implementing an on-slide intrinsic molecular classification of GC and the need of such classification.

II. The aim of the study and general objectives of the research

The lack of harmonization between all existing classifications, the complexity and unavailability of some of the tests required plus the demands on time and resources, all contribute to poor uptake in the diagnostic routine [17]. For this reason, we proposed modifications [18], including *harmonization of nomenclature* and *biomarkers* used, the introduction of an *indeterminate category*, the addition of the *predictive oncology* biomarkers currently required for therapy selection and *provision* for new biomarkers that inevitably will become mandatory [19]. The inclusion of an indeterminate category was part of the successful implementation of the new molecular classification for endometrial carcinoma [20–22] and we believe it is an important element for the effective clinical implementation.

In order to identify the advantages and disadvantages of the proposed classification we divided this study into three parts:

- the first part, where we need to understand whether the implementation of this classification is feasible in current Anatomical Pathology laboratory practice; thus we used a cohort of GC resections and performed a retrospective study by constructing paraffin blocks with tissue DNA microarrays (TMA); then we evaluated the biomarkers used and each case was classified into a specific molecular subtype [23];
- the second part was performed on a small batch of GC biopsies that were part of a retrospective study;
- the last part was performed on a batch of GC biopsies performing a prospective study.

It is important to note that for a significant proportion of patients diagnosed with GC, the only tissue available is endoscopic biopsies, as they will not be eligible for complete surgical resection (advanced disease stage or poor performance status/multiple comorbidities).

These present challenges such as identifying the invasive component versus high grade dysplasia (HG), limited tissue quantities and the ability to assess heterogeneity. In addition, the endoscopic biopsy is often a superficial mucosal sample with a different tumor microenvironment than invasive cancer from deeper portions of the gastric or esophageal wall. In an attempt to mimic the small amount of tissue in the biopsy, our first study was performed using the TMA technique. We are aware that the TMA cores were chosen from the most representative areas of the tumour and do not contain areas of normal mucosa. Therefore, in the

second and third parts of this thesis, we wanted to understand whether this classification can be achieved using endoscopic biopsies, with all the intrinsic limitations of these samples. Initially, we retrospectively tested a set of archival endoscopic biopsies (part two). Then, we assessed the challenges of implementing this classification prospectively; for this step, we used consecutive endoscopic biopsies from routine diagnostic work-up (part three).

III. General research methodology

The research database consisted of patients who presented at the University Emergency Hospital of Bucharest (SUUB).

- For the first study, adult patients (≥18 years) who underwent total or subtotal gastrectomy surgery with a diagnosis of adenocarcinoma (ADK) between 2013-2020 were included, thus composing the retrospective study.
- For the second study, patients who underwent upper gastrointestinal endoscopy (UGE) were included, following which biopsies were taken and histopathological diagnosis of ADK was established. These patients were included in a retrospective study, as the biopsies were taken in 2013.
- For the third study was included a small group of consecutive patients who underwent UGE in 2023, biopsies were taken and subsequently diagnosed as ADK; thus a prospective study was performed.

For each study, a database was compiled in Microsoft Excel (Microsoft 365 Office Home version) following the variables: demographic data (age, gender), tumour location and its histopathological subtype (according to WHO classification, 5th edition, 2019 and Laurén subtype). For the retrospective study performed on surgical resections, other aspects were also taken into consideration, such as: tumour size, resection limits, tumour grading, presence of lymphovascular as well as perineural invasion, and tumour staging, using the most recent (8th) edition of the American Cancer Society (AJCC).

Slides and blocks for the two retrospective studies were selected from the archive of the SUUB Pathology Department. All histopathological hematoxylin-eosin (H&E) stained slides of the selected patients were analyzed and reevaluated. They were digitized using the 3D-Histech PANNORAMIC 1000 (P1000) scanner with a primary magnification power of x36. They were subsequently viewed using CaseViewer v2.6 on 4K resolution monitors.

To carry out the first study, a representative slide was chosen from each case, it was digitized, two corresponding annotations were created, and then the TMAs were constructed using TMA-Grandmaster. The obtained paraffin blocks were subsequently sectioned using the microtome, H&E, IHC and ISH staining were performed. The markers used in all three studies are: EBER, MMR proteins (MLH-1 and MSH-2), E-cadherin, β-catenin, p53, Her2, DDISH,

PD-L1 (22C3) and Claudin18.2 For the two studies performed on endoscopic biopsies, the other two MMR proteins (PMS-2 and MSH-6) were also added. For the Her2 IHC, the UltraView DAB detection system was used.

Digitization, making TMAs blocks, all IHC and ISH tests as well as their visualization were performed at Poundbury Cancer Institute (PCI), Dorchester, UK, with consent and courtesy of Dr. Corrado D'Arrigo, Director of PCI, and its team.

All the data obtained were entered into a Microsoft Excel database. The statistical processing of the data was performed using the software provided by Microsoft Excel (Microsoft 365 Office Home version), but also the program R, version 4.4.0 (Copyright (C) 2024 The R Foundation for Statistical Computing, R Core Team (2024). R Foundation for Statistical Computing, Vienna, Austria), using the following additional packages: cluster and gtsummary. Other statistical tests used are: Fisher's exact test, ANOVA (one-way analysis of variance)/linear regression test, AIC (Akaike information criterion), BIC (Bayesian information criterion), log-likelihood, chi-square godness of fit as well as Bayesian LCA (latent class analysis).

IV. Special part – Summary of chapters

Chapter 5 is dedicated to the first retrospective study undertaken on resection specimens of GC. We first defined (*subsection 5.1*), the proposed molecular classification that contains six subtypes: GC-EBV (GC associated with Ebstein-Barr virus), GC-dMMR (GC associated with MMR deficient), GC-EMT (GC associated with epithelial to mesenchymal transition), GC-CIN (GC with chromosomal instability), GC-GS (GC genomically stable) and GC-NOS (GS- not otherwise specified). The classification of the present study used EBV-ISH, MMR status by IHC, E-cadherin and β-catenin IHC and p53 IHC, all of which are tests that can be provided by most histopathology laboratories. The classification presents a hierarchical approach to determine each subtype. After identification of EBV positive GC, dMMR and EMT, the use of p53 antibody separates CIN tumours from GS tumours (Figure 1).

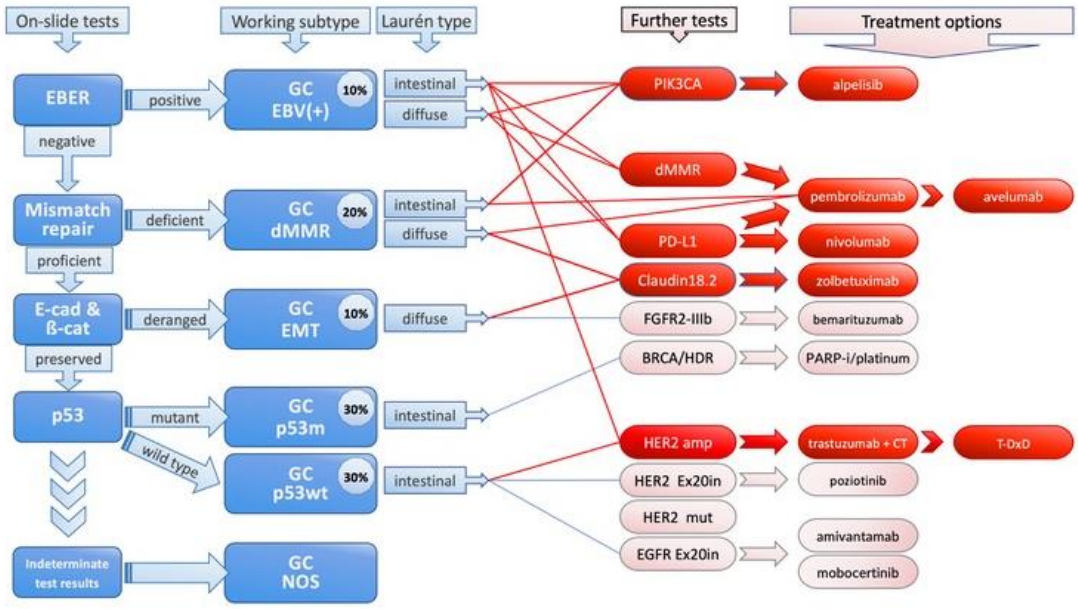

Figure 1. The proposed molecular classification

In order to identify the advantages and disadvantages of the proposed classification, it must be understood whether its implementation is feasible in the current practice of the pathology laboratory, the interpretation of each biomarker used and the hierarchical algorithm must be described (this was done in *subsection 5.1 and 5.2*). Thus we used a cohort of GC resections in a retrospective study and constructed paraffin blocks with DNA microarray (TMA)

tissue. Biomarkers were then evaluated and each case was classified into a molecular subtype. We also demonstrated that for a better TAT (turnaround time) and less burden on the laboratory staff, an approach of performing all the necessary tests upfront is better than the traditional "step by step approach". A first level of biomarkers performed on slides should probably provide prognostic data and guide a second level of predictive biomarkers for therapy (Figure 2).

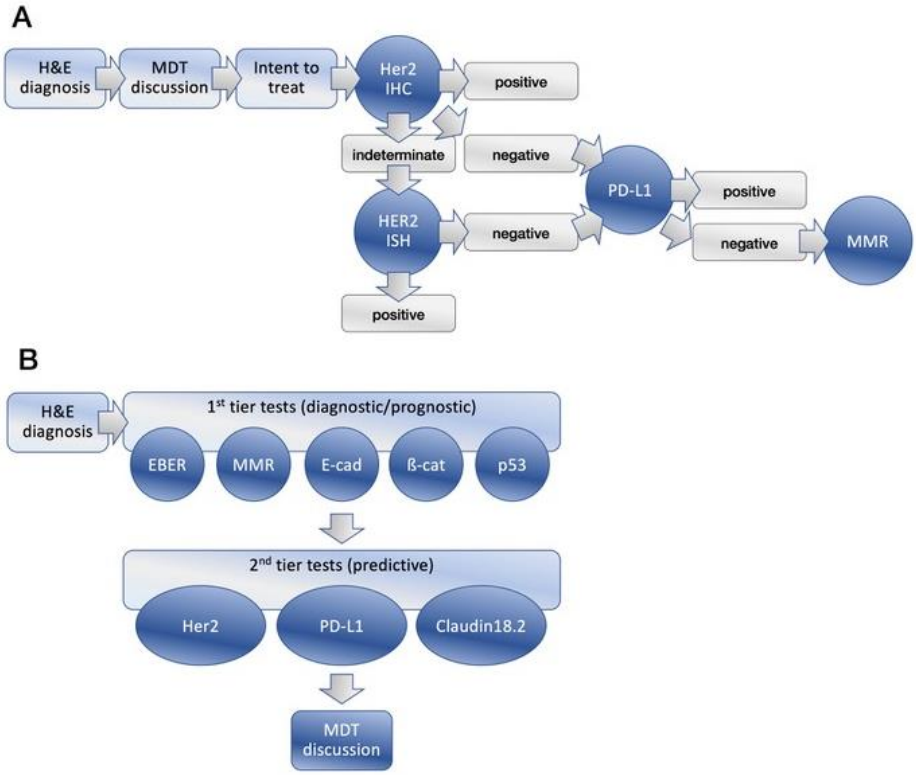

Figure 2. **A.** Current mandatory laboratory workflow, slow TAT. This is the current step by step approach often used. This leads to slow TAT because of the need to interpret each test before ordering the next one. **B.** Modified laboratory workflow, rapid TAT. After histological diagnosis of GC, all tier 1 tests are requested at the same time; tumours are classified using the hierarchical approach; second tier tests to predict response to specific treatments are then reflexed accordingly. Using this approach on other tumour types we are able to report routinely these datasets with two-four days TAT.

Following this, in the subsequent subsections *(5.2.1. and 5.2.2.)* we described our 79 cohort of GC resections, the definition and evolution of TMA, its technique and the advantages and disadvantages for using such equipment. Finally, we described this technique performed on our cohort as follows: during the planning and design phase of TMA, the following aspects were determined: from each patient, the representative histological region was investigated and our

target was for the invasive tumor center. Two tumor spots for each patient were included in the TMA block. We chose a core diameter of 1.5 mm for the TMA construction for all blocks. A distance of 0.4 mm between cores was also chosen for the design of this TMA. Thus, the TMA block contained 32 tumor biopsies (cores). For their orientation we used two reference biopsies from a liver and a blank, and each tumor formation was double sampled. In conclusion, five TMA blocks have been produced using TMA-Grandmaster (Figure 3).

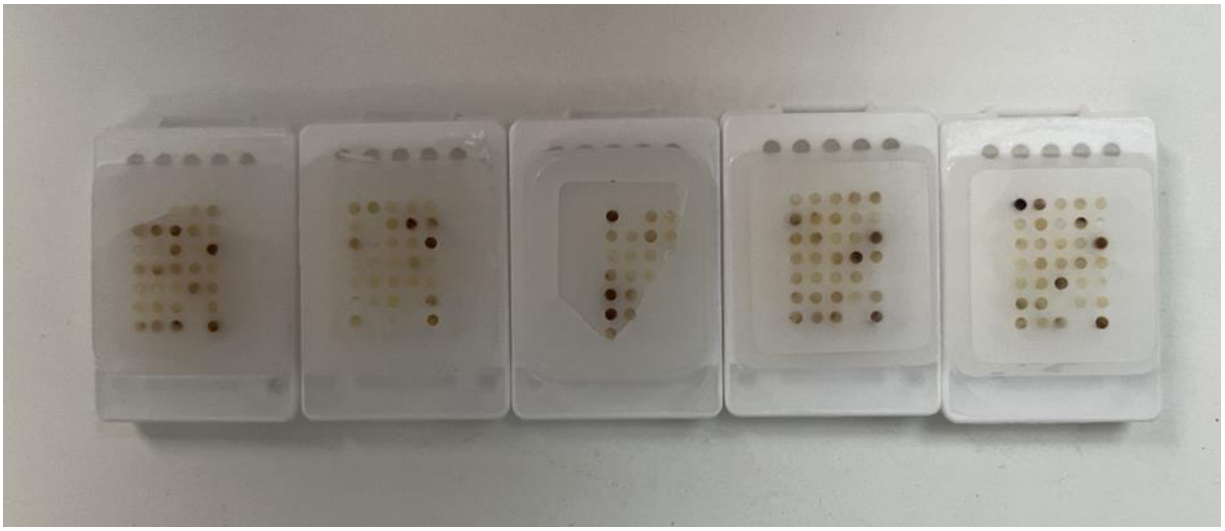

Figure 3. TMAs blocks performed

All these were then sectioned and slides were made for H&E staining, for IHC as well as for ISH. H&E stained slides from paraffin TMA blocks were compared with the original H&E sections obtained from the surgically excised tumor to validate the TMA libraries (Figure 4).

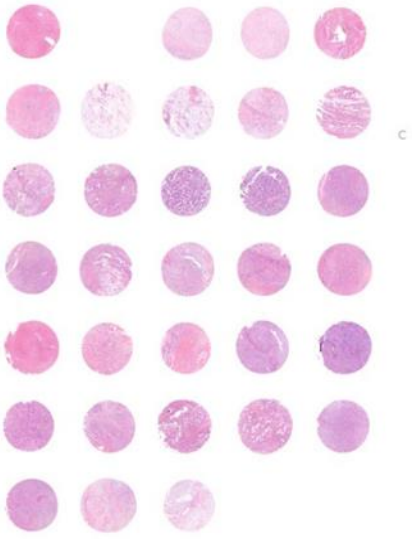

Figure 4. H&E TMA digitized slide, 0.5x.

In *subsection 5.2.4* we identified the molecular groups using the hierarchical algorithm. Initially, EBER status is assessed, and if positive, the case is classified as EBV (+) GC; all negative EBER GCs are then assessed for MMR status, and if deficient, the tumour formation is assigned to the GC-dMMR category. All MMR positive ("proficient") and EBER negative cases are assessed for the presence or absence of E-cadherin and β-catenin expression, so if aberrant expression is identified, the case is classified as GC-EMT. Finally p53 status is assessed for all cases where there is preservation of E-cadherin and β-catenin staining, are MMR proficient and EBER is negative. Those cases showing p53m pattern are classified as GC-CIN, while those with wild-type pattern are classified as GC-GS subtype (Figure 5).

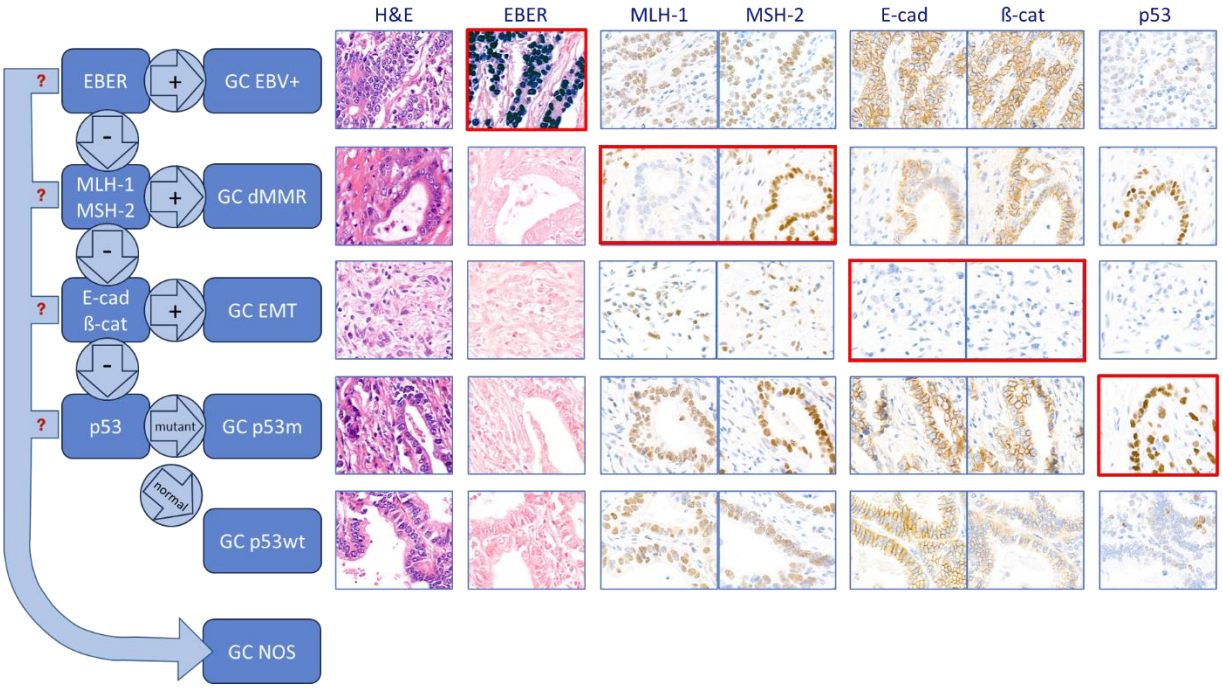

Figure 5. Hierarchical classification

We also assessed the prevalence of each subgroup along with the predictive biomarkers performed (Her2, PD-L1 and Claudin18.2). Following this, in *subsection 5.3.4* we performed a broad statistical analysis on the cohort examinated. In *5.4 subsection* we focused on the discussions about this first part of the study where our main aim was to define the parameters as well as the interpretation algorithm for each biomarker used and to identify the challenges in their evaluation. Therefore, we used tumor tissue from surgical resections of GC, making a batch of 79 cases, then constructing paraffin blocks with DNA microarray (TMA) tissue. We also

aimed to determine the relative proportion of each molecular category and associate them with the Laurén subtype, as well as with predictive biomarkers that are used in the treatment of GC patients. The findings have been discussed in detail with a focus on Claudin18.2 that is a new emerging biomarker in late-stage trials and is targeted by immunotherapy that appears to be highly effective in diffuse GC.

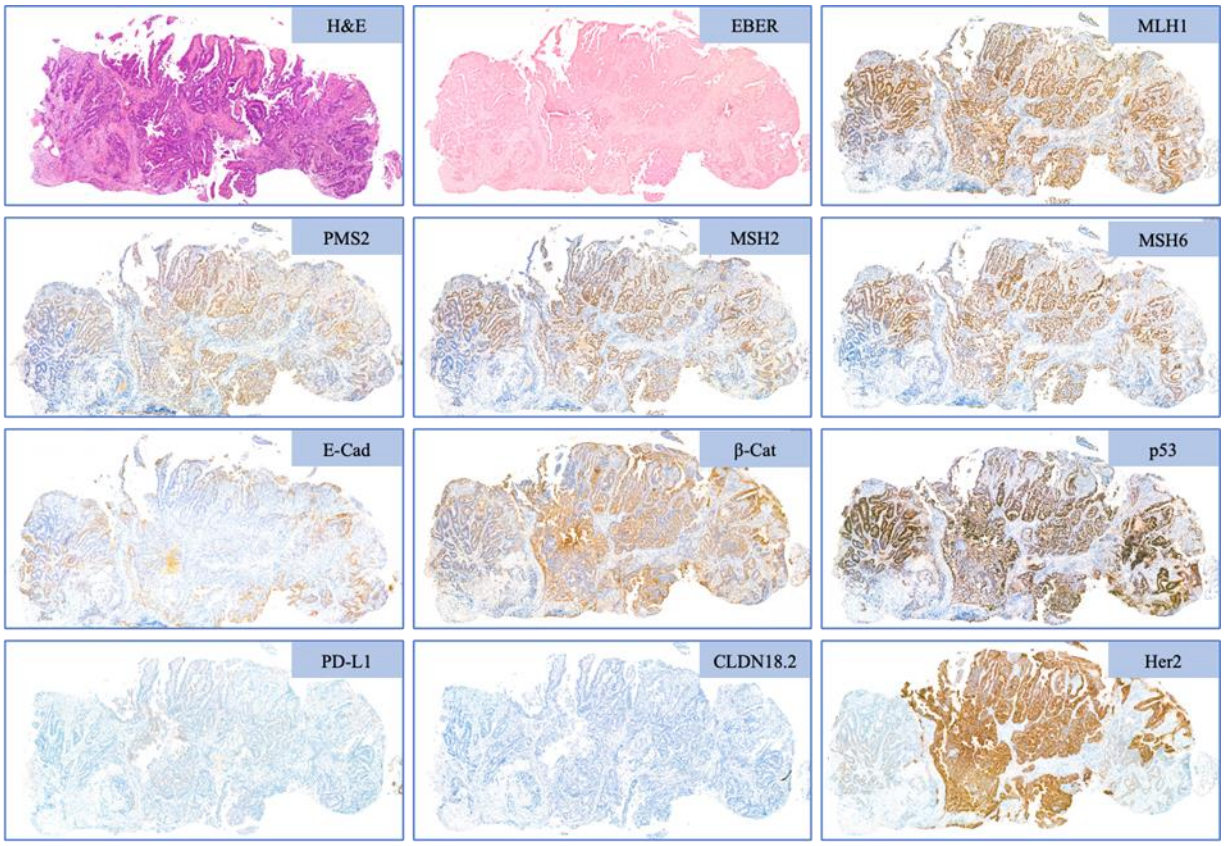

Figure 6. Example of biomarker evaluation for molecular classification. Case 2 from retrospective cohort. Final diagnosis: GC-CIN, intestinal Laurén type. H&E shows intestinal Laurén type; ISH for EBER is negative; IHC for MMR enzymes (MLH1, PMS2, MSH2 and MSH6) shows conserved nuclear expression; IHC for E-cadherin and β-catenin shows conserved membrane staining; IHC for p53 shows strong and diffuse nuclear staining in ≥80 of tumour cells (p53m). Additional CDx biomarkers: PD-L1 is negative (CPS < 5), Claudin18.2 is negative (no membrane staining) and Her2 is positive (IHC score 3+). All photomicrographs were taken at 3,5x digital magnification.

The next step in this project was to identify the feasibility of implementing this hierarchical classification in routine diagnosis using endoscopically collected biopsies. This has been performed in *chapter 6*. Therefore, for the retrospective part of this study we used 24 consecutive cases of gastric or oesophago-gastric junction (OGJ) ADK from endoscopic biopsies for which we had FFPE blocks with sufficient tissue to allow new sections to be cut. The prospective part of the study used 30 consecutive endoscopic biopsies of gastric ADK or OGJ confirmed as part of routine histopathological diagnosis between May and November 2023.

The biomarkers used and techniques were identical as the one used in the first part of this study (on resection specimens), except for TMA performed blocks (Figure 6). The two endoscopic studies has been described separately as well as combined.

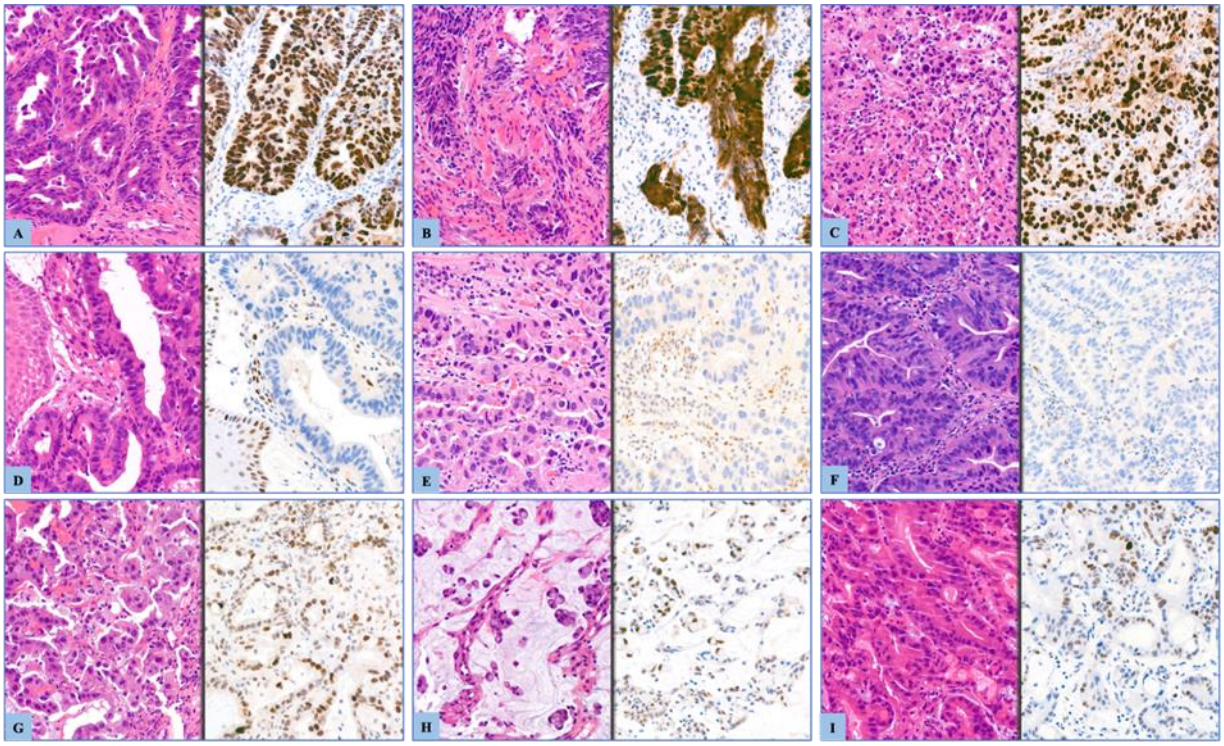

Figure 7. IHC staining patterns of p53. H&E and p53 IHC pairs. Top row (A-C): overexpressed p53m IHC model; middle row (D-F) null p53m IHC model; bottom row (G-I): p53wt model. All micrographs were taken at 20x digital magnification.

In the following subsections (*6.3 and 6.4*) we interpretated the results of the endoscopic study and we focused on the interpretation of p53 IHC since this is a challenging marker and the correct interpretation of each biomarker is essential for the successful implementation of this classification (Figure 7). In discussion section of this chapter (*6.5*) we concluded that this working classification performed only on histopathological (glass) slides can be used on small endoscopic biopsies, requires a minor modification of the current pathways in order to run a biopsy specimen in the Pathology Laboratory and can be delivered with a short TAT, meeting the requirements of cancer patients.

V. Conclusions and personal contributions

1. This doctoral research highlighted the need to implement a universally valid "molecular" classification that is easy to implement and can be delivered using the existing tools in any Pathology Laboratory.
2. The true molecular classifications remain available only to a minority of GC patients because it requires comprehensive high-cost genomics and transcriptomics, for which there is a conspicuous lack of capacity worldwide.
3. Several groups tried to deliver molecular classification based on small numbers of markers (Setia et al., Ramos et al., Ahn et al., Zhao et al.), however they used different nomenclature and often different tests or different interpretative algorithms. Therefore, there was a need for harmonization of the terminology, test repertoire and interpretation.
4. We proposed an inclusive working classification based on on-slide tests that can be delivered by Histopathology using existing resources.
5. The classification includes determination of Laurén's subtypes using H&E-stained sections and the status of 6 (EBER, MLH-1, MSH-2, E-cadherin, β-catenin and p53) on-slide biomarkers using ISH and IHC.
6. We used a cohort of 79 GC resection cases to assess the feasibility of delivering the classification of GC into one of 6 categories: GC associated with Epstein-Barr virus (GC-EBV), GC mismatch repair deficient (GC-dMMR), GC with epithelial-mesenchymal transition (GC-EMT), GC with chromosomal instability (GC-CIN), GC genomically stable (GC-GS) and GC-NOS/indeterminate.
7. We also assessed the feasibility of integrating into this classification a number of predictive on-slide companion diagnostic (CDx) tests required for the management of GC patients. These included Her2, PD-L1 and Claudin18.2.
8. The use of p53 antibody separates CIN tumours from GS tumours. Other biomarkers such as p21 can also be used for this separation.
9. We believe that the choice of nomenclature for CIN and GS is an improvement; it harmonizes research in GC as well as in other organs.

10. In order to identify cases of GC-EMT, we used β-catenin in addition to E-cadherin. Others who attempted molecular classification of GC with on-slide biomarkers limited testing to E-cadherin only.

11. We added GC-NOS category for those cases when a downstream biomarker of a tumour not yet classified couldn't be interpreted (indeterminate result), then the case was placed in the indeterminate category (GC-NOS).

12. We used an hierarchical approach for the attribution to the specific subtypes, having as a guide previous publications as well as the already recognised hierarchical classification of endometrial cancer.

13. With this hierarchical approach, once a tumour is assigned to a molecular subgroup, the downstream biomarkers are non-contributory for classification purposes.

14. We explained and exemplified the algorithm used in detail.

15. We defined all the parameters used, considering the most recent publications and interpretation guidelines accepted by ASCO/CAP (American Society of Clinical Oncology and College of American Pathologists), NICE (National Institute for Health and Care Excellence) and EMA (European Medicines Agency).

16. At the same time, taking into account that this classification that we have proposed is intended to be a "skeleton" to which other biomarkers can be added at any time, as a proof of concept we have used Her2, PD-L1 and Claudin18.2.

17. In the first study, we conducted a retrospective study with a cohort of 79 patients from a single hospital unit (SUUB) who underwent surgery - total or partial gastrectomy, in 2013 - 2020.

18. FFPE blocks corresponding to the cases were selected, and using the TMA-GrandMaster tool we made 5 recipient blocks with TMA cores. These were sectioned and subsequently stained with the specified markers.

19. All the slides obtained were digitized using the P1000 produced by 3D-Histech Ltd. with an initial magnification of 36x and then viewed on 4K resolution screens.

20. The TMA production, staining with IHC and ISH markers, digitalization and interpretation of the slides have been performed at Poundbury Cancer Institute, Dorchester, UK with the help and courtesy of Dr. Corrado D'Arrigo, Director of PCI, and its team.

21. Our results were similar with the one existing in the published literature.

22. We conducted a broad statistical analysis on the 79 resection specimens cohort.

23. We demonstrated that the markers chosen are the appropriate ones to describe each molecular group.

24. We demonstrated that this classification is an inexpensive and effective method that can be used by pathologists to provide prognostic information as well as to identify targeted treatment.

25. Many other predictive biomarkers will be needed in the near future, so this classification may provide insight to prioritize biomarkers needed for companion diagnostic tests.

26. We proposed an inclusive working molecular classification based on on-slides tests that provides harmonization and can be delivered by Histopathology using existing resources, however this study left a number of unanswered questions. For example, will endoscopic biopsies contain sufficient tissue for this proposed classification? Will pre-analytical conditions impact on test interpretation? How easy is the assessment of biomarkers on small and superficial samples of carcinoma?

27. Such questions are pertinent, since the endoscopic biopsy is the only tumour tissue available for a significant proportion of GC patients.

28. In an attempt to mimic the small amount of biopsy tissue, our previous study used tissue microarrays (TMA) but we are mindful that the TMA cores were chosen from the most representative areas of the tumour and did not contain normal mucosa.

29. In next part of the other two studies we therefore wanted to understand if this classification can be delivered using endoscopic biopsies, with all the intrinsic limitations of these samples. We used two different cohorts of patients with gastric cancer diagnosed on endoscopic biopsy. The retrospective cohort included 24 consecutive archival cases of gastric or oesophageal-gastric junction (OGJ) adenocarcinoma from which we had formalin-fixed paraffin-embedded (FFPE) blocks with sufficient tissue to allow for new sections to be cut. The prospective cohort comprised 30 consecutive patients with gastric or OGJ adenocarcinoma confirmed by endoscopic biopsy received in the diagnostic routine between May and November 2023. We used the same markers, the same interpretation and the same algorithm as the first study performed on resection specimens.

30. Each case was annotated for presence of sufficient diagnostic material and turnaround times (TAT). Discordant cases were reviewed in a medical conference and a consensus diagnosis was reached.

31. A time and motion study was undertaken to provide an indication on the additional resources needed to assess all the biomarkers and provide a final classification. This was done by measuring the time needed by a pathologist to examine a case that had been previously diagnosed as carcinoma and for which all the additional biomarkers were prepared and digitized.

32. Timely execution of our classification required coordination with laboratory staff.

33. We cut all the necessary sections upfront after a diagnosis of invasive carcinoma. We found that 16 spare sections were sufficient. We only had a single case (from a total of 54) where tissue was exhausted and a single test could not be performed (Claudin18.2).

34. We demonstrated that whilst a step-by-step approach would save reagent costs, it would increase time for both technicians and pathologists and would ultimately be more expensive to the service, therefore, planning of all tests upfront allows laboratory staff to optimize the use of immunostainers and minimize the impact on capacity, paradoxically even creating more capacity.

35. Correct interpretation of all the on slides tests is key for the successful implementation of this classification.

36. There is consensus on the scoring and interpretation of all of the biomarkers we used in the study apart from p53. That's why we stressed out the importance of this marker and how its interpretation should be more accurate.

37. We concluded that this on-slide working classification can be used with small endoscopic biopsies, requires minor modification on the existing tissue pathways and can be delivered with short TAT, fitting the requirements of cancer patients.

38. The major limitation of this study is the lack of survival outcomes and post-treatment effects.

39. Future studies focusing on the relationship between this proposed classification, which has the potential to be implemented in routine diagnosis in any Pathology department, and treatment outcomes, treatment effects and patient survival are needed.

40. Is worth to be noted that all our slides have been digitized and with the help of digital microscopy we can build a virtual library and finally carry out large-scale, inter-institutional studies.

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List of published scientific papers

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Active participation in scientific events

1. S. Costache, A.M. Ciongariu, A. Chefani, M. Costache, M. Sajin, The need to know more about gastric cancer: prevalence and features in a retrospective study, Virchows Archiv, Volume 479, Supplement 1, August 2021, E-PS-06-007 – abstract poster presentation at European Congress of Patology, 33rd edition, 29th-31st of August 2021

2. Costache S, Baltan A, Diaz McLynn S, Pegoraro M, De Havilland R, Porter M, Lerga A, D'Arrigo C, Wedden S, Billingham K, Molecular classification of gastric cancer using a small number of on-slide biomarkers for risk stratification and therapy selection – poster presentation at Tucson Symposium -Roche, 23rd-24t^{h} of April 2024, USA.