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### Barrett's esophagus and esophageal adenocarcinoma: from molecular pathogenesis to novel therapeutic targets

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# Chapter 1

## GENERAL INTRODUCTION & OUTLINE OF THE THESIS

Partly adapted from

*Immune signalling and regulation in esophageal cancer.* Ann N Y Acad Sci. 2014  
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## 1.1 ESOPHAGEAL CANCER

The recent advances in cancer genomics and the advent into the clinic of novel molecular targeted therapies are raising fresh enthusiasm and hope in the longstanding fight against cancer. It is now evident that only understanding both, tumor-cell intrinsic and extrinsic factors, we can effectively improve therapeutic responses. Despite latest improvements in diagnostics and therapeutics, Esophageal Cancer is still the sixth most frequent cause of death related to cancer worldwide<sup>1</sup>. Patients that suffer from this cancer have very poor prognosis, with 5-year survival rates of 15-20%<sup>2,3</sup>. This is mostly due to

late clinical presentation with advance disease. Esophageal cancer can be classified into two major histologic subtypes: Squamous Cell Carcinoma (ESCC) and Adenocarcinoma (EAC) (Figure1)<sup>4</sup>. The former occurs mainly in the upper and middle portions of the esophagus. EAC, instead, develops in the lower part of the esophagus, in proximity to the gastro-esophageal junction (GEJ). This classification has been recently confirmed and implemented at molecular level, where extensive genomic analysis of ESCC and EAC patients' biopsies, clearly defined the two cancer subtypes as distinct molecular entities<sup>5</sup>. Strikingly, especially in western countries, the incidence of EAC increased dramatically over the past twenty years<sup>6</sup>. Unfortunately, standard treatments, such as chemoradiotherapy and/or surgery, seem to be not effective. In this thesis I will outline the investigation of key molecular events that drive EAC pathogenesis, with a closer look to its immune microenvironment. Only a deep understanding of these molecular factors can lead the way to the development of effective targeted therapies.

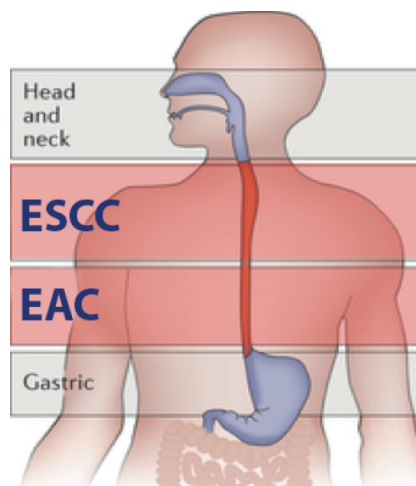


Figure 1. Adapted from *Romero D., Nat. Rev. Clinical Oncology, (2017)*

## 1.2 BARRETT'S ESOPHAGUS & ESOPHAGEAL ADENOCARCINOMA

Barrett's Esophagus (BE) is one of the major risk factor associated with the development of EAC. BE is a premalignant condition characterized by the replacement of the stratified squamous epithelium of the distal esophagus by intestinal-type columnar epithelium. Development of BE itself is strongly associated with the presence of gastro-esophageal reflux disease (GERD)<sup>7</sup>. Chronic reflux of gastric and duodenal acid and bile is the initial stimulus needed to drive the columnar phenotype. Although the cell of origin that give rise to this process has not been yet identified, most likely different stem cell lineages are responsible for the differentiate of the esophageal epithelium into neosquamous or columnar-like. The result of this process is the appearance of non-intestinal (non-IM) and intestinal metaplasia (IM), conditions that can evolve into low-grade (LGD) and high-grade dysplasia (HGD) and, finally into EAC. The risk of progression from BE to EAC is 0.12-0.7% per patient per year<sup>8,9</sup>. Although numbers are modest, the incidence of HGD and EAC increases up to 13,4% per year when LGD is present<sup>10</sup> and, up to 25% for patients diagnosed with HGD<sup>11</sup>. Stated the relationship between BE and risk of developing cancer, endoscopic surveillance is advised for BE patients in order to early detect the presence of HGD and/or EAC. Up do date, the Seattle protocol is the main endoscopic surveillance program recommended for BE patients. Biopsies are taken from four-quadrant random areas of the BE segment at 1-2cm intervals<sup>12</sup>. Additionally, in the presence of irregularities such as nodules, erythema and erosions, separate targeted biopsies are collected. The resulting histopathology analysis determines the surveillance interval for each case.

A lot of effort has been directed at finding molecular and genetic biomarkers in order to improve the efficacy of surveillance programs and diagnostic procedures. Despite these studies, for most biomarkers there is insufficient evidence for implementation into the clinic. Most data obtained are based on observational studies on transversal cohorts or case control series. Only a few longitudinal, long-term prospective follow up studies have been performed. From these studies several biomarkers seem to be promising. Chromosome instability has been associated with progression from BE to EAC<sup>13</sup>. In a 10-year prospective study, a biomarker panel detecting 9p loss of heterozygosity (LOH) (inactivation of the p16 tumor suppressor gene), 17p LOH (inactivation of the p53 tumor suppressor gene) and DNA content abnormalities (aneuploidy or tetraploidy), identified patients with high or low risk of progressing to

EAC<sup>14</sup>. In a population-based study, a panel comprising LGD, abnormal DNA ploidy and *Aspergillus oryzae* lectin, risk-stratified BE patients in progressors and non-progressors<sup>15</sup>. Our lab, in its place, has built a prediction model based on age, Barrett's length, p16, MYC and aneusomy with the ability to determine progression risk in non-dysplastic BE patients<sup>16</sup>. Moreover, Timmer and colleagues were also able to demonstrate that the risk of cancer development for patients with BE is predetermined by a baseline level of genetic diversity, which is higher in progressors and remains constant over time<sup>17</sup>. Detection of TP53 abnormalities may also serve as prognostic molecular biomarker. For instance, immunohistochemistry (IHC) of TP53 has resulted to be a predictive marker of progression<sup>18</sup>. Furthermore in a prospective follow-up study, our group has demonstrated that combining TP53 detection by DNA Fluorescence In Situ Hybridization to IHC, improves further the accuracy for detecting BE progressors<sup>19</sup>.

In this thesis, we approached the study of molecular key events involved in the development of both BE and EAC, from a therapeutic point of view.

### **1.2.1 THE ROLE OF BILE ACIDS IN THE DEVELOPMENT OF BARRETT'S ESOPHAGUS**

Understanding the signalling pathways activated by the chronic gastro-esophageal reflux exposure, can potentially unveil novel therapeutic targets at which preventive therapies can be directed.

The relationship between GERD and BE has been extensively studied in humans and animal models. First evidence of it occurred in 1970 from a study by Bremner and colleagues<sup>20</sup>. After denuding the distal esophagus of dogs, induction of chronic gastro-esophageal reflux favoured columnar re-epithelisation over squamous mucosa. In humans, by monitoring bilirubin and pH, Vaezi and colleagues showed that reflux episodes were more frequent in BE patients, followed by patients with GERD and ultimately normal controls<sup>21</sup>. Moreover, clinical evidence suggested that GERD duration linearly increases BE risk and exponentially EAC risk<sup>22</sup>. Interestingly, the observation that BE developed in patients that underwent total gastrectomy, suggested that acid was not the sole responsible for BE pathogenesis<sup>23</sup>.

Esophageal secretions and saliva, gastric secretions, such as acid and pepsin, and duodenal secretions, such as trypsin and bile salts, mainly compose the gastro-esophageal reflux. Within those components, bile salts and acid seem to play an

essential role in damaging the esophageal mucosa. This damage can then initiate BE development and/or stimulate its progression towards EAC<sup>24</sup>.

Several human studies using esophageal aspiration techniques aimed to identify the composition of the main refluxate constituents<sup>25,26</sup>. Nehra and colleagues, for instance, detected higher concentrations of primary and secondary conjugated bile in patients with BE compared to healthy controls. Interestingly, in the same study, they found a significant temporal relationship between reflux of taurine conjugates and time of acidic exposure, suggesting a synergistic critical role for these bile components and acidic pH in causing damage of the esophageal mucosa. The synergy between acid and biles seems to be the tuning mechanisms that determine the toxicity potential of the reflux. Bile components, in fact, can penetrate the cell membrane when present in a unionized and soluble state. Reason why unconjugated bile molecules (pKa ~7) are more harmful at neutral pH, while conjugated ones (pKa ~2) at acidic pH. Once inside the cell, bile components can act as signalling molecules by activating the nuclear receptor FXR $\alpha$  or the G-protein-coupled receptor TGR5. A progressive increased of FXR $\alpha$  expression has been observed in BE patients compared to healthy squamous epithelium<sup>27,28</sup>. The expression of TGR5, instead, has been found to be upregulated in human EAC tissues and seems to correlate with poor patients' overall survival<sup>29</sup>.

Our experimental approach aimed in characterizing the bile composition of the reflux of BE patients in order to identify the main components responsible for the esophageal damage. To this extend, we made use of a novel experimental mouse model, in which human BE reflux was given to mice in order to artificially simulate a GERD condition. Additionally, we supported our *in vivo* observations with extensive *in vitro* analysis, by using primary cells and established cell lines. We investigated the signalling pathways activated by the different bile components and underlined their role in driving BE development.

### **1.2.2 MOLECULAR PATHOGENESIS OF BARRETT'S ESOPHAGUS AND ESOPHAGEAL ADENOCARCINOMA**

The link between bile, acid and inflammation is a central factor in the pathogenesis of BE. Bile and acid, in tandem with esophagitis, have been associated to oxidative stress and free-radicals generation<sup>30-34</sup>. Increase in reactive-oxygen species and antioxidants depletions, such as glutathione and vitamin C, have been observed in intestinal

metaplasia<sup>35</sup>. Moreover, increased expression of inducible nitric oxide synthase and cyclooxygenase-2 (COX-2), has been detected in both BE and EAC<sup>36</sup>. Therefore, by inducing oxidative stress and consequently, DNA damage, all these elements can contribute to the molecular pathogenesis of BE and EAC.

Several experimental and clinical studies have been tried to dissect the cellular origin of BE. Until now different hypothesis have been proposed but results are still controversial. What is clear is that diverse signalling pathways are altered during the sequential progression from GERD to BE and finally to EAC. Remarkably, the majority of these pathways are physiologically involved in the embryonic differentiation of the foregut into esophagus and trachea, which are lined, respectively, by squamous epithelium and columnar epithelium<sup>37</sup>. Among them, alterations of the bone morphogenetic protein (BMP), Wingless-Type MMTV Integration Site Family (WNT), Hedgehog (HH), Notch and retinoic acid (RA) pathway, have been linked to BE development and its malignant transformation<sup>38</sup>. The main downstream effectors of these activated signalling cascades are transcription factors that, ultimately, regulate the differentiation of the esophageal epithelium into squamous- or columnar-type. Therefore, to interpret the role of these pathways in the development of intestinal metaplasia may be a logic strategy to consider their roles in embryogenesis. Studies in transgenic mouse models, for instance, have suggested that during the development of the foregut epithelium, the expression of the transcription factor NKX2.1 (NK2 Homeobox 1) is needed for the columnar differentiation, while activation of SOX2 (Sex Determining Region Y-Box 2) and p63 is required for squamous differentiation<sup>39,40</sup>. Interestingly, the expression of both SOX2 and p63, has been found to be progressively decreased during BE development, in favour of intestinal differentiation<sup>41,42</sup>. Additionally, *in vitro* exposure to bile and acid, induced SOX2 and p63 downregulation in normal esophageal cells<sup>43,44</sup>.

Our group has previously highlighted the crucial role of the BMP pathway in the pathogenesis of BE<sup>45</sup>. Specifically, BMP4 and its downstream target, phosphorylated mothers against decapentaplegic 1/5/8 (pSMAD1/5/8), resulted to be highly expressed in human biopsies of esophagitis and, to drive the expression of columnar type of genes. Furthermore, in a different *in vitro* study, the expression of BMP4 could be induced after exposing esophageal cells to bile and acid<sup>46</sup>, confirming its crucial role in the initiation phase of BE development. Additional evidence of the involvement of BMP4 in the pathogenesis of BE, comes from its observed association

with the HH signalling. In BE and EAC, the HH pathway and its downstream transcription factor GLI1, have been found to be aberrantly expressed compared to normal squamous epithelium<sup>47,48</sup>. Interestingly, HH signalling activation, have been observed to induce stromal expression of BMP4 and epithelial expression of the transcription factor SOX9<sup>49</sup>. Notably, SOX9 is an intestinal stem cell transcriptional regulator and, *in vitro*, can drive columnar differentiation of esophageal squamous cells<sup>50,51</sup>. Once again, these observations recall the role covered by these pathways in embryonic development, where the HH signalling has the ability to induce the activation of the BMP pathway<sup>52</sup>.

Another factor that seems to play a fundamental role in the development of BE metaplasia, in particular in the intestinal type of metaplasia, is the caudal-type homeobox transcription factor 2 (CDX2). It belongs to the caudal-related homeobox family and is a key regulator of intestinal development<sup>53</sup>. Moreover, a study on ~500 primary and metastatic adenocarcinomas has showed that CDX2 is an extremely sensitive and specific marker of intestinal carcinogenesis<sup>54</sup>. Notably, while CDX2 is not present in normal human squamous epithelium, it has been found to be expressed *de novo* in the intestinal epithelium of BE patients<sup>55,56,57</sup>. Furthermore the *in vitro* capacity of bile and acids in inducing CDX2 expression in esophageal cells, suggests once more a clear involvement in the pathogenesis of BE<sup>58,59</sup>. Intriguingly, ectopic CDX2 expression in the squamous esophageal epithelium of a mouse model did not induce columnar metaplasia<sup>60</sup>.

The above mentioned observations pointed out that all these molecular factors are insufficient, by their own, to induce the development of columnar epithelium. For this reason, our approach in this thesis was to study the combinatory effect of two crucial factors, BMP4 and CDX2, in the progression to intestinal metaplasia. To this extend we made use of a novel surgical mouse model, that simulated the presence of chronic gastro-esophageal reflux. Additionally, we analysed human squamous esophageal and BE tissues and, performed extensive *in vitro* analysis in order to get insights in the molecular relationship between those characterizing events of BE metaplasia: BMP4 pathway activation and CDX2 expression.



## **1.3 TARGETING THE IMMUNE SYSTEM IN ESOPHAGEAL ADENOCARCINOMA**

### **1.3.1 IMMUNOTHERAPY: BOOSTING THE IMMUNE RESPONSE**

The second part of this thesis will focus on the immunobiology of EAC. Our interest in the role of the immune system in EAC comes from the urgent need of novel and more effective therapeutic approaches, such as, immunotherapy. The first clinical indications for the potential use of cancer immunotherapy date back to 1891 when, Dr. William Coley, successfully treated inoperable sarcomas by injecting bacterial toxins<sup>61</sup>. Almost eighty years later, the link between cancer and immune system was first hypothesised by the Nobel laureate Sir Frank Macfarlane Burnet<sup>62,63</sup>. He defined the concept of immune surveillance as the ability of the immune system to overwhelm cancer development. When this thesis work begun, at the end of 2011, we could not predict how successful immunotherapy would prove to be in treating cancer. At that time, immunotherapy was surrounded by great scepticism due to the numerous clinical features that marked its past. Thanks to a “good” stubbornness, guided by a robust hypothesis-driven biological approach, our group always believed in the clinical potential of immunotherapy. Specifically, carrying on the work started by Francesca Milano on the potential of dendritic cell immunotherapy in EAC, we aimed, in this thesis, to unveil immunosuppressive mechanisms that characterize this cancer<sup>64</sup>. Paradoxically, in the same period, between 2010 and 2011, the US Food and Drug Administration approved the sipuleucel-T and the anti-cytotoxic T-lymphocyte associated protein 4 (CTLA-4) antibody (ipilimumab), for treating, respectively, castration-resistant prostate cancer and melanoma<sup>65,66</sup>. These unprecedented responses in patients with advanced-stage tumors boosted the field of immunotherapy and shed light on the importance of the immune checkpoint inhibitors in modulating anti-tumor immunity. Few years later, this molecular approach was further implemented by the approval of the anti-programmed cell-death protein 1 (PD-1) antibody<sup>67,68</sup>. CTLA-4 and PD-1 are immune checkpoints that, under physiological conditions, suppress the function of T cells, thus preventing autoimmunity. The clinical rationale for immune checkpoint inhibition arises from the fact that, tumor cells are able to overexpress them, in order to decrease T cell-driven anti-tumor response. However, until now, despite the remarkable clinical achievements, these immunotherapeutic approaches resulted to be beneficial only for a subset of cancer patients.

In order to circumvent this issue in EAC, we focused on its basic anti-tumor immunobiology. We tested novel methodologies for boosting the host's anti-tumor immune response and, we attempted to understand molecular mechanisms that can lead to tumor immune evasion.

Three sequential steps are thought to define the close relationship between tumor and immunity: "Elimination, equilibrium and escape"<sup>69</sup>. The "Elimination" phase is the first step in which the immune system gets activated in order to identify and kill the tumor cells. Most likely this phase takes place way before the tumor is clinically detectable. The "Equilibrium" phase consists of an intermediate stage in which the elimination phase induces immune selection, which stimulates part of the tumor cells to decrease their immunogenicity. In this context, a dynamic equilibrium is established, in which the immune system is not able anymore to clear all the tumor cells at the same time. Some of the tumor cells, in fact, become resistant and able to hide themselves from the immune effector cells. This eventually leads to the last phase of tumor "Escape".

Within the immune system, Dendritic Cells (DCs) play a crucial role in orchestrating anti-tumor immunity<sup>70</sup>. DCs are, in fact, specialized antigen presenting cells, with the unique ability of migrating to lymph nodes and initiating an anti-tumor immune response through activation of T helper cells, cytotoxic T cells and natural killer cells<sup>71</sup>. This potent and broad immune-stimulating function makes DCs ideal immunotherapeutic tools for cancer treatment. Moreover, several phase 1 clinical studies have highlighted their safety<sup>72</sup>. Despite the potential of DC-based immunotherapy in expanding T cell immunity even in patients with advanced-stage cancer, the clinical success until now has remained below expectations<sup>73</sup>. Further optimization is needed in the critical steps of *ex-vivo* generation and maturation of DCs such as, for instance, antigen loading and activating stimuli. Furthermore combinatory strategies that target the adverse immune-microenvironment are highly needed in order to boost the DC-mediated immune response<sup>74</sup>. In favour of this assumption, the combination of DC immunotherapy and immune-check point inhibitors, recently showed promising results in treating patients with advanced melanomas<sup>75</sup>, and parallel clinical trials are currently on going (NCT02677155, NCT01067287, and NCT01441765).

In this thesis we tested the combinatory potential of DC-based immunotherapy and curcumin treatment, in targeting esophageal adenocarcinoma cells, *in vitro*.

### 1.3.2 CURCUMIN AND CANCER



The rationale for including curcumin in our experimental settings comes from numerous studies that underlined its therapeutic potential in different disorders, including cancer<sup>76</sup>. Curcumin, or diferuloylmethane, is the main curcuminoid found in the rhizomes of the herbaceous perennial plant *Curcuma longa*

(Zingiberaceae family). Curcumin is not only responsible of the yellow colour of Turmeric, the spice derived by the dried rhizome powder of *Curcuma longa*, but is considered to be its most active components. The spice Turmeric has been used in traditional Indian Ayurveda medicine since centuries to treat different illnesses. Curcumin possesses a broad spectrum of activities such as, antioxidant, antimicrobial, hypoglycemic and wound healing<sup>77,78,79,80</sup>. Through its anti-inflammatory properties, curcumin seems to exert therapeutic effects against neurodegenerative, cardiovascular, pulmonary, metabolic, autoimmune and neoplastic diseases<sup>81</sup>. Curcumin has been observed to block NF- $\kappa$ B activation by inhibiting the I $\kappa$ B $\alpha$  kinase and AKT<sup>82</sup>. In different studies, the overall downregulation of NF- $\kappa$ B-regulated genes suppressed tumor cells proliferation and angiogenesis both, *in vitro* and *in vivo*<sup>83,84</sup>. Within the most studied genes targeted by curcumin, of note are the tumor necrosis factor (TNF), cyclooxygenase-2 (COX-2), vascular endothelial growth factor (VEGF), interleukin-1 (IL-1), IL-2, IL-6, IL-8, IL-12, matrix metalloproteinase (MMPs), CDK2<sup>85</sup>. Additionally, curcumin has been observed to induce apoptosis of cancer cells through p53 promotion<sup>86,87</sup>. Despite its safety and pleiotropic action, the main limitation of its effect resides in its poor bioavailability<sup>88</sup>. It gets poorly absorbed and rapidly metabolized and eliminated from the body. Pharmacodynamic and pharmacokinetic studies showed that, after oral intake, curcumin could be hardly detected in blood or urine, while it was recovered from feces<sup>89</sup>. However, intravenous administration in rats, improved its bioavailability in blood plasma suggesting that this route of administration should be considered also for human studies<sup>90</sup>. Several formulations of curcumin have been tested in order to increase availability and inhibit metabolic clearance<sup>91,92</sup>. Among them are, nano-formulations, Poly(lactic-co-glycolic acid) (PLGA), liposomal or cyclic oligosaccharides encapsulation, piperine combinations. All these formulations showed to considerably increase curcumin solubility and bioavailability.

In our study, we used a nano-curcumin formulation, highly dispersible in water, with a mean particle size of 0.19 $\mu$ m compared to 22.74 $\mu$ m of standard curcumin powder<sup>93</sup>. Compared to unformulated curcumin, it showed improved bioavailability in healthy human volunteers<sup>94</sup>. Additionally, systemic exposure to high concentration of this formulation did not induce adverse effect in cancer patients<sup>95</sup>.

### **1.3.3 MICRO-RNAS & ESOPHAGEAL ADENOCARCINOMA**

As explained before for Dendritic cell-based strategies, the success of any type of cancer immunotherapy ultimately relays on the absence of an unfavourable tumor immune microenvironment. Our efforts in identifying and overcome immune escape mechanisms in EAC, brought our attention on potent regulators of the immune system such as microRNAs (miRNAs). MiRNAs are small single-stranded noncoding RNAs that regulate mRNA expression at a post-transcriptional level. They mainly act by targeting the 3' untranslated region of the mRNA of a gene, resulting in either translational repression, mRNA degradation, or mRNA cleavage, depending on the complementarity between the miRNA and the target mRNA<sup>96</sup>.

In the last decade, miRNAs have been shown to regulate several processes in normal physiology, and miRNA dysregulation has been reported in many diseases, including cancer. Owing to their stable expression in serum, plasma, saliva, and other body fluids, they can be potentially good diagnostic, prognostic, and predictive biomarkers. Furthermore, since miRNAs act as oncogenes or tumor suppressor genes and regulate individual biological pathways by regulating the number of different downstream molecules, they make attractive candidates as therapeutic targets compared to approaches targeting single genes. To date, several studies have revealed distinct miRNA expression profiles between tumor and normal or premalignant tissue in both ESCC and EAC, identifying promising miRNAs with different roles at multiple steps of tumor progression.

Because of the increase in EAC incidence and the very poor prognosis, the identification of biomarkers is urgently needed in order to detect EAC precancerous lesions (e.g., BE) at an early and curable stage and to identify patients at risk for developing EAC. Feber et al.<sup>97</sup> performed miRNA expression arrays on a small cohort of patients that included esophageal tissues from normal squamous epithelium (NSE), high-grade dysplasia (HGD), ESCC, BE, and EAC. They found miRNA profiles that were distinct for each tissue type and could distinguish normal from malignant tissue. Additionally, they also found several miRNAs (e.g., miR-21, miR-192, miR-203) that

have been previously reported to be dysregulated in other types of cancer. Interestingly, the miRNA profile of NSE was found to be similar to ESCC, while that of EAC clustered closer to BE, reflecting the tissue specificity of miRNAs with the ability to discriminate between squamous and columnar tissue. The first microRNA profiling study carried out by Yang et al.<sup>98</sup> included paired tissue from various stages of BE and EAC. The resulting miRNA signatures discriminated between diseased tissues and paired normal tissues, and associated with the progression of EAC. A large multicenter study by Mathè et al.<sup>99</sup> reported microarray-based expression data of 100 EACs (including 63 patients diagnosed with BE) and the adjacent noncancerous tissues. They found miRNA differential expression in EAC patients and, interestingly, an association between miRNA miR-375 and survival of BE-associated EAC patients, highlighting the clinical utility of miRNAs as prognostic biomarkers. Furthermore, the combination of miR-375 expression and inflammatory risk score (IRS) was shown to be an improved prognostic classifier of BE-associated EAC patients<sup>100</sup>. Using northern blotting and in situ hybridization (ISH), Hu et al.<sup>101</sup> analyzed the expression of 10 miRNAs in four EAC cell lines and 158 ESCC and EAC tissue samples, finding associations of different miRNAs with tumor cell de-differentiation, lymph node metastasis (LNM), higher pathologic disease stage, poor overall survival, and disease-free survival. A miRNA signature of BE carcinogenesis has been found by Fassan et al.,<sup>102</sup> where microarray analysis of 14 NSE and 14 BE (7 LGD, 5 HGD, and 11 BE-associated) EAC tissues, followed by quantitative polymerase chain reaction (qPCR) and ISH validation, identified specific miRNAs that are involved in BE progression to EAC. Leidner et al.<sup>103</sup> used next generation sequencing in nine paired NSE–EAC tissues and identified 26 candidate miRNAs that were highly deregulated in EAC compared to NSE. Successful validation found two miRNAs (miR-31 and miR-375) to be potential markers of neoplastic progression in BE. NSG was also used by Bansal et al.,<sup>104</sup> where the miRNA transcriptome of five gastro esophageal reflux disease (GERD) and six BE patients showed differential expression of several miRNAs between the two groups. Wu et al.<sup>105</sup> published a large real-time PCR-based miRNA profile of 754 human miRNAs in 35 NSE, 34 BE (11 BE metaplasia, 13 LGD, 10 HGD), and 36 EAC tissues, confirming previously reported putative miRNA biomarkers and identifying new miRNAs differentially expressed at various stages of BE and EAC.

It is now clear that miRNAs play key roles in many processes of tumorigenesis in several types of cancer. This is also the case for EAC, where different studies have demonstrated miRNA expression signatures in the progression from non-dysplastic to

EAC with a high power of discrimination between different tissues and pathological phenotypes. Furthermore, many of the putative miRNAs identified in the above mentioned studies can function as oncogenes or tumor suppressor genes, playing important roles in the control of important biological processes, such as cell growth, differentiation, and apoptosis, and can therefore be used as potential diagnostic, prognostic, and therapeutic tools. However, miRNA profiling studies possess many limitations that need to be overcome in order to define EAC-specific signatures and valid biomarkers. Tissue contamination, the use of different profiling platforms, and strategies of data normalization are only a few of these. In addition, the small number of patients included in the studies makes it difficult to obtain consistent results and prevents any association with clinical variables. Different study protocols and clinical criteria between different hospitals further complicate the comparison of miRNA expression datasets in large multicenter studies. Another issue to take into consideration is the origin of the tissue analysed. miRNAs are highly tissue-specific and it is quite difficult to compare their expression in cross-sectional studies, where tissues affected by progressive grades of cancer are obtained from different patients. Novel high-throughput methodologies, such as RNA sequencing, should be routinely used instead of miRNA microarrays, since they have a higher degree of sensitivity and can lead to discovery of yet unknown miRNAs. In conclusion, miRNAs play important roles in EAC carcinogenesis, and their clinical utility looks extremely promising, but reproducible detection methods, standardized sample selection and preparation protocols, and improved and consistent data analysis methods need to be adapted before we can assess and take full advantage of their clinical utility.

Our approach in this thesis was to study if any miRNA, in EAC, could regulate an essential pathway of anti-tumor immunity, such as the MHC-I antigen presentation. It has been recently demonstrated that miRNAs can indeed target MHC-I genes and, that the integrity of the MHC-I pathway is indispensable for proper antigen presentation and tumor recognition by the effector T cells<sup>107</sup>. To this aim, we first characterized the expression of MHC-I pathway in several EAC patients' derived biopsies. We then established an *in vitro* model using EAC cell lines, in which we screened the expression of several miRNAs known to be involved in immunopathology pathways. Finally we validated our findings by performing robust *in vitro* functional studies and, analysing patients' biopsies by immunohistochemistry and miRNA *in situ* hybridization.

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