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At the intersection of Diabetes and Cancer: the role of angiogenesis and inflammation

Ana Rita da Costa Rocha

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INSTITUTO DE CIÊNCIAS BIOMÉDICAS ABEL SALAZAR

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AT THE INTERSECTION OF DIABETES AND CANCER:

THE ROLE OF ANGIOGENESIS AND INFLAMMATION

Dissertação de Candidatura ao grau de Mestre em Oncologia – Especialização em Oncologia Molecular submetida ao Instituto de Ciências Biomédicas de Abel Salazar da Universidade do Porto.

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TÍTULO: At the intersection of Diabetes and Cancer: the role of angiogenesis and inflammation

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Aos meus pais.

“I prefer to be a dreamer among the humblest,

with visions to be realized,

than lord among those without dreams and desires.”

Khalil Gibran

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Resumo

Diabetes *mellitus* tipo 2 (DM2) é um importante problema de saúde, com 415 milhões de pessoas diagnosticadas em todo o mundo. Foram descritas evidências quanto à sua associação com vários tipos de cancro, incluindo o cancro gástrico. Foram sugeridas algumas hipóteses para explicar como a DM2 poderia aumentar o risco de desenvolvimento de cancro, como por exemplo a hiperglicemia, hiperinsulinemia, stress oxidativo, distúrbios vasculares e um estado de inflamação crónica basal. O cancro gástrico é o quinto cancro mais comum no mundo e é a terceira principal causa de morte por cancro. O cancro gástrico é frequentemente associado à infeção por *Helicobacter pylori,* e essa inflamação desempenha um papel central no processo carcinogénico. Esse estado inflamatório crónico, relacionado com o desequilíbrio angiogénico, stress oxidativo e com a sinalização metabólica, sugere que a DM2 pode ser um importante fator de risco na iniciação e progressão do cancro gástrico, no entanto serão necessários mais estudos.

Foi estudada uma coorte constituída por dois grupos de pacientes, um grupo com cancro gástrico e DM2 (n=22) e outro grupo controlo com cancro gástrico e sem DM2 (n=21). Foi realizada uma análise imunohistoquímica utilizando um anticorpo contra CD31, para avaliar a densidade dos vasos. Foi também realizada uma coloração histoquímica com Sirius Red, para determinar a percentagem de fibrose no tumor e na mucosa normal adjacente. Com base na avaliação do infiltrado de células inflamatórias no tumor e da percentagem de estroma no tumor, realizou-se uma avaliação semi-quantitativa denominada "Glasgow Microenvironment Score". Além disso, o score para o prognóstico de Glasgow, que é amplamente conhecido como um marcador baseado na inflamação sistémica, foi determinado para cada paciente. Os grupos foram estabelecidos de forma a que as características clinico-patológicas dos pacientes fossem semelhantes e a principal diferença fosse a presença ou ausência de diabetes. Relacionou-se a diabetes com a sobrevida de cada paciente, e também de forma estratificada pelos diferentes scores de Glasgow Microenvironment Score e Glasgow Prognostic Score.

Verificamos não existirem diferenças significativas tanto nas características clinico-patológicas, como na inflamação sistémica e tumoral de cada paciente e

também na sobrevida a dois e cinco anos, nem entre grupos nem quando estratificados pelos diferentes scores. Na avaliação da densidade vascular e da fibrose, verificou-se apenas diferenças significativas entre a área adjacente normal dos controlos e a área tumoral dos diabéticos.

Mais investigação será necessária para verificar estes resultados e perceber se estes marcadores poderão ser bons indicadores de prognostico destas patologias.

Palavras-chave: cancro gástrico; diabetes *mellitus;* inflamação; angiogénese; fibrose.

Abstract

Type 2 Diabetes mellitus (DM2) is a major health problem, with 415 million people diagnosed worldwide. Evidence regarding its association with various types of cancer has been reported, including GC. Some hypotheses have been suggested to explain how DM2 could enhance the risk of cancer development, such as hyperglycemia, hyperinsulinemia, oxidative stress, vascular disturbances and a chronic low inflammation state. Gastric cancer (GC) is the fifth most common cancer worldwide and ranks as the third leading cause of cancer-related death. GC is frequently associated with infection by *Helicobacter pylori* and inflammation plays a central role in the carcinogenic process. Such chronic inflammatory state, linked with angiogenesis imbalance, oxidative stress and metabolic signaling, suggests that also DM2 might be a major risk factor in initiation and progression of GC, demanding further investigation.

A series of GC from DM2 (n=22) and control (n=21) patients were studied. Immunohistochemistry (IHC) using antibodies against CD31 performed, to assess density of vessels. Histochemical staining with Sirius red was performed to determine the percentage of fibrosis in the tumor and non-neoplastic adjacent mucosa. Based on assessment of tumor inflammatory cell infiltrate and tumor stroma percentage, a semi-quantitative evaluation of Glasgow Microenvironment Score was performed. Also, Glasgow Prognostic Score, that is widely known as a systemic inflammatory-based marker, was determined for each patient. Additionally, the Glasgow prognostic score, which is widely known as a marker based on systemic inflammation, was determined for each patient. The groups were established so that the clinical-pathological characteristics of the patients were similar and the main difference was the presence or absence of diabetes. Diabetes was related to the survival of each patient, and also stratified by the different scores, Glasgow Microenvironment Score and Glasgow Prognostic Score.

We verified that there were no significant differences in the clinical and pathological characteristics, as well as in the systemic and tumoral inflammation of each patient and also in the survival at two and five years, neither between groups nor when stratified by the different scores. In assessing vascular density

and fibrosis, there were only significant differences between the normal adjacent area of the controls and the tumor area of the diabetics.

More research will be needed to verify these results and to see if these markers could be good indicators of the prognosis of these pathologies.

Keywords: gastric cancer; diabetes *mellitus*; inflammation; angiogenesis; fibrosis.

Poster presentation under the scope of this thesis

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List of Abbreviations

AJCC	American Joint Committee on Cancer
AKT	Protein kinase B
AMP	Adenosine Monophosphate-activated Protein
ATP	Adenosine triphosphate
BMI	Body mass index
CC1	Cell conditional 1
CD31	Cluster of differentiation 31
CRP	C-reactive protein
CT	Computed tomography
DAB	3,3'-diaminobenzidine
DM	Diabetes mellitus
DNA	Deoxyribonucleic acid
EBV	Epstein-barr virus
EC	Endothelial cells
ECM	Extracellular matrix
ERK	Extracellular Signal-regulated Kinase
FPG	Fasting plasma glucose
GC	Gastric cancer
GCA	Gastric cardia
GLUT	Glucose transporter
GMS	Glasgow microenvironment score
GNCA	Gastric non-cardia

GPS	Glasgow prognostic score
HbA1c	Hemoglobin A1c
HE	Hematoxylin and eosin
HER2	Human epidermal growth factor Receptor
HRP	Horseradish peroxidase
IGF	Insulin-like growth factor
IL	Interleukin
IR	Insulin resistense
KM	Klintrup–Mäkinen
LKB1	Liver kinase B1
MALT	Mucosa-associated lymphoid tissue
MAPK	Mitogen-activated protein kinase
MRI	Magnetic resonance imaging
mTOR	Mammalian target of rapamycin
NIDDM	Non-insulin-dependent diabetes mellitus
OGTT	Oral glucose tolerance test
PFKFB3	6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 3
PI3K	Phosphoinositide 3-kinase
PlGF	Placental growth factor
PPAR	Peroxisome Proliferator-Activated Receptor
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
SRC	Signet ring cell
TNF	Tumor necrosis factor
TNM	Tumor-Node-Metastasis

TSP	Tumor stroma percentage
TZD	Thiazolidinediones
UICC	International Union for Cancer Control
US	Ultrasonography
VEGF	Vascular endothelial growth factor
VEGFR	Vascular endothelial growth factor receptor
WHO	World Health Organization

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Introduction

1. Diabetes *mellitus*

Diabetes *mellitus* (DM) is one of the most prevalent chronic diseases worldwide and one of the main global health emergencies of the 21st century. Recent studies estimate that approximately 415 million people worldwide suffer from DM, and its prevalence is expected to rise to 642 million by 2040 [1]. In 2015, it was estimated that DM affects 59,8 million in Europe, with Portugal accounting for approximately 1 million (13% of its population) [1].

There are three major types of diabetes:

Type 1 DM (DM1), accounting for approximately 7 to 12% of the total cases[1]. It is characterized by an autoimmune destruction of pancreatic *β*-cells, leading to absolute insulin deficiency and, consequently, to the total dependence on exogenous insulin to sustain life [2]. In the past, DM1 was also referred to as juvenile onset diabetes, with a peak incidence between the ages of 8 and 14 [3,4];

Type 2 DM (DM2), accounting for almost 87-91% of the diabetic population[1], also known as non-insulin-dependent diabetes mellitus (NIDDM) or as adult-onset diabetes, is characterized by insulin resistance which may be combined with relatively reduced insulin secretion levels [5,6]. DM2 is a complex pathology involving multiple genetic and environmental factors, such as sedentary lifestyle, eating habits, and some genetic polymorphisms related to *β*-cells function and insulin secretion. The disease is usually associated with obesity, insulin resistance, metabolic syndrome and dysfunction of *β*-cells [7,8]. In DM2, the use and storage of carbohydrates are altered, causing compensatory hyperglycemia and hyperinsulinemia which later results in progressive deficiency of insulin secretion [6,7,9]. Persistent hyperglycemia may lead to glucotoxicity, a critical factor in the pathogenesis of long-term complications of diabetes. Associated mechanisms include the harmful effects of advanced glycosylation products and activation of protein kinase C [7,8].

Gestational DM, which only occurs during pregnancy and is related to a degree of glucose intolerance [10,11]. Represents 17.8% of all pregnancies [12], and it is also an important risk factor for type 2 diabetes in women that have been diagnosed with gestational diabetes [13,14].

Glucose levels are usually regulated by insulin at the following restricted limits: 60-100 mg/dl. Diabetes *mellitus* is not a simple pathology, but rather a set of metabolic alterations that share a common characteristic: hyperglycemia. This pathology is caused by changes in insulin secretion and/or action [2,6-8]. Since hyperglycemia and hyperinsulinemia coexist for a long-time due to insulin resistance (IR) in peripheral tissues, hyperglycemia is the most prominent clinical syndrome that characterizes DM2. Patients require insulin treatment only when their cells are out-of-function and cause a deficiency of endogenous insulin [15].

There are many ways that are used to diagnose diabetes [2], glycated hemoglobin (HbA1c), Fasting Plasma Glucose (FPG) and Oral Glucose Tolerance Test (OGTT).

Measurement of glycated hemoglobin is essential to the supervision of patients with diabetes. HbA1c is used to screen long-term glycemic control, adjust therapy, assess the quality of diabetes care and predict the risk for the development of complications. HbA1c is a specific glycated hemoglobin that results from the attachment of glucose to the N-terminal valine of the hemoglobin β-chain [16].The HbA1c test measures the average blood glucose for the past 2 to 3 months, and diabetes is diagnosed when HbA1c is greater than or equal to 6.5% [2].

FPG test measures fasting blood glucose levels, which means that the subjects had nothing to eat or drink (except water) for at least 8 hours before the test. Diabetes is suspected if fasting blood glucose is higher than or equal to 126 mg/dl.

The OGTT test is used to determine whether the body has difficulty metabolizing the intake of sugar/carbohydrates. The patient is asked to ingest a high glucose drink and their blood glucose level is measured before and at time points after the sugary drink is taken. Further blood samples may be taken either at regular intervals of say 30 or 60 minutes or one single test after 2 hours. Diabetes is diagnosed at 2h blood glucose of upper than or equal to 200 mg/dl [2].

Table 1- Blood glucose levels to diagnose diabetes mellitus [17].

	HbA1c	FPG	OGTT
Normal	< 5.7% (< 39 mmol/L)	< 100 mg/dl (< 5.6 mmol/L)	< 140 mg/dl (< 7.8 mmol/L)
Prediabetes	5.7-6.4% (39-47 mmol/L)	100-125 mg/dl (5.6-6.9 mmol/L)	140-199 mg/dl (7.8-11.0 mmol/L)
Diabetes	≥ 6.5% (> 48 mmol/L)	≥ 126 mg/dl (> 7.0 mmol/L)	≥ 200 mg/dl (> 11.1 mmol/L)

HbA1c = glycated hemoglobin; FPG = Fasting Plasma Glucose; OGTT = Oral Glucose Tolerance Test

Chronic hyperglycemia and the occurrence of metabolic imbalances may be associated with secondary lesions in several organs [6-8]. This metabolic imbalance is caused by two main factors, the pancreas not being able to produce enough insulin or the cells not responding to the insulin produced by the pancreas, or both. It is about quantity and quality of insulin that the pancreas produces [10,18]. On the other hand, intermediate hyperglycemia consists of the presence of high glucose levels that do not reach the values of diabetes but greatly increase the risk of developing the disease [6-8]. The chronic hyperglycemia of diabetes is associated with long-term damage, dysfunction and failure of various organs, macro- (damage to larger arteries) and microvasculature (damage to small blood vessels), eyes, kidneys, nerves, heart and blood vessels [10,15]. Therefore, more work is needed to better understand the mechanisms in diabetic patients to allow earlier diagnoses and provide better strategies for diabetes treatment [15]. Also, associated with this condition are the high triglyceride and cholesterol levels in blood (dyslipidemia), that could increase the vascular wall injury and the pro-thrombotic state. When the hemostasis of the body is out of balance, high pressure in the arteries occurs and a pro-inflammatory condition is installed in the organs [19].

Moreover, DM2 is strongly associated with cardiovascular disease and various kinds of cancer as found by several epidemiological and experimental

studies and meta-analyses [20]. There is still much unknown regarding the mechanisms by which DM2 enhances the risk of developing cancer. However, some hypotheses have been presented, such as the presence, in these patients, of hyperglycemia, hyperinsulinemia, oxidative stress, vascular disturbances and a chronic low inflammation state. There may be also site-specific mechanisms in some particular organs, that promote the development of cancer in a specific organ. In fact, there is a growing number of papers that associate diabetes with gastric cancer [21-23].

2. Gastric cancer

Results from GLOBOCAN [24] show that, in 2012, 14.1 million new cases of cancer were diagnosed worldwide (excluding non-melanoma skin cancer) and there were 8.2 million deaths associated to cancer. The incidence and cancer-related deaths were slightly more frequent among men, with a prevalence of 53% and 57%, respectively, than women.

Cancer involves a pathological failure in the processes that controls cell proliferation, differentiation and death. Commonly, the malignant cells form a tumor arise from epithelial tissue – carcinoma – or from glandular epithelium – adenocarcinoma. While having certain characteristics in common, different types of cancers may have very different causes and show widely different responses to treatment [25].

Gastric cancer (GC) is the fifth most common cancer worldwide, only after cancers of the lung, breast, colorectal and prostate, and it ranks as the third leading cause of cancer-related death. Based on GLOBOCAN, almost one million new cases of stomach cancer were estimated to have occurred in 2012 (952,000 cases, 6.8% of the total) [24]. Most GCs are gastric adenocarcinomas, which are malignant epithelial neoplasms. However, GC is a highly heterogeneous entity with respect to patterns of architecture and growth, cell differentiation, histogenesis and molecular pathogenesis [26].

Tumor heterogeneity can be due to genetic alterations, epigenetic events, interactions between tumor cells and the microenvironment, and also interactions

between the different tumor cell clones/populations within the tumor [27]. The heterogeneity of GC is known to vary widely across geographical regions [24], macroscopic and microscopic features, and also molecular alterations [27]. Additionally, phenotypic and molecular variety includes not only interpatient variations, but also intratumor variations [27].

Incidence of GC remains very high in numerous countries from Asia, Latin America and Central and Eastern Europe, whereas in North America and in most Western European countries it is no longer a common cancer [28,29]. In Portugal, despite a steady decline in mortality, [28,30] gastric cancer rates [24] are among the highest in Europe, especially in the North of the country [31].

The fundamental reasons for these geographical differences are likely multi-factorial, and may involve differences in infectious etiology (e.g., *Helicobacter pylori*, Epstein-Barr Virus), environmental risk factors (diet, obesity), and population-specific differences in host genetic polymorphisms [32]. If gastric cancer is detected at an early stage, the 5-years survival is approximately 90% [33], but because there are no specific symptoms at this stage when the disease is surgically curable, most patients have locally advanced or metastatic disease, which has a median survival of 24 month and 5-years survival of less than 15% [32,34,35].

Adequate surgical resection, or endoscopic resection in selected early-stage tumors [36] , are the mainstays of the curative approach. Fluoropyrimidine- and platinum-based chemotherapeutic regimens are recommended in the neoadjuvant or adjuvant setting, and as the first-line treatment in patients with advanced and unresectable GC [37]. Trastuzumab (anti-HER2; human epidermal growth factor receptor 2) [38] and ramucirumab (anti-VEGFR2; vascular endothelial growth factor receptor 2) [39,40] monoclonal antibodies have recently been approved as clinically validated molecular targeted therapies in the treatment of advanced/metastatic GC. However, these therapies offer only a limited survival advantage of a few months (1.5–2.2 months) [38-40] and, to date, except for HER2, there are no predictive biomarkers of tumor response to targeted therapies in GC [41].

The epidemiological and molecular features of gastric cancer differ according to the histological type and location of the tumor [35,42]. Among clinical risk factors for GC, which include smoking, high-salt diet, high intake of meats, and bile reflux, infection with *Helicobacter pylori* is a leading factor [43,44]. On the basis of

improved estimates from prospective studies, 89% of new non-cardia GC cases are attributable to *H. pylori* worldwide [45].

Incidence rates showed differences according to tumor topography [46,47], with upward trends in cardia incidence [48,49]. Cardia tumors, the upper part of the stomach contiguous to esophagus, may be related to gastroesophageal reflux, whereas the majority of non-cardia gastric cancers are attributable to chronic mucosal infection by *Helicobacter pylori* [46]. Apart from *H. pylori* infection, nutritional exposures are implicated as risk factors for noncardia gastric cancer. Consumption of salt is associated with increased incidence, whereas consumption of fresh fruits and vegetables protective role [50].

Lin *et al.* [51] showed, in a population-based cohort study, conducted in the United States, a statistically significant 89% increased risk of gastric cardia (GCA) in diabetics, but no increase in risk of gastric non-cardia (GNCA). Obesity-mediated hyperinsulinemia could be one of the possible mechanisms for this increased risk [52,53] However, this study also showed that the risk remained significantly higher even after adjusting for body mass index (BMI) or when stratified by BMI categories. This result suggests a different carcinogenic mechanism, independent of obesity, for diabetes-related GCA. Recent studies have shown that *H. pylori* infection may be associated with higher risk of GCA also [54]. However, the role of *H. pylori* in GCA was not evaluated.

H. pylori is classified as a carcinogenic agent by the WHO [24], which states that colonization of the stomach epithelia leads to an inflammatory precancerous cascade consisting of chronic gastritis, atrophic gastritis, intestinal metaplasia, dysplasia and finally adenocarcinoma [35]. The prevalence differs with the geographic regions, age, socio-economic status, education level, living environment and occupation [55]. Among infected individuals, approximately 10% develops peptic ulcer disease, 1–3% progresses to GC, and 0.1% develops mucosa-associated lymphoid tissue (MALT) lymphoma [42,56].

Gastric cancers can be divided into different subtypes at the histological level, of which the two most popular classification systems were proposed by Laurén in 1965 [57] and by World Health Organization in 2010 [58] (Table 2). Other classifications were also proposed by Ming and Goseki [59].

2.1. Laurén classification

Since 1965, the Laurén classification of gastric cancer has been the most frequently used and the most studied classification for gastric adenocarcinoma. Laurén separated the histology of gastric cancer (Figure 1) into two groups, the **intestinal type** (clearly defined glandular structures) and the **diffuse type** (consisting of individually infiltrating neoplastic cells). Later, the **indeterminate type** was included to describe an uncommon/mixed histology [57]. Signet ring cell (SRC) carcinoma is included in the diffuse type [59]. In some studies, the intestinal type has been described to be the most common, followed by the diffuse and finally by the indeterminate type [60,61].

The intestinal type is more frequently observed in older patients and follows multifocal atrophic gastritis, that is usually accompanied by intestinal metaplasia and leads to cancer *via* dysplasia. Thus, intestinal metaplasia is considered a dependable morphological marker for gastric cancer risk. The diffuse type occurs more commonly in young patients, can be multifocal, is not often accompanied by intestinal metaplasia and can be hereditary [44].

2.2. World Health Organization (WHO) classification

In 2010, the World Health Organization (WHO) released a classification that appears to be the most detailed of all pathohistological classification systems. This classification includes not only the adenocarcinoma of the stomach but also other types of tumors of the digestive system [58].

The gastric adenocarcinoma type is divided into papillary, tubular, mucinous and poorly cohesive, which includes the signet ring cell carcinoma, and that can be compared to the indeterminate type in the Laurén classification [58]. In the WHO classification, tubular adenocarcinoma is the most common type of gastric cancer, followed by the papillary and mucinous types. The signet-ring cell carcinoma accounts for approximately 10% of gastric cancers and is defined by the presence of signet ring cells in over 50% of the tumor [58].

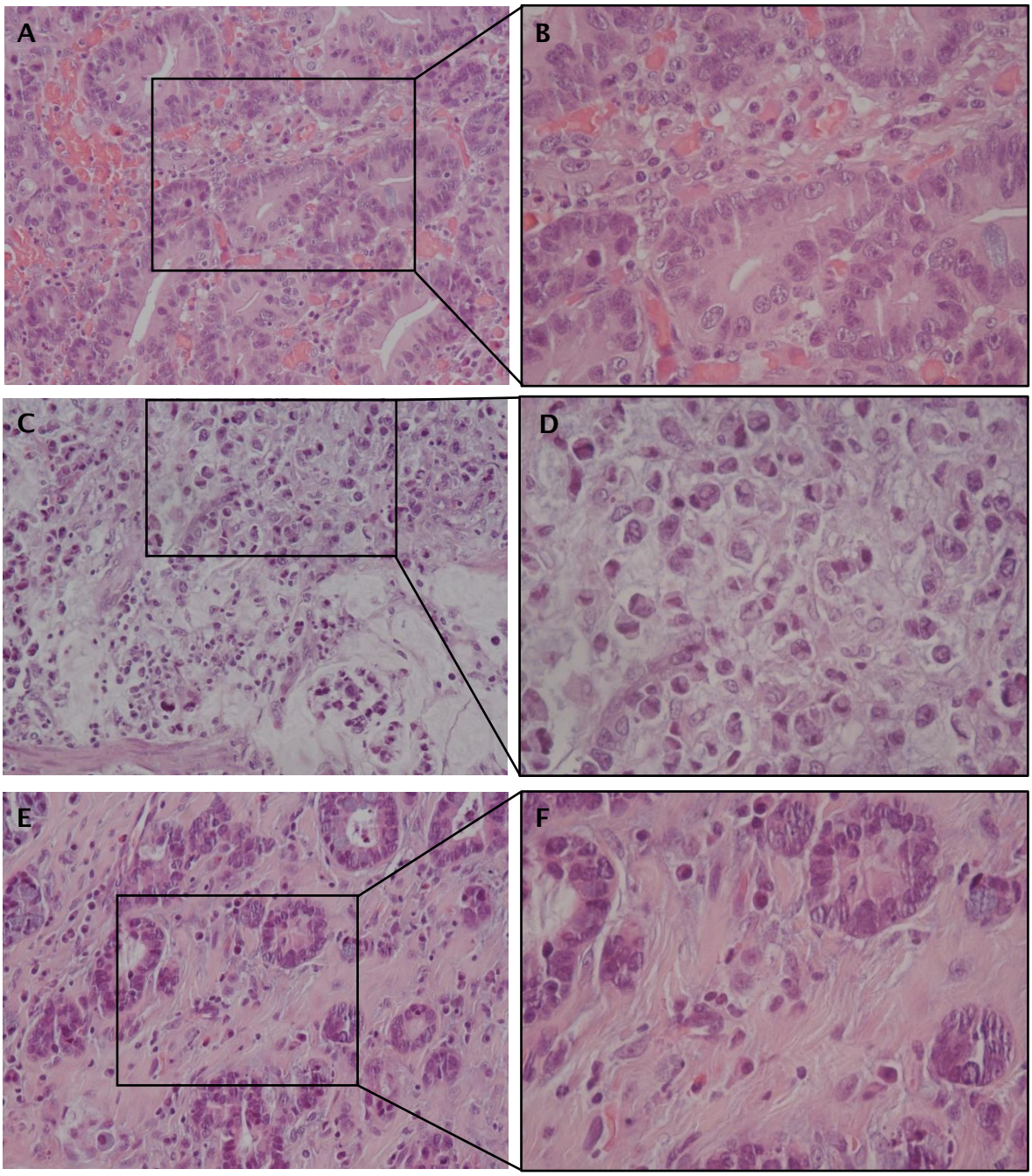


*Figure 1 - Morphological heterogeneity in GC. Several histological patterns are represented according to the Laurén Classification. **A** – intestinal type. HE. Original magnification: x200; **B** – intestinal type. HE. Original magnification: x400. **C** - diffuse type. HE. Original magnification: x200; **D** – diffuse type. HE. Original magnification: x400; **E** - mixed type. HE. Original magnification: x200; **F** – mixed type. HE. Original magnification: x400.*

Table 2 – Heterogeneity of histopathological classification systems in gastric cancer by Laurén and World Health Organization. Adapted from [27].

Laurén Classification	WHO Classification
Intestinal type	Papillary adenocarcinoma
	Tubular adenocarcinoma
	Mucinous adenocarcinoma
Diffuse type	Poorly cohesive, SRC phenotype
	Poorly cohesive, other cell types
Mixed type	Mixed type
Indeterminate type	Undifferentiated
	Rare variants

2.3. Prognostic and predictive biomarkers for gastric cancer

The **tumor-node-metastasis** (TNM), is the global standard to stage solid tumors, extensively used by clinicians, accepted by the American Joint Committee on Cancer (AJCC) and the International Union for Cancer Control (UICC). The system codes the extent of the primary tumor (T), regional lymph nodes involvement (N), and distant metastases (M) and categorized a stage grouping based on T, N, and M [62,63]. TNM classification has become the principal method for prognosis assessment in gastric cancer (Table 3) [64]. Although these variables are, indeed, effective and are widely applied, they cannot provide sufficient prognostic information [65].

Histologically, cancer tissues are composed of parenchyma and stroma, and tumor stroma constitutes a microenvironment for cancer cells. Accumulating evidence proposes that tumor stroma significantly contributes to tumor growth and dissemination [66,67], angiogenesis [68], immune evasion [69,70] and chemotherapy resistance [71]. Stroma to parenchyma ratio in tumor could reflect patient's prognosis, and a high **tumor stroma percentage (TSP)** was found to be associated

with reduced survival in colorectal cancer [72], breast cancer [73] and esophageal cancer[74]. Klintrup *et. al.* and Park *et. al.*, validated the **Klintrup–Mäkinen (KM) grade**, a semi-quantitative evaluation for immune cells at invasive front of tumor, as a useful prognostic marker in colorectal cancer [75,76]. Tumor-infiltrating lymphocytes were found to be associated with a favorable prognosis in gastric cancer with Epstein–Barr virus (EBV) infection [77]. As TSP and KM grade both were effective in prognosis evaluation, Park *et al.* combined these two markers and provide the **Glasgow microenvironment score (GMS)** to increase prognostic utility, and its prognostic value was validated in colorectal cancer [76]. A study from 2017, showed that GMS in gastric cancer was a useful prognostic factor, with a low GMS as a manifestation of better prognosis and an inflammatory tumor microenvironment [65].

There is increasing evidence that host inflammatory response plays an important role in the development and progression of cancer, in particular, by an elevation of C-reactive protein (CRP) levels in serum, that is a key factor in the progression of a variety of common solid tumors [78] and represents a prognostic marker in gastro-esophageal [79,80], urinary bladder [81], pancreatic [82], renal [83,84], and non–small-cell lung [85] cancers, independent of tumor stage [86]. Also, hypoalbuminemia has been reported to be associated with poor survival in advanced cancers [87,88]. McMillan described the combination of these two biomarkers as **Glasgow Prognostic Score (GPS),** that associated with tumor stage, as part of the assessment of the patient with cancer, will highlight the need not only to treat the tumor, but also the systemic inflammatory response [86].

The standard imaging modalities used for the preoperative staging of gastric cancer include computed tomography (CT) and endoscopic ultrasonography (US). Endoscopic US is regarded as the most accurate imaging tool for evaluating tumor depth, and CT is the principal imaging modality used for staging because of its ability to detect distant metastases. Magnetic resonance imaging (MRI) and diagnostic laparoscopy are other imaging tools that can be successfully used to stage gastric cancer [89].

Since the different biological behavior between intestinal type and diffuse type, researchers were concentrated in the epigenetic regulation and prognostic biomarkers between these two categories. Currently, a clinically molecular marker for GC is genomic amplification and overexpression of HER2, that occurs in around

15% of patients with GC, with the proportion of 33% in intestinal type and 6% in diffuse type [26,32,60]. The prognostic value of HER2 positivity in advanced GC is still a controversial issue. Some studies indicated that HER2 amplification is associated with a poor prognosis and aggressive disease [90-93], while others showed no difference in prognosis when comparing with HER2 positive and negative tumors [94-96]. Qui and colleagues suggested that the combination of Laurén classification and HER2 status is a better prognostic factor in gastric cancer patients. HER2 negative intestinal type gastric cancer patients presented a better survival comparing to HER2 positive diffuse type gastric cancer patients [97]. Investigations of biological biomarkers and genetic therapeutic targets should be continuous in gastric cancer treatment [98].

Table 3 - T-staging of gastric cancer.

TX	**Primary tumor cannot be assessed**
T0	No evidence of primary tumor
Tis	Carcinoma *in situ*: intraepithelial tumor without invasion of the lamina propria
T1	Tumor invades the lamina propria, muscularis mucosae, or submucosa
T1a	Tumor invades the lamina propria or muscularis mucosae
T1b	Tumor invades the submucosa
T2	Tumor invades the muscularis propria
T3	Tumor penetrates the subserosal connective tissue without invasion of the visceral peritoneum or adjacent structures. T3 tumors also include those extending into the gastrocolic or gastrohepatic ligaments or into the greater or lesser omentum, without perforation of the visceral peritoneum covering these structures
T4	Tumor invades the serosa (visceral peritoneum) or adjacent structures
T4a	Tumor invades the serosa (visceral peritoneum)
T4b	Tumor invades adjacent structures, such as the spleen, transverse colon, liver, diaphragm, pancreas, abdominal wall, adrenal gland, kidney, small intestine, and retroperitoneum

3. Diabetes and gastric cancer

Cancer and DM are very common diseases that have heterogeneous developmental pathways. Increasing incidence trends of both these diseases may at least partly be due to the shared risk factors and mechanistic pathways [99]. Shared risk factors include both non-modifiable (age, gender and race) and modifiable risk factors (obesity, diet and smoking). Experimental and epidemiological evidence indicates that DM is associated with several types of cancer [20], including gastric [100,101], breast [102-104], ovarian [105], liver [106,107], pancreas [108-112], esophageal [113], colorectal [114-117], endometrial [118,119], kidney [120,121], non-Hodgkin lymphoma [122,123] and urinary bladder [124].

Shared risk factors, such as obesity [125-127], hyperinsulinemia [128,129], diet and smoking [130-132], **hyperglycemia** [133,134], ***H. pylori* infection** [130,131,135], **salt intake** [130,136], and **medications** [130,137], may be the possible link between diabetes and cancer.

DM2 is characterized by metabolic changes in the body (hyperglycemia, dyslipidemia, insulin resistance), which associates with pathological processes such as chronic inflammation, oxidative stress, angiogenic imbalances and tissue fibrosis that could be some of the shared mechanistic processes between DM and GC [101,138-140]. Hyperinsulinemia, between these factors, is considered to be the primary mechanism of shared risk of DM and cancer. Insulin has been shown to have both metabolic and mitogenic capabilities. Hyperinsulinemia, either through insulin resistance or through insulin-like growth factor (IGF) 1 system, may result in up-regulation of the mitogenic and anti-apoptotic effect, thus mediating the cancer initiation and progression [138]. Hyperglycemia, on the other hand, has been shown to support carcinogenesis indirectly by increasing insulin production and by providing glucose for energy metabolism of cancer cells. Obesity has also been shown to indirectly increase the risk of cancers due to increased hyperglycemia, insulin resistance and inflammation [101]. Most of these shared factors and mechanisms are not site specific and would be common to many cancer sites. If the association between DM and cancer is only because of these common factors, the increased risk should be consistent across most anatomical sites [131].

3.1. Shared risk factors

Obesity is often linked with glucose intolerance, insulin resistance and dyslipidemia [141]. Serum insulin and IGF-1 are thus higher in obese people as compared to healthy lean individuals, and have been reported to increase the risk of some types of cancer [142]. In fact, patients with cancer that have pre-existing DM2 have worse prognosis and have higher mortality rates than ones who had a normal glycemic values [143]. The mechanisms by which these molecules affect cancer are yet undetermined. However, a cross talk between these and PI3K/mTOR and Ras signaling pathways, two transduction pathways often implicated in cancer cells, was established [144], explaining their effects in inhibiting apoptosis, and stimulating tumor proliferation [145]. The fact that insulin and IGF-1 are overexpressed in obesity, leads us to assume that obese patients are much more predisposed to develop more aggressive tumors than lean subjects.

Hyperinsulinemia, especially when patients had insulin resistance, is one more possible mechanism of gastric carcinogenesis in DM [146-148]. Since insulin has a mitogenic effect, it is tempting to speculate that hyperinsulinemia may be involved in gastric mucosal proliferation where genetic alterations are inducible. Increased insulin levels will activate mitogenic pathways through its binding to its receptor (IR) and to insulin and IGF-1 hybrid receptors (IR/IGF-1R). The binding of the hormone to these receptors in cancer cells will eventually activate MAPK and the PI3K/Akt oncogenic pathway [149]. Moreover, hyperinsulinemia may also have an indirect effect in the development of cancer, by increasing IGF-1, an important growth factor in cancer, which role is regulating the cellular growth and the survival of transformed cells [150] and decreasing IGF binding proteins (IGFBs). In fact, it is thought that the mitogenic effects of insulin are mediated by IGF-1, since it's the activation of IGF-1R that stimulates cell growth and proliferation.

Smoking is another risk factor for DM [151] and GC [152]. Because of the higher risk of gastric cancer associated with diabetes is still significant after adjustment for smoking. The link between these diseases can also be independent of smoking [133,134].

3.2. Hyperglycemia

Hyperglycemia may also promote carcinogenesis *via* increasing reactive oxygen species resulting in DNA damage [153] or increasing the expression of vascular endothelial growth factor, which is correlated with tumor vascularity and metastasis [154]. The VEGF/VEGFR system is also been showed to interact with the IGF/IGF-IR axis in various tumors including gastrointestinal ones [155,156]. As cancer cells have lesser capacity in using glucose for energy expenditure, and they may consume more glucose than normal cells (the Warburg effect), hyperglycemia provides a more suitable condition for tumor cells to grow [157].

Some studies, *in vivo* and *in vitro*, support the association between hyperglycemia and gastric cancer, possible *via* β-catenin acetylation with increased Wnt signaling [158], which is also characteristic of gastric cancer [159]. Expression of pro-inflammatory cytokine such as interleukin-1, interleukin-6 and tumor necrosis factor-α had an increased expression in DM patients [160]. It is also shown that these factors may upregulate and activate the Wnt/β-catenin pathway [161].

Additionally, an interaction between hyperglycemia and *H. pylori* infection was reported to markedly increase the risk of gastric cancer [21].

3.3. *H. pylori* infection

H. pylori infection is well known as a risk factor for gastric ulcer and cancer [152,162,163]. *H. pylori* may increase the risk of GC either directly through mutagenic or protein modulatory effect on the epithelium or indirectly by induction of inflammatory process in the epithelium [164-166]. However, not all individuals infected with *H. pylori* develop GC, suggesting the requirement of other cofactors to aid *H. pylori* mediated carcinogenic process. Patients with diabetes may have a higher infection rate, a lower eradication rate, and/or a higher reinfection rate of *H. pylori* [167-169]. *H. pylori*, through gastrin secretion, can increase the glucose-stimulated insulin release, thereby resulting in hyperinsulinemia [170]. It has also been shown that *H. pylori* independently promotes insulin resistance and related oxidative stress, thereby also possibly facilitating the hyperinsulinemia-mediated carcinogenic process in the gastric mucosa [171,172] Insulin resistance, induced by *H. pylori* infection, may also accelerate β-cell loss and lead to diabetes. Therefore,

insulin deficiency, as well as insulin resistance, might be seen in chronic *H. pylori* infection [173,174].

On the other hand, the active chronic inflammation induced by *H. pylori* infection may also increase the risk of DM2, because it may affect the normal secretion and function of insulin leading to glucose dysregulation [171,173,175].

3.4. Salt intake

Evidence has been provided some strong associations between gastric cancer and a few dietary factors, among which is the dietary salt content and the habitual consumption of salt-rich foods [176]. Many case-control studies detected an adverse effect of high salt consumption on the risk of gastric cancer [177].

A recent meta-analysis of these studies has shown that dietary salt intake is directly associated with the risk of gastric cancer, with progressively increasing risk across increasing levels of habitual consumption [178]. This epidemiological evidence is supported by the results of clinical and experimental studies which found that high salt intake may alter the viscosity of the gastric protective mucous barrier [179] and increase the colonization by *Helicobacter pylori* , a recognized risk factor for gastric cancer [180]. High intra-gastric sodium concentrations were shown to cause mucosal damage and inflammation, which in turn has been reported to increase cell proliferation and endogenous mutations [181,182].

3.5. Anti-diabetic drugs and cancer

The function of the major classes of DM drugs is replace the circulating insulin and reduce hyperglycemia by different mechanisms [183]. Agents which treat or prevent DM2 might be expected to influence risk of cancer favorably. The insulin sensitizers, metformin, and thiazolidinediones (TZDs) are promising cancer therapies [139].

In particular, metformin, the drug of choice for the management of DM2 [184], reduces levels of both circulating glucose and insulin in patients with insulin resistance and hyperinsulinemia, by altering signaling through the AKT/mTOR pathway [185,186]. The mechanisms for the anti-tumor effects of metformin include an inhibition of cell proliferation, decrease cancer proliferation, with partial cell-cycle

arrest in oncogenic cell lines with the activation of 5' adenosine monophosphate-activated protein (AMP) and AMP-kinase (AMPK) [101]. AMPK is an essential mediator of the tumor suppressor LKB1. Because of its properties, AMPK could be used to suppress cancer cells containing loss-of-function LKB1 mutations, active B-Raf mutations, or in cancers associated with metabolic syndrome [139].

Thiazolidinediones, bind to peroxisome proliferator-activated receptor (PPAR) gamma (γ) receptor molecules inside the cell nucleus which, when activated, result in transcription of a variety of genes. PPAR γ is an adipocyte transcription factor, stimulating differentiation of adipocytes as well as inhibiting inflammatory cytokine production [187]. This class of drugs reduces insulin levels by enhancing insulin action. PPAR-γ activation results in reduced free fatty acids and eicosanoids and inhibition of VEGF-induced angiogenesis, amongst other actions [188]. Like metformin, TZDs inhibit cancer cell growth, potentiation, and proliferation, inducing apoptosis, at the in vitro level [101,189].

However, some studies duggest that anti-diabetic drugs may also have effects on the risk for cancer. Some studies shows that insulin sensitizers such as metformin and TZDs are associated with prostate cancer [190] and HER2-positive breast cancer [191] among diabetic patients. Nonetheless, it is more expected that diabetic patients, who are treated with insulin or insulin secretors, develop cancer than those with metformin [192-195].

3.6. Biological links between diabetes and gastric cancer

Chronic inflammation, angiogenesis, fibrosis and oxidative stress are also considered to be some of the shared mechanistic processes between diabetes and cancer, *via* their influence on neoplastic processes [99,196].

Clinical studies also showed an increased risk of diabetes being associated with different types of cancers [197]. In diabetic patients with a deregulated glucose metabolism, were usually found increased levels of chronic inflammatory markers, e.g., interleukin (IL)-1, IL-6, and tumor necrosis factor (TNF)-α, and emerging evidence has highlighted activation of the immune response in the progression and development of cancer cells. Consequently, uncontrolled proinflammatory responses could possibly create a chronic inflammatory state, promoting a

satisfactory microenvironment for tumor and potentially promoting immune over-activation and cancer growth [15].

Chronic inflammatory condition associated with diabetes, which usually persists for years to decades, may increase inflammation-caused ROS by reducing intracellular antioxidant activities [198]. Oxidative stress, has long been recognized as an adverse event for promoting tumorigenesis and cancer progression [199,200]. Exacerbated levels of ROS can irreversibly modify protein, lipid and DNA molecules, and permanently or temporarily change their cellular behavior, leading to the accumulation of somatic DNA mutations, proto-oncogenes activation and epigenetic alterations. Conversely, increased signaling *via* AKT, RAS\RAF\ERK and NF-κB pathways, constitutively activated signaling cascades in most malignant cells, stimulate endogenous ROS production, further enhancing oxidative damages within the tumor microenvironment [201,202]. Furthermore, various types of cells present in the tumor microenvironment can generate not only ROS but also reactive nitrogen species (RNS). Tumor associated macrophages are effective producers of ROS and RNS and further contribute to a pro-oxidant environment [203]. Another potential mechanism is related to a proinflammatory and pro-tumorigenic cytokine - TNF-α -, produced by adipose tissues [204], that plays a significant role in cancer formation and cancer development by upregulating the transcription factor, NF-κB [205]. NF-κB is known to be involved in the association between cancer and inflammation, by manipulating cytokine production and even the angiogenesis potential in immune-responsive cells.

Studies which showed the correlation between chronic inflammation and hyperglycemia [206] portray possible mechanisms at the intersection of obesity, hyperglycemia, diabetes, chronic inflammation, and cancer development. Over-activated proinflammatory cytokines continuously drive cell survival and induce a proinflammatory response, which could conceivably create a chronic inflammatory state, resulting in a pro-tumor microenvironment.

Angiogenesis is a global term associated with the physiological process involving the growth of new blood vessels or neovascularization. Angiogenesis is an important step in the transition of tumors from a confined local to malignancy [207]. Neovascularization or angiogenesis has also been interchangeably associated with *vasculogenesis* which primarily refers to developmental formation of vascular structures from circulating or tissue-resident endothelial progenitor cells that

proliferate into *de novo* endothelial cells. Angiogenesis predominantly relates to formation of endothelium-lined microvasculature with supportive cells. These processes require several biochemical and physiological factors to stimulate vessel sprouting and remodeling of the primitive vascular network, which in turn establish stable and functional blood vessel networks. There are several angiogenic factors which are involved in stimulation, promotion, and stabilization of new blood vessels such as the family of vascular endothelial growth factors (VEGFs) which comprises VEGF-A, VEGF-B, VEGF-C, VEGF-D and placental growth factor (PlGF) [208].

VEGF-A is the most potent angiogenic factor of VEGF family, and exerts its action through the activation of its main receptor, VEGFR-2 and target proteins promoting vascularization [209,210]. Hypoxia, inflammation and oxidative stress, are among the principal angiogenic stimuli presented in both diabetes and cancer. In response to angiogenic stimuli, endothelial cells (EC) proliferate and migrate to avascular areas. In this situation, EC at the front of migrating vessel rely on glycolysis to produce ATP for rapid generation of energy and sprout [211]. The EC glucose uptake is mediated through the activation of the PI3K-AKT pathway, promoting the expression of glucose transporters, mainly GLUT-1 [212]. It has been reported that the 6- phosphofructo-2-kinase-2/fructose-2,6-bisphosphatase 3 (PFKFB3) enzyme expression is induced by hypoxia in avascular areas in several cell lines [213] and human cancer cell [214]. PFKFB3 is a bifunctional enzyme abundant in EC, that plays an important role in ensuring the high glycolytic flux, essential for vessel growth[211].

Recently, Schoors and collaborators demonstrated the therapeutic potential of endothelial PFKFB3 regulation by inhibiting PFKFB3 *in vitro* and silencing PFKFB3 *in vivo* and the resultant effect was a 30-40% decrease in the glycolytic pathway sufficient to reduce angiogenesis [215]. These data suggest that anti-angiogenic therapies could be based on the inhibition of this metabolic target, starving pathological vessels. Thus, PFKFB3 expression emerges as an important regulator of the endothelial phenotype, postulating that a metabolic switch induces the angiogenic switch and could be an important strategy to counteract the increased tumour vascularization.

Fibrotic tumour microenvironment is associated to poor prognostic, influencing tumour progression and invasion, and hampering the efficacy of chemotherapy [216-218]. Different cell types contribute to the fibrosis stage tumour

microenvironment, including fibroblasts, leucocytes such as neutrophils, lymphocytes and macrophages, and adipocytes [219]. In a fibrotic tumor stroma, the abnormal deposition of collagen and impairment in degradation of these proteins leads to decrease in flexibility and increased stiffness and density of ECM, which lead to changes mechanical forces and in cellular signaling, facilitating survival and migration of the cancer cells [217,220].

Angiogenesis imbalance, chronic Inflammation, oxidative stress, that are some of the putative biological links between DM2 and GC, could be the key of the development of new therapeutics against DM2 and GC.

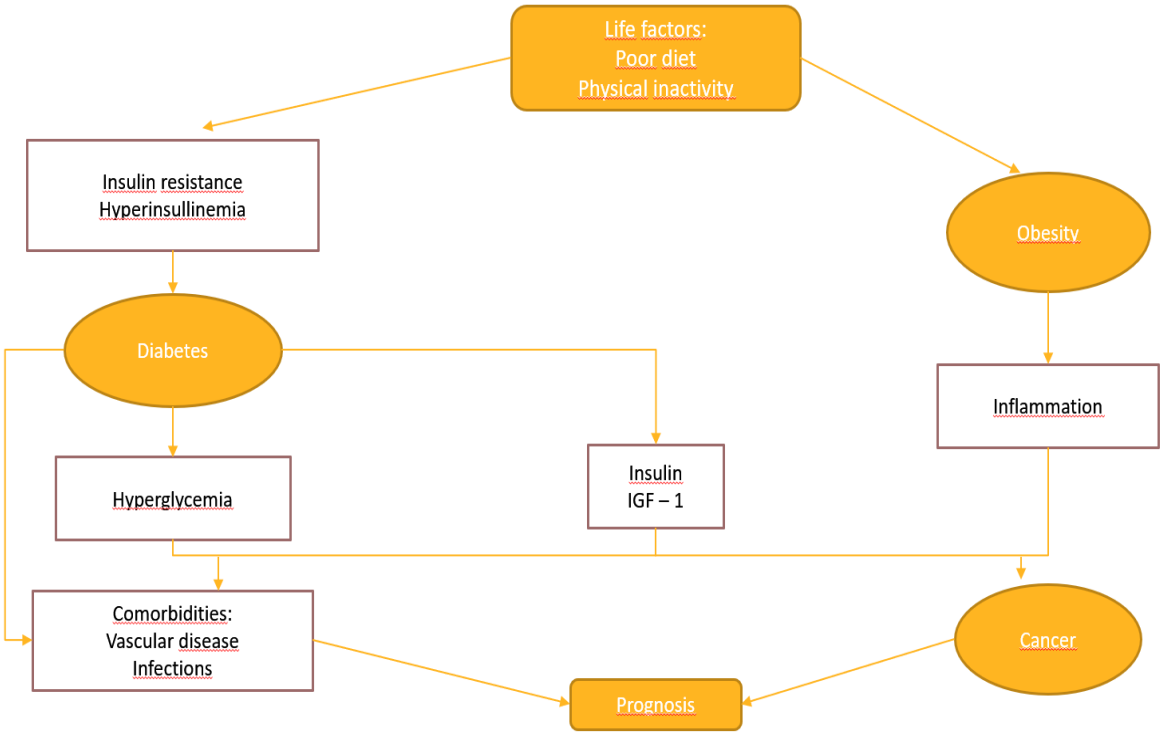


Figure 2 - Interrelationship between pathological mechanisms and modifiable and non-modifiable risk factors involved in diabetes, obesity and cancer. IGF, insulin-like growth factor.

Aims

The main aim of this thesis was to investigate the mechanisms that may justify the association of DM2 and GC, and possibly helps us identify a sub-group of GC patients that is highly associated with DM2, and may in the future, beneficiate from more metabolic focused therapies.

Taking advantage of the hospital tumor bank, we will identify inflammatory and angiogenic pathways, often imbalanced in diabetic subjects, that are differentially expressed in GC patients with DM2.

Investigate the role of a new prognostic score associated to diabetes and gastric cancer patients.

Material and Methods

1. Study design

Approval for this study was obtained from the Ethical Committee of Centro Hospitalar São João, Porto, Portugal.

This study included 43 individuals diagnosed with gastric cancer. The diagnosis was confirmed pathologically, after the histopathological examination of either the tissue or the endoscopic biopsy. Of these, 22 patients had DM2 clinically diagnosed by an endocrinologist and were on at least one antidiabetic oral drug (metformin). All the patients with a confirmed gastric adenocarcinoma and DM2 were included in the study. Gastric tissues of each patients were obtained from Tumor Bank of Centro Hospitalar São João, Porto, Portugal.

A retrospective review of our gastric surgical database from July 2008 to September 2014 was performed. Pathological and clinical data from our patients were collected.

Two groups were established, one group of patients with only gastric cancer (control group), and other group with gastric cancer and DM2 (diabetic group).

Each patient was extensively characterized regarding both the tumor pathological features and the available clinical data. Inclusion criteria for both groups were not insulin-dependent, did not present any other previous neoplasia, did not receive neoadjuvant chemotherapy, radiotherapy, or both. For diabetic group, additional criteria were only the clinical diagnostic DM2 and prescribed anti-diabetic medication but not have more than two anti-diabetic drugs, simultaneously.

All patient's glycaemias at the moment of surgery and at random time-points were checked to make sure the metabolic state of the patients was accurate. This approach was used to assure that the key difference between groups was the presence or absence of diabetes.

2. Histology review

Full sections of 43 cases of gastric cancer were stained with hematoxylin & eosin (HE) and were scanned in a microscope (Nikon Eclipse 50i, Germany) by a pathologist specialized in gastric cancer. Tumor Stroma Percentage (TSP) was then semi-quantitatively assessed according to the criteria previously reported by Park *et al.* [76]. Briefly, the TSP evaluation was performed by using the most invasive tumor area. Thereafter, TSP was calculated as the percentage of stroma relative to the whole tumor area, and tumors were subgrouped as high TSP (>50%) or low TSP (≤50%). A score 0 was defined as low TSP and score 1 as a high TSP.

For each case, the Klintrup–Mäkinen (KM) grade was evaluated as previously described by Klintrup *et al.* [75] and Park *et al.* [76]. Briefly, overall inflammatory reaction at the invasive margin was scored as:

- 0, no increase of inflammatory cells;
- 1, mild and patchy aggregation of inflammatory cells;
- 2, markedly increased inflammatory cells at the invasive margin formed a band, and some cancer cells might be destructed by inflammatory cells;
- 3, very prominent inflammatory cell infiltration formed a florid-like zone, and destructed cancer cell could be observed invariably.

A score of 0 or 1 was defined as low KM grade, while a score of 2 or 3 was high KM grade. Then, a score 0 was define as low KM and a score 1 as high KM.

In the present study, all cases of gastric cancer were reviewed in a double-blinded fashion by a pathologist with subspecialty training and experience in gastric pathology.

2.1. Assessment of Glasgow Microenvironment Score

The GMS was a combination of the TSP and KM grade, as described previously by Park *et al.*: 0 score, a high KM grade with high or low TSP; 1 score, low TSP with low KM grade; 2 score, high TSP with low KM grade. According to GMS, the 43 cases were stratified into three subgroups.

3. Assessment of GPS

All values of C-reactive protein and albumin were collected and grouped as previously described by [221].

The resultant prognostic score (0, 1, 2) was defined as follows:

- Patients with both an elevated C-reactive protein (>10 mg/l) and hypoalbuminemia (<35 g/l) were allocated a score 2;
- Patients in whom only one of these biochemical abnormalities was present were allocated of 1;
- Patients in whom neither of these abnormalities was present allocated a score 0.

4. Fibrosis evaluation

To label collagen components within cancer tissues, the tissue sections were stained with 0.5% Sirius Red (Direct Red 80; Sigma-Aldrich). Then the slides were observed and images were taken using Nikon Eclipse 50i microscopy. Thereafter, images of each tissue core were thresholded with ImageJ (version 1.48v, NIH, Bethesda USA), and pixel densities of collagen components were detected.

5. Microvessel density evaluation

5.1. Immunohistochemistry

CD31 has cytoplasmic, membranous expression in non-neoplastic and neoplastic vascular endothelial cells [222]. It has been used as a tool to identify the vascular origin of neoplasms such as angiosarcomas, Kaposi sarcomas and epithelioid hemangioendothelioma [222,223]. Immunohistochemical study against CD31 has also been shown useful to detect areas of tumor lymphovascular invasion [224]. Additionally, detection of weak diffuse cytoplasmic CD31 immunoreactivity has

been seen in cases of various carcinomas with occasional membranous staining in ductal carcinomas of the breast as well as in intratumoral macrophages [225,226]

The gastric tissue sections were processed on an automatic staining equipment BenchMark ULTRA System (Ventana Medical Systems) using the mouse monoclonal antibody, anti-CD31 (Cell Marque (JC70)). Each slide was individually processed, starting by heating the blades to 72˚C, followed by deparaffinized with the EZprep (Ventana) buffer solution. For antigen-retrieved was used the Cell Conditional 1 (CC1) buffer solution, consisting of Tris/Borate and Ethylenediaminetetraacetic acid (EDTA), pH 8.4, 24 minutes at 100˚C. Inhibition of endogenous peroxidase was carried out with a commercial solution of 0.04% hydrogen peroxide (H_2O_2) in phosphate-buffer solution (Ventana). Thereafter, the tissue sections were incubated with the primary antibody for 44 minutes at 37˚C. Subsequently, washes were performed with the reaction buffer (Ventana), a wash buffer solution, pH 7.6, used for the washes between the various processing steps. The tissue sections were then incubated with the detection kit OptiView Universal Diaminobenzidine Tetrahydrochloride (DAB) (Ventana). The OptiView Universal DAB (Ventana) use a set of synthetic molecules, to detect the antigen-antibody interaction. This system consists of 6 reagents: OptiView peroxidase inhibitor, OptiView HQ universal linker, OptiView HRP multimer, OptiView H_2O_2 and OptiView Copper. The kit uses first the OptiView HQ universal linker, a cocktail of secondary antibodies (anti-rat and anti-rabbit), which binds to the primary antibody previously linked to the antigen. The HRP multimer OptiView, a tertiary antibody, also referred to as multimer, is then conjugated to an HRP peroxidase enzyme which recognizes the secondary antibody. HRP enzymes conjugated to the multimer react with the hydrogen peroxide substrate (OptiView H_2O_2), and with OptiView Copper, oxidize the OptiView DAB chromogen, giving rise to a brown precipitate. Subsequently, the tissue sections were contrasted with Hematoxylin for 8 minutes and Bluing Reagent (Ventana, Roche) for 4 minutes. Bluing reagent is an alkaline solution that enhances the nuclear contrast, making the nuclei more bluish.

5.2. Immunohistochemistry Scoring

The number of vessels was counted in three photos for each of three hotspots of vessels in tumor and adjacent area of the tissue sections for each patient. A negative control was included. Any positive-stained EC or cluster that was separated from adjacent vessels was considered an individual vessel.

6. Statistical analysis

Qualitative variables were expressed as number of cases and percentage (%), and the quantitative variables are expressed as mean and standard error of the mean. The D'Agostino-Pearson test was used to verify the normality of the data.

The difference between two independent experimental groups was evaluated using the unpaired Student t test for normally distributed variables, and the Mann-Whitney U test for variables that did not meet the normal parameters.

To compare 3 or more independent groups with normal distribution we used a simple analysis of variance (one-way ANOVA) with post-hoc Tukey's test. Kruskal-Wallis ANOVA with Dunns post hoc was used to compare 3 or more groups when a sample did not meet the criteria of normality.

To compare 2 or more nominal variables, we used a chi-square test. To evaluate whether diabetes status (yes versus no) might be associated with gastric cancer survival at 2 and 5 years, odds ratios and their 95% confidence intervals were calculated. We performed Kaplan-Meier (K-M) analysis to test if diabetic status, GPS and GMS were significant for prognosis or prediction for 2 and 5-year survival.

A p value <0.05 was considered statistically significant and <0.01 very significant. All statistical analyses were performed with the aid of the GraphPad Prism software version 7.00 and IBM SPSS Statistics version 24, both for Windows.

Results

1. Cohort characteristics

In the studied cohort, patients were paired by their demographic and clinicopathologic features, except on diabetic parameters' such as blood glucose (Figure 3) and diabetic specific medication.

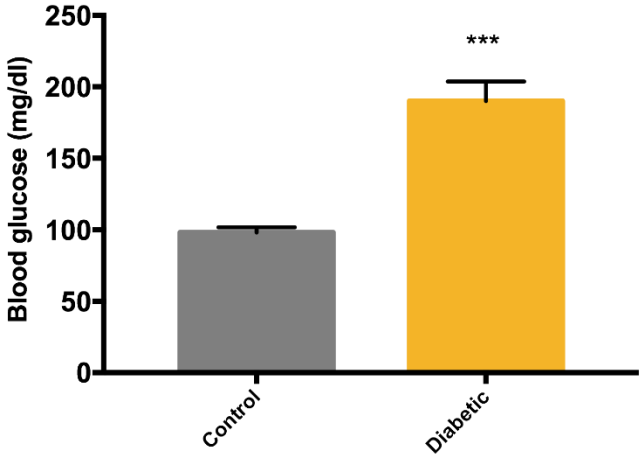


*Figure 3 - Blood glucose at the surgery. Data represents mean ± SE. *** p < 0.01.*

Patients characteristics are shown in Table 4.

In the control group, 66.7% had more than 70 years and 66.7% was female. Of the 21 patients, 52.4% had intestinal type of gastric cancer. 4.8% had diffuse type, 33,3% had mixed type and 9.5% had indeterminate type. The lower part of the stomach was the most affected in this group (71.4%), and 71.4% of tumors was bigger than 5 cm. Only 3 (14.3%) of the 21 patients had recurrence of the disease.

In diabetic group, 81.8% had more than 70 years and 68.2% was female. Of the 22 patients, 40.9% had intestinal type of gastric cancer, 4.5% had diffuse type, 27.3% had mixed type and 27.3% had indeterminate type. The lower part of the stomach was also the most affected in this group (68.2%). 31.8% of the diabetic patients had recurrence of the disease.

Table 4 – Demographic and clinicopathological characteristics of the studied cohort (n=43).

		Group				
		Control		Diabetic		*p*
		N	%	N	%	
Gender	**Female**	14	66.7%	15	68.2%	ns
	Male	7	33.3%	7	31.8%	
Age	**<70**	7	33.3%	4	18.2%	ns
	>70	14	66.7%	18	81.8%	
Laurén	**Intestinal**	11	52.4%	9	40.9%	ns
	Diffuse	1	4.8%	1	4.5%	
	Mixed	7	33.3%	6	27.3%	
	Indeterminate	2	9.5%	6	27.3%	
pT stage	**T1**	1	4.8%	2	9.1%	ns
	T2	2	9.5%	2	9.1%	
	T3	13	61.9%	12	54.5%	
	T4	5	23.8%	6	27.3%	
pN stage	**N0**	6	28.6%	10	45.5%	ns
	N1	3	14.3%	4	18.2%	
	N2	4	19.0%	3	13.6%	
	N3	8	38.1%	5	22.7%	
M stage	**Mx**	20	95.2%	18	81.8%	ns
	M1	1	4.8%	4	18.2%	
Stage	**I**	2	9.5%	3	13.6%	ns
	II	6	28.6%	9	40.9%	
	III	13	61.9%	10	45.5%	
Lymphatic invasion	**No**	5	23.8%	5	22.7%	ns
	Yes	16	76.2%	17	77.3%	
Vascular invasion	**No**	7	33.3%	9	40.9%	ns
	Yes	14	66.7%	13	59.1%	
Neural invasion	**No**	8	38.1%	8	36.4%	ns
	Yes	13	61.9%	14	63.6%	
Location	**Upper**	2	9.5%	1	4.5%	ns
	Middle	3	14.3%	4	18.2%	
	Lower	15	71.4%	15	68.2%	
	Diffuse	1	4.8%	2	9.1%	
Size	**<5 cm**	6	28.6%	7	31.8%	ns
	>5 cm	15	71.4%	15	68.2%	
R state	**R0**	18	85.7%	20	90.9%	ns
	R1	3	14.3%	1	4.5%	
	R2	0	0.0%	1	4.5%	
Recurrence	**No**	18	85.7%	15	68.2%	ns
	Yes	3	14.3%	7	31.8%	

2. Significance of DM2 for GMS, GPS and outcome of GC

There is increasing evidence that systemic inflammation and inflammatory processes in the tumor microenvironment play an important role in the development of several tumors [227,228]. In brief, in this study we investigated the prognostic relevance of GMS, GPS and evaluated the patients' outcome.

2.1. DM2 and GMS

GMS was a combination of KM and TSP markers, such as described in Material and Methods chapter.

The scoring of TSP and KM to assess the GMS score was shown in Table 5.

In the KM score distribution between control and diabetic group appears to be skewed in the opposite direction, in score "0" (36.4% vs 61.9% respectively), and in score "1" (38.1% vs 63.6%, respectively), although the difference is not significant.

In the TSP score, the distribution of diabetic patients through different scores, 0 and 1, was statistically different compared with control group (p=0.021). In control group, the distribution of patients seems to be more homogeneous between the TSP scores (57.1% vs 42.9%, for "0" and "1", respectively). In diabetic patients, this distribution tends to score "1" compared with score "0" (77.3% vs 22.7%, respectively).

In the GMS score, the distribution of diabetic patients through different scores, 0, 1and 2, was statistically different compared with the control group (p=0.045). In the control group, the distribution of patients seems to be more homogeneous between the different scores (38.1% vs 33.3% vs 28.6%, for "0", "1" and "2", respectively). In diabetic patients, this distribution tends to score "0" compared with score "1" and "2" (63.6% vs 4.5% vs 31.8%, respectively).

Table 5 - Assessment of Glasgow Microenvironment Score of control group (n=21) and diabetic group (n=22).

		Group				*p*
		Control		Diabetic		
		N	%	N	%	
KM	0	13	61.9%	8	36.4%	ns
	1	8	38.1%	14	63.6%	
TSP	0	12	57.1%	5	22.7%	0.021
	1	9	42.9%	17	77.3%	
GMS	0	8	38.1%	14	63.6%	0.045
	1	7	33.3%	1	4.5%	
	2	6	28.6%	7	31.8%	

2.2. DM2 and GPS

To assess the GPS score we analyzed the systemic albumin and C-reactive protein values of each patients and results are showed in Table 6.

The patients' distribution by the different scores of GPS, in both groups of the studied cohort, seems to be similar.

Table 6 - GPS scoring of control group (n=21) and diabetic group (n=22)

		Group				*p*
		Control		Diabetic		
		N	%	N	%	
GPS	0	8	38.1%	8	36.4%	ns
	1	5	23.8%	4	18.2%	
	2	8	38.1%	10	45.5%	

2.3. DM2 and GC outcome

DM2 does not appear to be a risk factor for patient survival at 2 and 5 years (Figure 4).

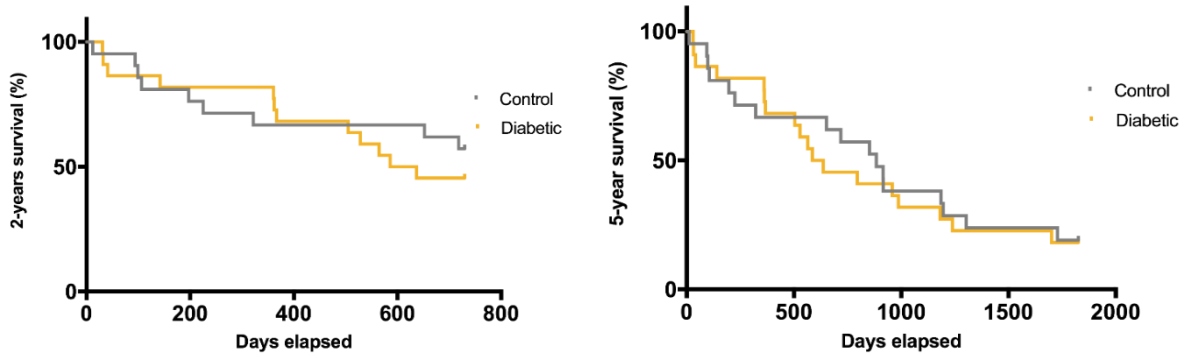


Figure 4 – Kaplan-Meier survival curves of the studied cohort at 2 and 5-years

Table 7 - Overall survival of the studied cohort

Group	N	2-year OS (%)	Odds ratio (95% CI)	p	5-year OS (%)	Odds ratio (95% CI)	p
Control	21	57.14	1.60 (0.48 – 5.34)	ns	19.05	1.06 (0.23– 4.92)	ns
Diabetic	22	45.45			18.18		

In stratification of the patients by GMS score, the difference of patients' survival at 2 and 5 years, was not significant.

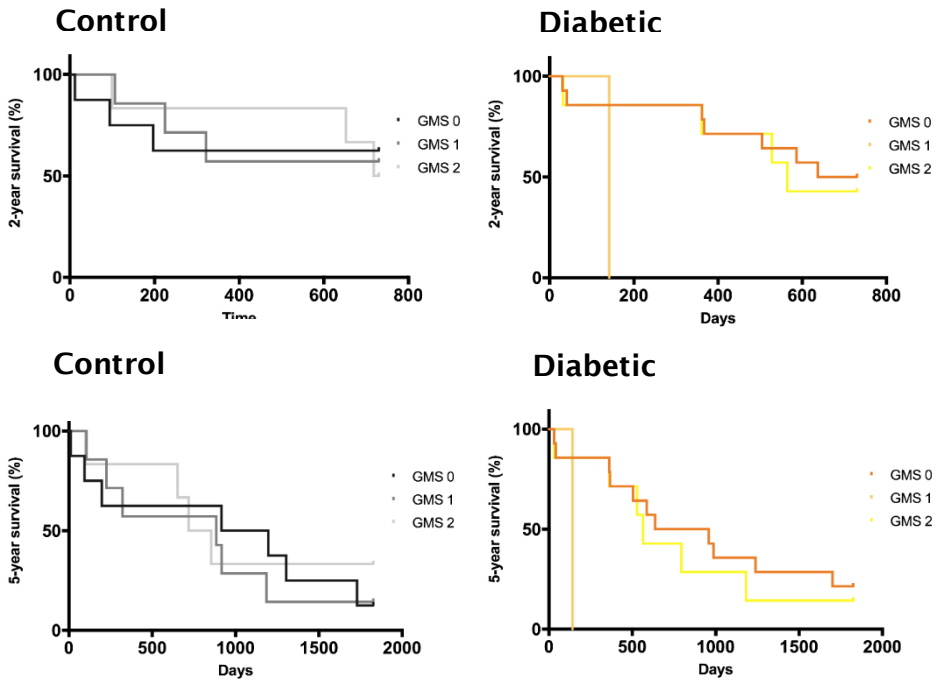


Figure 5 – Kaplan-Meier survival curves at 2 and 5-years, of all patients in study, stratified by GMS score.

In stratification of the patients by GPS score, the difference of patients' survival at 2 and 5 years, was not significant.

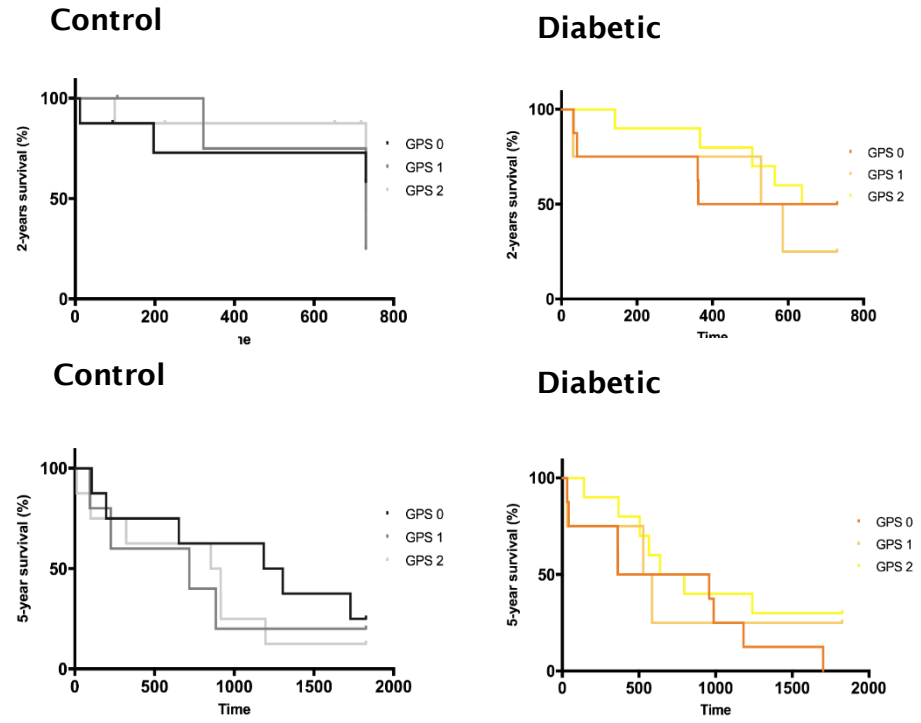


Figure 6 – Kaplan-Meier survival curves at 2 and 5-years, of all patients in study, stratified by GPS score.

3.Fibrotic status

Erro! A origem da referência não foi encontrada. shows the macroscopic d ifferences in fibrosis staining in control and diabetic patient, respectively.

Erro! A origem da referência não foi encontrada. shows the fibrotic status o f tumor microenvironment in control group (n=21) and diabetic group (n=22).

The fibrotic status assessed by Sirius Red histological staining higher in diabetic group when compared with the control group both in normal adjacent area (12.288 ± 1.53 vs 10.975 ± 1.076) and in the tumor area (9.362 ± 1.337 vs 7.165 ± 1.012).

In the control group, the tumor area was more fibrotic when compared to the normal adjacent area (7.165 ± 1.012 vs 10.975 ± 1.076).

In the diabetic group, the tumor area was also more fibrotic then the normal adjacent area (9.362 ± 1.337 vs 12.288 ± 1.53).

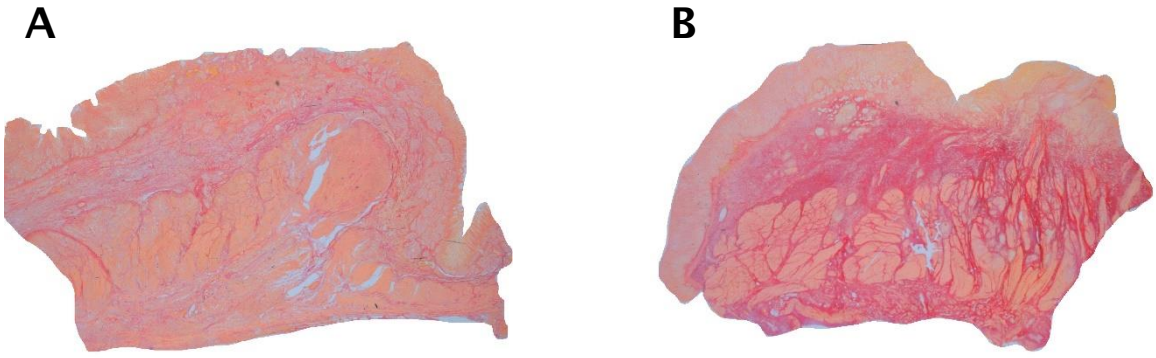


Figure 7 - Fibrosis staining in (A) control patient and (B) diabetic patient.

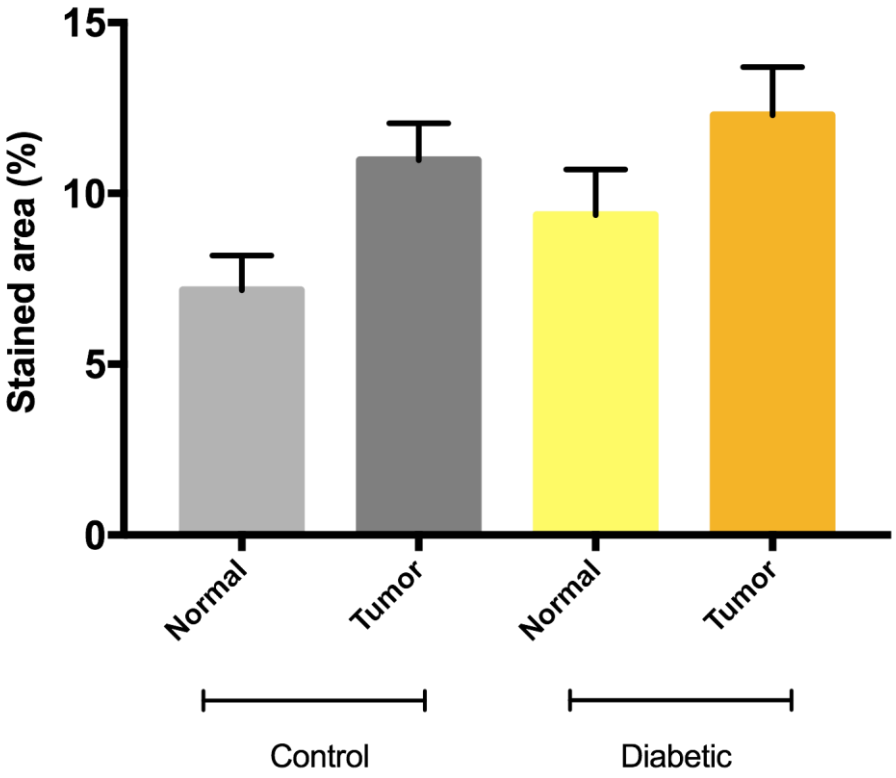


Figure 8 - Fibrotic status of control (n=21) and diabetic (n=22) gastric cancer groups, by Sirius Red staining. Control group: 7.165 ± 1.012 vs 10.975 ± 1.076. Diabetic group: 9.362 ± 1.337 vs 12.288 ± 1.531. Data represents mean ± SE.

4.CD31 staining analysis

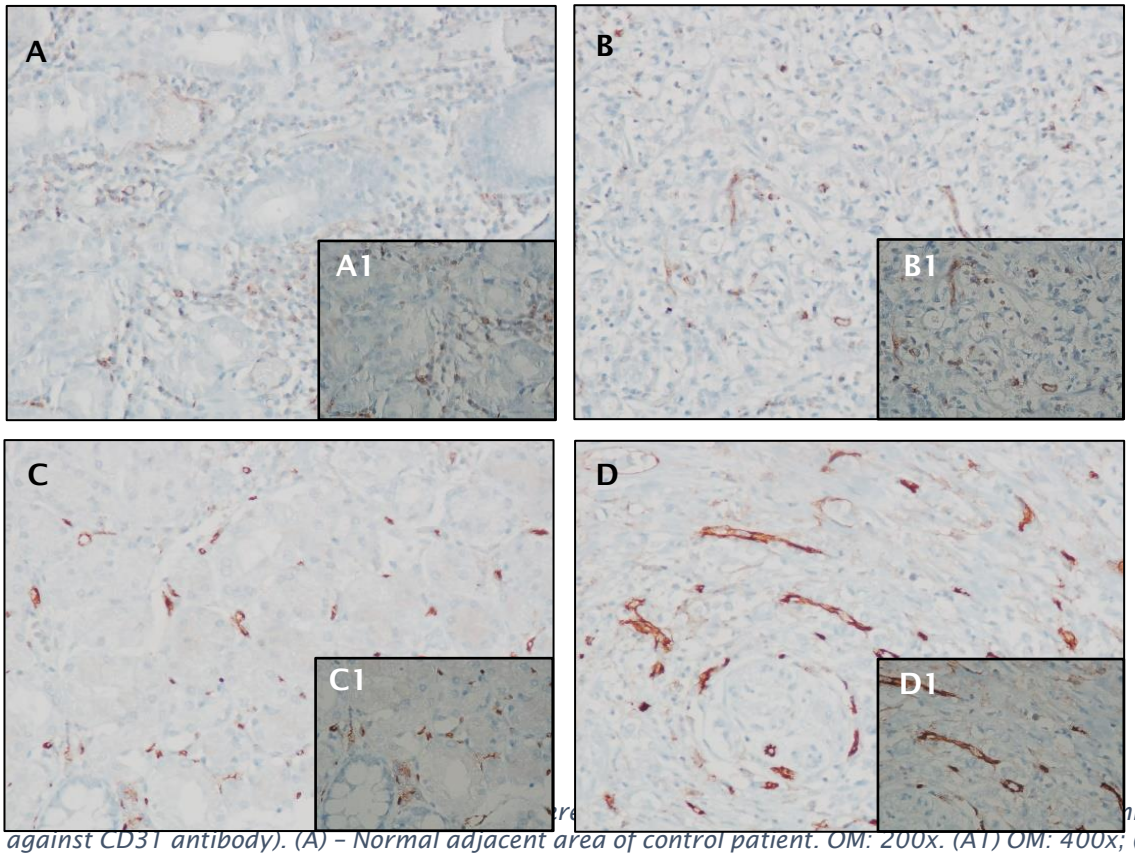
The development of DM2 and cancer comprises distinct extracellular matrix remodeling and deregulated angiogenesis in different organs. We next evaluated whether these diseases interfere with the number of vessels in diabetic (n=22) and control patients (n=21), in normal adjacent area and tumor area **Erro! A origem da r eferência não foi encontrada.**.

Results are shown in **Erro! A origem da referência não foi encontrada.**.

The microvessel density was higher in control group compared with the diabetic group, both in normal adjacent area (17.55 ± 2.084 vs 12.63 ± 1.029), and in tumor area (16.27 ± 1.463 vs 11.56 ± 0.639).

In the control group, normal adjacent area had more density of microvessels when compared with tumor area (17.55 ± 2.084 vs 16.27 ± 1.463).

In the diabetic group, normal adjacent area had higher density of microvessels when compared to the tumor area (12.63 ± 1.029 vs 11.56 ± 0.639).



against CD31 antibody). (A) – Normal adjacent area of control patient. OM: 200x. (A1) OM: 400x; (B)) tumor area of control patient. OM: 200x. (B1) OM: 400x; (C) Normal adjacent area of diabetic patient. OM: 200x. (C1) OM: 400x; (D) tumor area of diabetic patient. OM: 200x. (D1) OM 400x.

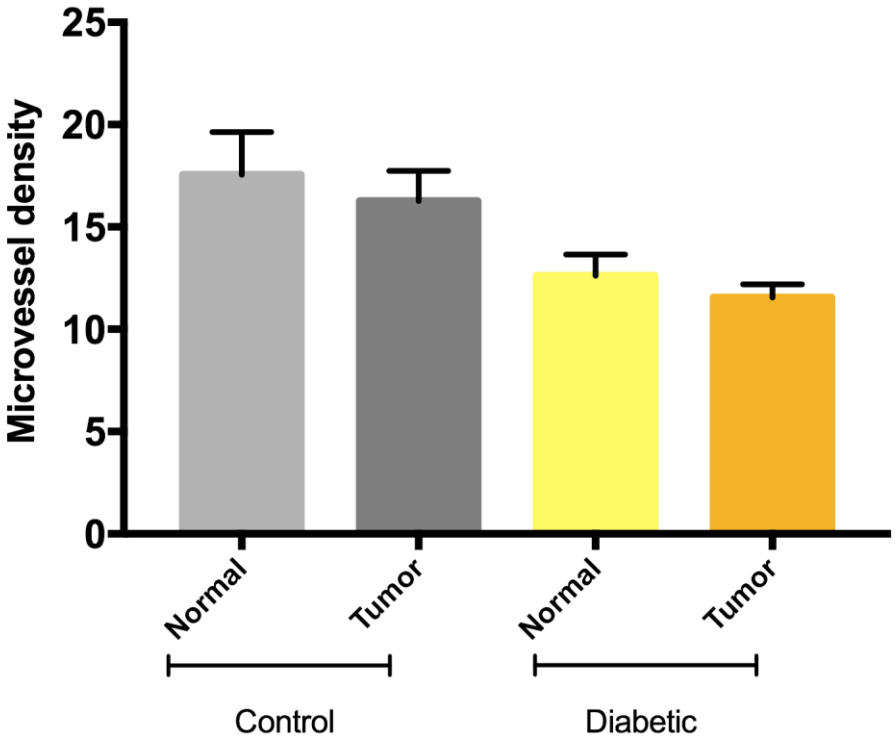


Figure 10 - Gastric cancer microvessel density of control (n=21) and diabetic group (n=22) assessed by CD31 staining. Control group: 17.55 ± 2.084 vs 16.27 ± 1.463. Diabetic group: 12.63 ± 1.029 vs 11.56 ± 0.639. Data represents mean ± SE.

Discussion

With the industrialization of the modern World, diseases such as obesity and its co-related comorbidities such as DM2 have increased its prevalence dramatically. The overall influence of these metabolically associated pathologies in the development and progression of multiple malignancies have been under the scope of researchers for a long time. It is becoming increasing evident that there is a substantial increase in cancer incidence in diabetic patients[130]. However, the mechanisms by which this association occurs it is not fully understood. There are lots of biopathological factors that may play an important role in the process of cancer initiation and progression and that are also present in diabetes, such as angiogenesis imbalance, chronic inflammation and oxidative stress, among others[99].

Various malignancies have been associated with metabolic disturbances, and one of these is gastric cancer [99]. In many studies, it has been suggested that, not only, does metabolic disease appear to promote the development of cancer but it also appears to be associated with a worst prognosis.

The main aim of this thesis was to investigate the mechanisms that may justify the association of DM2 and gastric cancer and possibly helps us identify a sub-group of gastric cancer patients that is highly associated with DM2, and may, in the future, beneficiate from more metabolic focused therapies. Taking advantage of the collaboration of the Unit of Biochemistry, of the Department of Biomedicine of the Faculty of Medicine of the University of Porto, with the tumor bank of the Centro Hospitalar São João, Porto, Portugal, we gained access to formalin-fixed, paraffin embedded tissue samples, and also had access to several clinical and pathological information associated with each patient.

We started by establishing two experimental groups from the lists of available samples: a group of patients with gastric cancer that have DM2 (n=22) and a control group with patients with gastric cancer but no known metabolic disease (n=21). In brief, to avoid confounding variables, the diabetic group was initially defined and only patients who were not insulin-dependent, did not present any other previous neoplasia, did not receive neoadjuvant chemotherapy or radiotherapy, or both, and were not on more than 2 anti-diabetic drugs simultaneously were included. In order to establish the control group, patients who had similar clinical and pathological parameters were chosen. Although all diabetic patients were taking at least one anti-diabetic drug, the glycaemia in the moment

of surgery and from random time-points were checked to make sure the metabolic state of the patients was accurate. This approach was used as to assure that the key difference between groups was the presence or absence of DM2. The available clinical and pathological data of the patients was collected to better characterize the patients. Unfortunately, we were not able to fully characterize the metabolic landscape of the patients, as several values regarding body weight, height (which would allow us to calculate the BMI), Hba1c, insulin, lipid metabolism, among others were missing from the clinical record we had access to, as the patients may have been followed in private practice.

Although the diabetic patients were clinically diagnosed beforehand, and all of them were on anti-diabetic drugs, their glycaemias was not totally under control. At the moment of surgery, the diabetic group presents a significantly higher blood glucose levels when compared to the control group (190.1 ± 13.6 mg/dL vs 98.2 ± 3.6 mg/dL, $p<0.001$, respectively). As there were very few values of HbA1c available, we cannot infer about the glycemic status of the patients. An insufficient control of the diabetic status may not be enough to avoid diabetic associated complications.

Paraffin embedded tumor samples were used to evaluate fibrosis and vascular density. We have performed and quantified the Sirius red stained area to access fibrosis, and have performed an anti-CD31 immunohistochemistry to access the microvascular density in both groups. An experienced pathologist evaluated both the Klintrup-Mäkinen (KM) grade, a semi-quantitative evaluation for immune cells, and the tumor stroma percentage (TSP), which allowed us to access the Glasgow Microenvironment Score (GMS). Also, a modified Glasgow Prognostic Score was calculated, based on hematological components, to give a systemic inflammation-prognostic score.

It is described in the literature that the incidence of gastric cancer is higher in male than in female patients [131]. However, in the cohort that was available to our study, and that fulfilled our inclusion criteria, were more women than men (Table 4), which was a consequence of the availability of information regarding the possible patient. There were no significant differences between the mean age of the patients at the time of the diagnostics.

Focusing on the pathological parameters, the histological type, the T-state, the stage and the localization of the tumors are very similar between the

experimental groups (Table 4). In our cohort, as it is described in the literature, the intestinal type of gastric tumor was the most frequent [27]. However, when focusing on the localization of the tumor, the current worldwide trends in gastric cancer indicate that gastric cardia tumors are increasing [49], probably due to an increase in obesity and metabolic disturbances. These tumors are highly associated with gastroesophageal reflux which is a known consequence of obesity. Nonetheless, in Portugal and Asia, the most frequent tumors are the ones in the lower part of the stomach, which is reflected in our cohort [28,29]. These tumors are more associated with H. Pylori infection. Our cohort corroborates the data available in the literature that states that gastric cancer is often diagnosed late, as it early stages do not present clinical symptoms, and were diagnosed in an advanced stage [33].

Recently, a paper was published by Zhou, *et al*, that aimed to evaluate the prognostic value of the Glasgow Microenvironment Score in gastric cancer [65]. The authors indicate that a lower GMS value was associated with a better prognostic. Guided by this paper, an experienced pathologist evaluated the inflammatory status, given by the KM score and the tumor stoma percentage. These scores were used to further assess the GMS of our cases. We found that the diabetic and control patients have significantly different distribution profiles according to the GMS value. Our diabetic group presented, more frequently, a lower value for GMS. This is due to a high inflammatory profile of these samples, associated with a high tumor percentage of stoma. It is not unexpected that diabetic patients could have an increased inflammatory status, consequence of diabetes itself. A chronic inflammatory status in the diabetic group, may lead to an increase in fibrotic tissue, which could justify the increased stroma found in these patients. It is accepted that metabolic impaired patients may have worse prognosis to a variety of conditions, as is the case of cancer. However, we did not find any significant differences in the overall survival between diabetic and non-diabetic patients both in a follow up period of 2 and 5 years. Also, we did not find any correlation between overall survival and the GMS status of the patients. This is probably due to low number of patients included in our cohort. Also, as our lower GMS values is mainly associated with the diabetic patients, with increased TSP, it may suggest that the tumor microenvironment is desmoplastic which may complicate the treatment options, and may demand a more aggressive therapeutic strategy, such as more extensive surgery. In this paper, an inflammatory status, translated by a high KM is regarded as a more beneficial environment and associated with a better prognosis. The

worse prognosis of diabetic patients which is described in the literature, despite the higher inflammatory microenvironment, may be due to a macrophage polarization towards a M2 phenotype.

We also thought of evaluating the systemic inflammation through the assessment of the Glasgow Prognostic Score, which combines albumin and C-reactive protein into a risk stratification score for predicting clinical outcome in patients with cancer [88]. We did not find any significant differences between the experimental groups and in the overall survival. This is probably due to, although the diabetic patients present a low chronic inflammatory state this is overcome by the tumor-associated inflammation, present in both groups.

We have found lower microvessel density in the diabetic group when compared to the control groups. And, more surprisingly, we found that the tumors, in both experimental groups, had lower microvessel density than the normal adjacent mucosa. This is in accordance with the results obtained for the TSP involvement in the tumors. A more desmoplastic tumor environment is associated with lower vascularization and higher fibrotic tissue, which was found with the Sirius Red staining [65,229]. However, we would like to further confirm the CD31 results with different evaluation methods such as another endothelial marker (i.e. CD34), or different image assessment method.

Although we have found no significant differences in overall survival between diabetic and non-diabetic patients we believe that this is mainly due to the low number of patients in our experimental groups and the lack of representation of more cases of gastric cardia tumors. Furthermore, when the patients were subdivided according to their GMS or GPS score, the N of the experimental groups further decreased, decreasing also the strength of the analysis. Further work is needed in order to confirm the obtained data and increase the statistical significance of the obtained results.

Conclusion and future perspectives

In conclusion, although not significant, we observe differences between the groups and between the tumor and the normal adjacent areas. DM2 changes both systemic and tumoral inflammatory profiles of the patients, as well as the angiogenic profile in tumor and peri-tumoral areas. This work raised many questions, and will need further research in order to fully understand the effect of metabolic disturbances in the development and progression of gastric cancer.

We will continue our work by:

- increasing our cohort;

- confirming the results obtained by CD31 staining, through another endothelial cell marker, or measure of the vessel size or diameter, or even evaluate the microvessel density with another imaging technique;

- evaluating oxidative stress, by immunohistochemistry against 3-Nitrotyrosine (currently in process of optimization of the antibody);

- evaluating the polarization of macrophages (M0, M1 and M2) to verify if the population of immune cells in the tumor have a pro- or anti-inflammatory profile.

References

1 Ogurtsova, K. *et al.*, **2017**, IDF Diabetes Atlas: Global estimates for the prevalence of diabetes for 2015 and 2040, Diabetes Res Clin Pract, 128 40-50

2 American Diabetes, A., **2017**, 2. Classification and Diagnosis of Diabetes, Diabetes Care, 40 (Suppl 1), S11-S24

3 Melton, L. J., 3rd *et al.*, **1983**, Incidence of diabetes mellitus by clinical type, Diabetes Care, 6 (1), 75-86

4 Krolewski, A. S. *et al.*, **1987**, Epidemiologic approach to the etiology of type I diabetes mellitus and its complications, N Engl J Med, 317 (22), 1390-1398

5 Wang, J. *et al.*, **2008**, Dietary energy density predicts the risk of incident type 2 diabetes: the European Prospective Investigation of Cancer (EPIC)-Norfolk Study, Diabetes Care, 31 (11), 2120-2125

6 Amorosa L. , L. E., Swee D., **2012**, Diabetes Mellitus Textbook of Family Medicine, 8th, Elsevier Saunders,

7 Maitra, A., **2010**, The Endocrine System, Vinay Kumar, Abul K. Abbas, and Jon C. Aster. Robbins & Cotran Pathologic Basis of Disease, 8th,

8 Hall, J. E., **2010**, Insulin, Glucagon, and Diabetes Mellitus, Guyton and Hall Textbook of Medical Physiology, 78, 12th,

9 Gaddam S., F. F., Whitlatch H., **2013**, Diabetes Mellitus, Ferri F. Ferri's Clinical Advisor 2013: 5 Books in 1. , 1st,

10 Alberti, K. G. & Zimmet, P. Z., **1998**, Definition, diagnosis and classification of diabetes mellitus and its complications. Part 1: diagnosis and classification of diabetes mellitus provisional report of a WHO consultation, Diabet Med, 15 (7), 539-553

11 McCance, D. R., **2011**, Pregnancy and diabetes, Best Pract Res Clin Endocrinol Metab, 25 (6), 945-958

12 Sacks, D. A. *et al.*, **2012**, Frequency of gestational diabetes mellitus at collaborating centers based on IADPSG consensus panel-recommended criteria: the Hyperglycemia and Adverse Pregnancy Outcome (HAPO) Study, Diabetes Care, 35 (3), 526-528

13 Ali, S. & Dornhorst, A., **2011**, Diabetes in pregnancy: health risks and management, Postgrad Med J, 87 (1028), 417-427

14 Kim, C. *et al.*, **2002**, Gestational diabetes and the incidence of type 2 diabetes: a systematic review, Diabetes Care, 25 (10), 1862-1868

15 Chang, S. C. & Yang, W. V., **2016**, Hyperglycemia, tumorigenesis, and chronic inflammation, Crit Rev Oncol Hematol, 108 146-153

16 Organization, W. H. **2011**.Use of Glycated Haemoglobin (HbA1c) in the Diagnosis of Diabetes Mellitus

17 Melander, A. *et al.*, **1999**, 35th Annual Meeting of the European Association for the Study of Diabetes : Brussels, Belgium, 28 September-2 October 1999, Diabetologia, 42 (Suppl 1), A1-A330

18 Ahmed, I. & Goldstein, B., **2006**, Diabetes mellitus, Clin Dermatol, 24 (4), 237-246

19 Petersen, K. F. *et al.*, **2007**, The role of skeletal muscle insulin resistance in the pathogenesis of the metabolic syndrome, Proc Natl Acad Sci U S A, 104 (31), 12587-12594

20 Vigneri, R., **2009**, Diabetes: diabetes therapy and cancer risk, Nat Rev Endocrinol, 5 (12), 651-652

21 Ikeda, F. & Kiyohara, Y., **2015**, Helicobacter pylori infection and Hyperglycemia/Diabetes are associated with an increased risk of Gastric Cancer, Gan To Kagaku Ryoho, 42 (5), 529-533

22 Shen, Z. *et al.*, **2015**, Glycemic changes after gastrectomy in non-morbidly obese patients with gastric cancer and diabetes, Hepatogastroenterology, 62 (137), 245-250

23 Sekikawa, A. *et al.*, **2014**, Diabetes mellitus increases the risk of early gastric cancer development, Eur J Cancer, 50 (12), 2065-2071

24 Ferlay, J. *et al.* **2013**, Cancer Incidence and Mortality Worldwide: IARC CancerBase No. 11, <http://globocan.iarc.fr>

25 Stewart, B. & Wild, C. P. **2014**.World cancer report 2014.

26 Lordick, F. *et al.*, **2014**, Unmet needs and challenges in gastric cancer: the way forward, Cancer Treat Rev, 40 (6), 692-700

27 Gullo, I. *et al.*, **2017**, Heterogeneity in Gastric Cancer: From Pure Morphology to Molecular Classifications, Pathobiology,

28 Bertuccio, P. *et al.*, **2009**, Recent patterns in gastric cancer: a global overview, Int J Cancer, 125 (3), 666-673

29 Peleteiro, B. *et al.*, **2014**, Model-based patterns in stomach cancer mortality worldwide, Eur J Cancer Prev, 23 (6), 524-531

30 Lunet, N. *et al.*, **2004**, Regional trends in Portuguese gastric cancer mortality (1984–1999), European journal of cancer prevention, 13 (4), 271-275

31 Curado, M.-P. *et al.*, **2007**, Cancer incidence in five continents, Volume IX, IARC Press, International Agency for Research on Cancer,

32 Tan, P., **2014**, Gastric cancer—a convergence of genomic heterogeneity, Translational Gastrointestinal Cancer, 4 (2), 118-122

33 Cooke, C. L. *et al.*, **2013**, Biomarkers of Helicobacter pylori-associated gastric cancer, Gut Microbes, 4 (6), 532-540

34 Dicken, B. J. *et al.*, **2005**, Gastric adenocarcinoma: review and considerations for future directions, Ann Surg, 241 (1), 27-39

35 Padmanabhan, N. *et al.*, **2017**, How to stomach an epigenetic insult: the gastric cancer epigenome, Nat Rev Gastroenterol Hepatol,

36 Smyth, E. *et al.*, **2016**, Gastric cancer: ESMO Clinical Practice Guidelines for diagnosis, treatment and follow-up, Annals of Oncology, 27 (suppl_5), v38-v49

37 Tan, I. B. *et al.*, **2011**, Intrinsic subtypes of gastric cancer, based on gene expression pattern, predict survival and respond differently to chemotherapy, Gastroenterology, 141 (2), 476-485, 485 e471-411

38 Bang, Y. J. *et al.*, **2010**, Trastuzumab in combination with chemotherapy versus chemotherapy alone for treatment of HER2-positive advanced gastric or gastro-oesophageal junction cancer (ToGA): a phase 3, open-label, randomised controlled trial, Lancet, 376 (9742), 687-697

39 Fuchs, C. S. *et al.*, **2014**, Ramucirumab monotherapy for previously treated advanced gastric or gastro-oesophageal junction adenocarcinoma (REGARD): an international, randomised, multicentre, placebo-controlled, phase 3 trial, The Lancet, 383 (9911), 31-39

40 Wilke, H. *et al.*, **2014**, Ramucirumab plus paclitaxel versus placebo plus paclitaxel in patients with previously treated advanced gastric or gastro-oesophageal junction adenocarcinoma (RAINBOW): a double-blind, randomised phase 3 trial, The lancet oncology, 15 (11), 1224-1235

41 Fuchs, C. S. *et al.*, **2016**, Biomarker analyses in REGARD gastric/GEJ carcinoma patients treated with VEGFR2-targeted antibody ramucirumab, British journal of cancer, 115 (8), 974

42 Noto, J. M. & Peek, R. M., **2012**, Helicobacter pylori: An Overview, Helicobacter Species: Methods and Protocols, Humana Press, 7-10

43 Liu, X. & Meltzer, S. J., **2017**, Gastric Cancer in the Era of Precision Medicine, Cell Mol Gastroenterol Hepatol, 3 (3), 348-358

44 Milne, A. N. *et al.*, **2009**, Nature meets nurture: molecular genetics of gastric cancer, Hum Genet, 126 (5), 615-628

45 Plummer, M. *et al.*, **2015**, Global burden of gastric cancer attributable to Helicobacter pylori, Int J Cancer, 136 (2), 487-490

46 Anderson, W. F. *et al.*, **2010**, Age-specific trends in incidence of noncardia gastric cancer in US adults, JAMA, 303 (17), 1723-1728

47 Camargo, M. C. *et al.*, **2011**, Divergent trends for gastric cancer incidence by anatomical subsite in US adults, Gut, 60 (12), 1644-1649

48 Steevens, J. *et al.*, **2010**, Trends in incidence of oesophageal and stomach cancer subtypes in Europe, Eur J Gastroenterol Hepatol, 22 (6), 669-678

49 Abrams, J. A. *et al.*, **2013**, Diverging trends in the incidence of reflux-related and Helicobacter pylori-related gastric cardia cancer, J Clin Gastroenterol, 47 (4), 322-327

50 Forman, D. *et al.*, **2006**, The associations between food, nutrition and physical activity and the risk of stomach cancer and underlying mechanisms, Food, nutrition, physical activity, and the prevention of cancer: a global perspective: World Cancer Research Fund,

51 Lin, S. W. *et al.*, **2011**, Prospective study of self-reported diabetes and risk of upper gastrointestinal cancers, Cancer Epidemiol Biomarkers Prev, 20 (5), 954-961

52 Abnet, C. C. *et al.*, **2008**, A prospective study of BMI and risk of oesophageal and gastric adenocarcinoma, Eur J Cancer, 44 (3), 465-471

53 Merry, A. H. *et al.*, **2007**, Body mass index, height and risk of adenocarcinoma of the oesophagus and gastric cardia: a prospective cohort study, Gut, 56 (11), 1503-1511

54 Cavaleiro-Pinto, M. *et al.*, **2011**, Helicobacter pylori infection and gastric cardia cancer: systematic review and meta-analysis, Cancer Causes Control, 22 (3), 375-387

55 Wang, F. *et al.*, **2014**, Helicobacter pylori-induced gastric inflammation and gastric cancer, Cancer Letters, 345 (2), 196-202

56 Magalhaes, A. *et al.*, **2015**, Helicobacter pylori chronic infection and mucosal inflammation switches the human gastric glycosylation pathways, Biochim Biophys Acta, 1852 (9), 1928-1939

57 Lauren, P., **1965**, The Two Histological Main Types of Gastric Carcinoma: Diffuse and So-Called Intestinal-Type Carcinoma. An Attempt at a Histo-Clinical Classification, Acta Pathol Microbiol Scand, 64 31-49

58 Bosman, F. T. *et al.*, **2010**, WHO classification of tumours of the digestive system, World Health Organization,

59 Berlth, F. *et al.*, **2014**, Pathohistological classification systems in gastric cancer: diagnostic relevance and prognostic value, World J Gastroenterol, 20 (19), 5679-5684

60 Chen, Y. C. *et al.*, **2016**, Clinicopathological Variation of Lauren Classification in Gastric Cancer, Pathol Oncol Res, 22 (1), 197-202

61 Dobru, D. *et al.*, **2004**, An epidemiological study of gastric cancer in the adult population referred to gastroenterology medical services in Romania -- a multicentric study, Rom J Gastroenterol, 13 (4), 275-279

62 Sobin, L. H. *et al.*, **2011**, TNM classification of malignant tumours, John Wiley & Sons,

63 Compton, C. *et al.*, **2010**, AJCC cancer staging manual, Chapter, 14 143-164

64 Aurello, P. *et al.*, **2007**, Classification of lymph node metastases from gastric cancer: comparison between N-site and N-number systems. Our experience and review of the literature, Am Surg, 73 (4), 359-366

65 Zhou, Z. H. *et al.*, **2017**, The prognostic value and pathobiological significance of Glasgow microenvironment score in gastric cancer, J Cancer Res Clin Oncol, 143 (5), 883-894

66 Alexander, J. & Cukierman, E., **2016**, Stromal dynamic reciprocity in cancer: intricacies of fibroblastic-ECM interactions, Curr Opin Cell Biol, 42 80-93

67 Joyce, J. A. & Pollard, J. W., **2009**, Microenvironmental regulation of metastasis, Nat Rev Cancer, 9 (4), 239-252

68 Moserle, L. & Casanovas, O., **2013**, Anti-angiogenesis and metastasis: a tumour and stromal cell alliance, J Intern Med, 273 (2), 128-137

69 Fearon, D. T., **2014**, The carcinoma-associated fibroblast expressing fibroblast activation protein and escape from immune surveillance, Cancer Immunol Res, 2 (3), 187-193

70 Harper, J. & Sainson, R. C., **2014**, Regulation of the anti-tumour immune response by cancer-associated fibroblasts, Semin Cancer Biol, 25 69-77

71 Muerkoster, S. *et al.*, **2004**, Tumor stroma interactions induce chemoresistance in pancreatic ductal carcinoma cells involving increased secretion and paracrine effects of nitric oxide and interleukin-1beta, Cancer Res, 64 (4), 1331-1337

72 Park, J. H. *et al.*, **2014**, The relationship between tumour stroma percentage, the tumour microenvironment and survival in patients with primary operable colorectal cancer, Ann Oncol, 25 (3), 644-651

73 Gujam, F. J. *et al.*, **2014**, The relationship between the tumour stroma percentage, clinicopathological characteristics and outcome in patients with operable ductal breast cancer, Br J Cancer, 111 (1), 157-165

74 Wang, K. *et al.*, **2012**, Tumor-stroma ratio is an independent predictor for survival in esophageal squamous cell carcinoma, J Thorac Oncol, 7 (9), 1457-1461

75 Klintrup, K. *et al.*, **2005**, Inflammation and prognosis in colorectal cancer, Eur J Cancer, 41 (17), 2645-2654

76 Park, J. H. *et al.*, **2015**, Evaluation of a tumor microenvironment-based prognostic score in primary operable colorectal cancer, Clin Cancer Res, 21 (4), 882-888

77 Kang, B. W. *et al.*, **2016**, Prognostic value of tumor-infiltrating lymphocytes in Epstein-Barr virus-associated gastric cancer, Ann Oncol, 27 (3), 494-501

78 Roxburgh, C. S. & McMillan, D. C., **2010**, Role of systemic inflammatory response in predicting survival in patients with primary operable cancer, Future Oncol, 6 (1), 149-163

79 Ikeda, M. *et al.*, **2003**, Significant host- and tumor-related factors for predicting prognosis in patients with esophageal carcinoma, Ann Surg, 238 (2), 197-202

80 Crumley, A. B. *et al.*, **2006**, An elevated C-reactive protein concentration, prior to surgery, predicts poor cancer-specific survival in patients undergoing resection for gastro-oesophageal cancer, Br J Cancer, 94 (11), 1568-1571

81 Hilmy, M. *et al.*, **2005**, The relationship between the systemic inflammatory response and survival in patients with transitional cell carcinoma of the urinary bladder, Br J Cancer, 92 (4), 625-627

82 Jamieson, N. B. *et al.*, **2005**, Systemic inflammatory response predicts outcome in patients undergoing resection for ductal adenocarcinoma head of pancreas, Br J Cancer, 92 (1), 21-23

83 Lamb, G. W. *et al.*, **2006**, The relationship between the preoperative systemic inflammatory response and cancer-specific survival in patients undergoing potentially curative resection for renal clear cell cancer, Br J Cancer, 94 (6), 781-784

84 Karakiewicz, P. I. *et al.*, **2007**, C-reactive protein is an informative predictor of renal cell carcinoma-specific mortality: a European study of 313 patients, Cancer, 110 (6), 1241-1247

85 Hara, M. *et al.*, **2007**, Preoperative serum C-reactive protein level in non-small cell lung cancer, Anticancer Res, 27 (4C), 3001-3004

86 McMillan, D. C., **2008**, An inflammation-based prognostic score and its role in the nutrition-based management of patients with cancer, Proc Nutr Soc, 67 (3), 257-262

87 Deans, D. A. *et al.*, **2009**, The influence of systemic inflammation, dietary intake and stage of disease on rate of weight loss in patients with gastro-oesophageal cancer, Br J Cancer, 100 (1), 63-69

88 Forrest, L. M. *et al.*, **2003**, Evaluation of cumulative prognostic scores based on the systemic inflammatory response in patients with inoperable non-small-cell lung cancer, Br J Cancer, 89 (6), 1028-1030

89 Choi, J. I. *et al.*, **2014**, State-of-the-art preoperative staging of gastric cancer by MDCT and magnetic resonance imaging, World J Gastroenterol, 20 (16), 4546-4557

90 Gravalos, C. & Jimeno, A., **2008**, HER2 in gastric cancer: a new prognostic factor and a novel therapeutic target, Ann Oncol, 19 (9), 1523-1529

91 Tanner, M. *et al.*, **2005**, Amplification of HER-2 in gastric carcinoma: association with Topoisomerase IIalpha gene amplification, intestinal type, poor prognosis and sensitivity to trastuzumab, Ann Oncol, 16 (2), 273-278

92 Bar-Sela, G. *et al.*, **2013**, The incidence and prognostic value of HER2 overexpression and cyclin D1 expression in patients with gastric or gastroesophageal junction adenocarcinoma in Israel, Oncol Lett, 5 (2), 559-563

93 Barros-Silva, J. D. *et al.*, **2009**, Association of ERBB2 gene status with histopathological parameters and disease-specific survival in gastric carcinoma patients, Br J Cancer, 100 (3), 487-493

94 Aizawa, M. *et al.*, **2014**, Evaluation of HER2-based biology in 1,006 cases of gastric cancer in a Japanese population, Gastric Cancer, 17 (1), 34-42

95 Gordon, M. A. *et al.*, **2013**, Assessment of HER2 gene amplification in adenocarcinomas of the stomach or gastroesophageal junction in the INT-0116/SWOG9008 clinical trial, Ann Oncol, 24 (7), 1754-1761

96 Grabsch, H. *et al.*, **2010**, HER2 expression in gastric cancer: Rare, heterogeneous and of no prognostic value - conclusions from 924 cases of two independent series, Cell Oncol, 32 (1-2), 57-65

97 Qiu, M. Z. *et al.*, **2014**, HER2-positive patients receiving trastuzumab treatment have a comparable prognosis with HER2-negative advanced gastric cancer patients: a prospective cohort observation, Int J Cancer, 134

98 Ang, Y. L. *et al.*, **2016**, Translating gastric cancer genomics into targeted therapies, Crit Rev Oncol Hematol, 100 141-146

99 Xu, C. X. *et al.*, **2014**, Diabetes and cancer: Associations, mechanisms, and implications for medical practice, World J Diabetes, 5 (3), 372-380

100 Arcidiacono, B. *et al.*, **2012**, Insulin resistance and cancer risk: an overview of the pathogenetic mechanisms, Exp Diabetes Res, 2012 789174

101 Giovannucci, E. *et al.*, **2010**, Diabetes and cancer: a consensus report, Diabetes Care, 33 (7), 1674-1685

102 Zhou, Y. *et al.*, **2015**, Influence of diabetes mellitus on mortality in breast cancer patients, ANZ J Surg, 85 (12), 972-978

103 Boyle, P. *et al.*, **2012**, Diabetes and breast cancer risk: a meta-analysis, Br J Cancer, 107 (9), 1608-1617

104 Larsson, S. C. *et al.*, **2007**, Diabetes mellitus and risk of breast cancer: a meta-analysis, Int J Cancer, 121 (4), 856-862

105 Lee, J. Y. *et al.*, **2013**, Diabetes mellitus and ovarian cancer risk: a systematic review and meta-analysis of observational studies, Int J Gynecol Cancer, 23 (3), 402-412

106 Wang, P. *et al.*, **2012**, Diabetes mellitus and risk of hepatocellular carcinoma: a systematic review and meta-analysis, Diabetes Metab Res Rev, 28 (2), 109-122

107 Chen, J. *et al.*, **2015**, Effect of type 2 diabetes mellitus on the risk for hepatocellular carcinoma in chronic liver diseases: a meta-analysis of cohort studies, Eur J Cancer Prev, 24 (2), 89-99

108 Mao, Y. *et al.*, **2015**, Effect of Diabetes Mellitus on Survival in Patients with Pancreatic Cancer: A Systematic Review and Meta-analysis, Sci Rep, 5 17102

109 Bosetti, C. *et al.*, **2014**, Diabetes, antidiabetic medications, and pancreatic cancer risk: an analysis from the International Pancreatic Cancer Case-Control Consortium, Ann Oncol, 25 (10), 2065-2072

110 Song, S. *et al.*, **2015**, Long-Term Diabetes Mellitus Is Associated with an Increased Risk of Pancreatic Cancer: A Meta-Analysis, PLoS One, 10 (7), e0134321

111 Batabyal, P. *et al.*, **2014**, Association of diabetes mellitus and pancreatic adenocarcinoma: a meta-analysis of 88 studies, Ann Surg Oncol, 21 (7), 2453-2462

112 Ben, Q. *et al.*, **2011**, Diabetes mellitus and risk of pancreatic cancer: A meta-analysis of cohort studies, Eur J Cancer, 47 (13), 1928-1937

113 Huang, W. *et al.*, **2012**, Risk of esophageal cancer in diabetes mellitus: a meta-analysis of observational studies, Cancer Causes Control, 23 (2), 263-272

114 Tseng, C. H., **2012**, Diabetes but not insulin is associated with higher colon cancer mortality, World J Gastroenterol, 18 (31), 4182-4190

115 Mills, K. T. *et al.*, **2013**, Diabetes mellitus and colorectal cancer prognosis: a meta-analysis, Dis Colon Rectum, 56 (11), 1304-1319

116 Tseng, C. H., **2012**, Diabetes, metformin use, and colon cancer: a population-based cohort study in Taiwan, Eur J Endocrinol, 167 (3), 409-416

117 Jiang, Y. *et al.*, **2011**, Diabetes mellitus and incidence and mortality of colorectal cancer: a systematic review and meta-analysis of cohort studies, Eur J Epidemiol, 26 (11), 863-876

118 Friberg, E. *et al.*, **2007**, Diabetes mellitus and risk of endometrial cancer: a meta-analysis, Diabetologia, 50 (7), 1365-1374

119 Cote, M. L. *et al.*, **2015**, Risk factors for endometrial cancer in black and white women: a pooled analysis from the Epidemiology of Endometrial Cancer Consortium (E2C2), Cancer Causes Control, 26 (2), 287-296

120 Larsson, S. C. & Wolk, A., **2011**, Diabetes mellitus and incidence of kidney cancer: a meta-analysis of cohort studies, Diabetologia, 54 (5), 1013-1018

121 Bao, C. *et al.*, **2013**, Diabetes mellitus and incidence and mortality of kidney cancer: a meta-analysis, J Diabetes Complications, 27 (4), 357-364

122 Tseng, C. H., **2012**, Diabetes, insulin use, and non-Hodgkin lymphoma mortality in Taiwan, Metabolism, 61 (7), 1003-1009

123 Castillo, J. J. *et al.*, **2012**, Increased incidence of non-Hodgkin lymphoma, leukemia, and myeloma in patients with diabetes mellitus type 2: a meta-analysis of observational studies, Blood, 119 (21), 4845-4850

124 Zhu, Z. *et al.*, **2013**, Risk of bladder cancer in patients with diabetes mellitus: an updated meta-analysis of 36 observational studies, BMC Cancer, 13 310

125 Klil-Drori, A. J. *et al.*, **2017**, Cancer, obesity, diabetes, and antidiabetic drugs: is the fog clearing?, Nat Rev Clin Oncol, 14 (2), 85-99

126 Vigneri, P. *et al.*, **2006**, Obesity and cancer, Nutr Metab Cardiovasc Dis, 16 (1), 1-7

127 Calle, E. E. *et al.*, **2003**, Overweight, obesity, and mortality from cancer in a prospectively studied cohort of U.S. adults, N Engl J Med, 348 (17), 1625-1638

128 Pollak, M., **2008**, Insulin and insulin-like growth factor signalling in neoplasia, Nat Rev Cancer, 8 (12), 915-928

129 Pollak, M., **2012**, The insulin and insulin-like growth factor receptor family in neoplasia: an update, Nat Rev Cancer, 12 (3), 159-169

130 Tseng, C. H. & Tseng, F. H., **2014**, Diabetes and gastric cancer: the potential links, World J Gastroenterol, 20 (7), 1701-1711

131 Marimuthu, S. P. *et al.*, **2011**, Diabetes mellitus and gastric carcinoma: Is there an association?, J Carcinog, 10 30

132 Lauby-Secretan, B. *et al.*, **2016**, Body Fatness and Cancer--Viewpoint of the IARC Working Group, N Engl J Med, 375 (8), 794-798

133 Tian, T. *et al.*, **2012**, Diabetes mellitus and incidence and mortality of gastric cancer: a meta-analysis, Exp Clin Endocrinol Diabetes, 120 (4), 217-223

134 Yoon, J. M. *et al.*, **2013**, Pre-existing diabetes mellitus increases the risk of gastric cancer: a meta-analysis, World J Gastroenterol, 19 (6), 936-945

135 Ikeda, F. *et al.*, **2009**, Hyperglycemia increases risk of gastric cancer posed by Helicobacter pylori infection: a population-based cohort study, Gastroenterology, 136 (4), 1234-1241

136 Ge, S. *et al.*, **2012**, Association between Habitual Dietary Salt Intake and Risk of Gastric Cancer: A Systematic Review of Observational Studies, Gastroenterol Res Pract, 2012 808120

137 Currie, C. J. *et al.*, **2009**, The influence of glucose-lowering therapies on cancer risk in type 2 diabetes, Diabetologia, 52 (9), 1766-1777

138 Cannata, D. *et al.*, **2010**, Type 2 diabetes and cancer: what is the connection?, Mt Sinai J Med, 77 (2), 197-213

139 Sun, G. & Kashyap, S. R., **2011**, Cancer risk in type 2 diabetes mellitus: metabolic links and therapeutic considerations, J Nutr Metab, 2011 708183

140 Nicolucci, A., **2010**, Epidemiological aspects of neoplasms in diabetes, Acta Diabetol, 47 (2), 87-95

141 Wang, Y. C. *et al.*, **2011**, Health and economic burden of the projected obesity trends in the USA and the UK, Lancet, 378 (9793), 815-825

142 Pollack, M. N., **2007**, Insulin, insulin-like growth factors, insulin resistance, and neoplasia, Am J Clin Nutr, 86 (3), s820-822

143 Barone, B. B. *et al.*, **2008**, Long-term all-cause mortality in cancer patients with preexisting diabetes mellitus: a systematic review and meta-analysis, JAMA, 300 (23), 2754-2764

144 Fukumura, D. *et al.*, **2016**, Obesity and cancer: an angiogenic and inflammatory link, Microcirculation, 191-206

145 Nimptsch, K. & Pischon, T., **2016**, Obesity Biomarkers, Metabolism and Risk of Cancer: An Epidemiological Perspective, Recent Results Cancer Res, 208 199-217

146 Cohen, D. H. & LeRoith, D., **2012**, Obesity, type 2 diabetes, and cancer: the insulin and IGF connection, Endocr Relat Cancer, 19 (5), F27-45

147 Shikata, K. *et al.*, **2013**, Diabetes mellitus and cancer risk: review of the epidemiological evidence, Cancer Sci, 104 (1), 9-14

148 Mussig, K. *et al.*, **2011**, Type 2 diabetes mellitus and risk of malignancy: is there a strategy to identify a subphenotype of patients with increased susceptibility to endogenous and exogenous hyperinsulinism?, Diabet Med, 28 (3), 276-286

149 Pollak, M. N. *et al.*, **2004**, Insulin-like growth factors and neoplasia, Nat Rev Cancer, 4 (7), 505-518

150 Brahmkhatri, V. P. *et al.*, **2015**, Insulin-like growth factor system in cancer: novel targeted therapies, Biomed Res Int, 2015 1-24

151 Bi, Y. *et al.*, **2012**, Advanced research on risk factors of type 2 diabetes, Diabetes Metab Res Rev, 28 Suppl 2 32-39

152 Nagini, S., **2012**, Carcinoma of the stomach: A review of epidemiology, pathogenesis, molecular genetics and chemoprevention, World J Gastrointest Oncol, 4 (7), 156-169

153 Ma, Y. *et al.*, **2013**, Relation between gastric cancer and protein oxidation, DNA damage, and lipid peroxidation, Oxid Med Cell Longev, 2013 543760

154 Mahdy, R. A. *et al.*, **2010**, The role of vascular endothelial growth factor in the progression of diabetic vascular complications, Eye (Lond), 24 (10), 1576-1584

155 Warren, R. S. *et al.*, **1996**, Induction of vascular endothelial growth factor by insulin-like growth factor 1 in colorectal carcinoma, J Biol Chem, 271 (46), 29483-29488

156 Akagi, Y. *et al.*, **1998**, Regulation of vascular endothelial growth factor expression in human colon cancer by insulin-like growth factor-I, Cancer Res, 58 (17), 4008-4014

157 Warburg, O., **1956**, On the origin of cancer cells, Science, 123 (3191), 309-314

158 Chocarro-Calvo, A. *et al.*, **2013**, Glucose-induced beta-catenin acetylation enhances Wnt signaling in cancer, Mol Cell, 49 (3), 474-486

159 Wu, W. K. *et al.*, **2010**, Dysregulation of cellular signaling in gastric cancer, Cancer Lett, 295 (2), 144-153

160 Wieser, V. *et al.*, **2013**, Inflammation, cytokines and insulin resistance: a clinical perspective, Arch Immunol Ther Exp (Warsz), 61 (2), 119-125

161 Katoh, M., **2007**, Dysregulation of stem cell signaling network due to germline mutation, SNP, Helicobacter pylori infection, epigenetic change and genetic alteration in gastric cancer, Cancer Biol Ther, 6 (6), 832-839

162 Shimizu, H. *et al.*, **2010**, Effects of twice-daily injections of premixed insulin analog on glycemic control in type 2 diabetic patients, Yonsei Med J, 51 (6), 845-849

163 Yamagata, H. *et al.*, **2000**, Impact of Helicobacter pylori infection on gastric cancer incidence in a general Japanese population: the Hisayama study, Arch Intern Med, 160 (13), 1962-1968

164 Chiba, T. *et al.*, **2008**, Mechanism for gastric cancer development by Helicobacter pylori infection, J Gastroenterol Hepatol, 23 (8 Pt 1), 1175-1181

165 Farinati, F. *et al.*, **2008**, Helicobacter pylori, inflammation, oxidative damage and gastric cancer: a morphological, biological and molecular pathway, Eur J Cancer Prev, 17 (3), 195-200

166 Peleteiro, B. *et al.*, **2012**, The role of Helicobacter pylori infection in the web of gastric cancer causation, Eur J Cancer Prev, 21 (2), 118-125

167 Ojetti, V. *et al.*, **2010**, Helicobacter pylori infection and diabetes, Minerva Med, 101 (2), 115-119

168 Ojetti, V. *et al.*, **2005**, The role of H. pylori infection in diabetes, Curr Diabetes Rev, 1 (3), 343-347

169 Gasbarrini, A. *et al.*, **1999**, Insulin-dependent diabetes mellitus affects eradication rate of Helicobacter pylori infection, Eur J Gastroenterol Hepatol, 11 (7), 713-716

170 Acbay, O. *et al.*, **1996**, Does Helicobacter pylori-induced gastritis enhance food-stimulated insulin release?, Dig Dis Sci, 41 (7), 1327-1331

171 Gunji, T. *et al.*, **2009**, Helicobacter pylori infection significantly increases insulin resistance in the asymptomatic Japanese population, Helicobacter, 14 (5), 144-150

172 Aslan, M. *et al.*, **2006**, Insulin resistance in H pylori infection and its association with oxidative stress, World J Gastroenterol, 12 (42), 6865-6868

173 Donath, M. Y. & Shoelson, S. E., **2011**, Type 2 diabetes as an inflammatory disease, Nat Rev Immunol, 11 (2), 98-107

174 Stumvoll, M. *et al.*, **2005**, Type 2 diabetes: principles of pathogenesis and therapy, Lancet, 365 (9467), 1333-1346

175 Bener, A. *et al.*, **2007**, Association between type 2 diabetes mellitus and Helicobacter pylori infection, Turk J Gastroenterol, 18 (4), 225-229

176 D'Elia, L. *et al.*, **2014**, Dietary salt intake and risk of gastric cancer, Cancer Treat Res, 159 83-95

177 Research, A. I. f. C., **2007**, Food, nutrition, physical activity, and the prevention of cancer: a global perspective, Amer Inst for Cancer Research, 1, 517

178 D'Elia, L. *et al.*, **2012**, Habitual salt intake and risk of gastric cancer: a meta-analysis of prospective studies, Clin Nutr, 31 (4), 489-498

179 Tatematsu, M. *et al.*, **1975**, Effects in Rats of Sodium Chloride on Experimental Gastric Cancers Induced by N-Methyl-N′-nitro-N-nitrosoguanidine or 4-Nitroquinoline-1-oxide 2, Journal of the National Cancer Institute, 55 (1), 101-106

180 Fox, J. G. *et al.*, **1999**, High-salt diet induces gastric epithelial hyperplasia and parietal cell loss, and enhances Helicobacter pylori colonization in C57BL/6 mice, Cancer research, 59 (19), 4823-4828

181 Charnley, G. & Tannenbaum, S. R., **1985**, Flow cytometric analysis of the effect of sodium chloride on gastric cancer risk in the rat, Cancer research, 45 (11 Part 2), 5608-5616

182 Furihata, C. *et al.*, **1996**, Cause and effect between concentration-dependent tissue damage and temporary cell proliferation in rat stomach mucosa by NaCl, a stomach tumor promoter, Carcinogenesis, 17 (3), 401-406

183 Onitilo, A. A. *et al.*, **2012**, Diabetes and cancer II: role of diabetes medications and influence of shared risk factors, Cancer Causes Control, 23 (7), 991-1008

184 Shimizu, T. *et al.*, **2012**, [Medical management of hyperglycemia in type 2 diabetes: a consensus algorithm for the initiation and adjustment of therapy: a consensus statement of the American Diabetes Association and the European Association for the Study of Diabetes], Nihon Rinsho, 70 Suppl 3 591-601

185 Norwood, P. *et al.*, **2012**, Safety of exenatide once weekly in patients with type 2 diabetes mellitus treated with a thiazolidinedione alone or in combination with metformin for 2 years, Clin Ther, 34 (10), 2082-2090

186 Stein, S. A. *et al.*, **2013**, A review of the efficacy and safety of oral antidiabetic drugs, Expert Opin Drug Saf, 12 (2), 153-175

187 Majuri, A. *et al.*, **2007**, Rosiglitazone treatment increases plasma levels of adiponectin and decreases levels of resistin in overweight women with PCOS: a randomized placebo-controlled study, Eur J Endocrinol, 156 (2), 263-269

188 Vijay, S. K. *et al.*, **2009**, Effect of pioglitazone and rosiglitazone on mediators of endothelial dysfunction, markers of angiogenesis and inflammatory cytokines in type-2 diabetes, Acta Diabetol, 46 (1), 27-33

189 Wolf, I. *et al.*, **2005**, Diabetes mellitus and breast cancer, The lancet oncology, 6 (2), 103-111

190 He, X. X. *et al.*, **2011**, Thiazolidinediones and metformin associated with improved survival of diabetic prostate cancer patients, Ann Oncol, 22 (12), 2640-2645

191 Martin-Castillo, B. *et al.*, **2010**, Incorporating the antidiabetic drug metformin in HER2-positive breast cancer treated with neo-adjuvant chemotherapy and trastuzumab: an ongoing clinical-translational research experience at the Catalan Institute of Oncology, Ann Oncol, 21 (1), 187-189

192 Evans, J. M. *et al.*, **2005**, Metformin and reduced risk of cancer in diabetic patients, BMJ, 330 (7503), 1304-1305

193 Libby, G. *et al.*, **2009**, New users of metformin are at low risk of incident cancer: a cohort study among people with type 2 diabetes, Diabetes Care, 32 (9), 1620-1625

194 Landman, G. W. *et al.*, **2010**, Metformin associated with lower cancer mortality in type 2 diabetes: ZODIAC-16, Diabetes Care, 33 (2), 322-326

195 Bowker, S. L. *et al.*, **2006**, Increased cancer-related mortality for patients with type 2 diabetes who use sulfonylureas or insulin: Response to Farooki and Schneider, Diabetes Care, 29 (8), 1990-1991

196 Reuter, S. *et al.*, **2010**, Oxidative stress, inflammation, and cancer: how are they linked?, Free Radic Biol Med, 49 (11), 1603-1616

197 Vigneri, P. *et al.*, **2009**, Diabetes and cancer, Endocr Relat Cancer, 16 (4), 1103-1123

198 Federico, A. *et al.*, **2007**, Chronic inflammation and oxidative stress in human carcinogenesis, Int J Cancer, 121 (11), 2381-2386

199 Fried, L. & Arbiser, J. L., **2008**, The reactive oxygen-driven tumor: relevance to melanoma., Pigment Cell Melanoma Res., 21 (2), 117--122

200 Fruehauf, J. P. & Trapp, V., **2008**, Reactive oxygen species: an Achilles' heel of melanoma?, Expert Rev. Anticancer Ther., 8 (11), 1751--1757

201 Meyskens, F. L. *et al.*, **1999**, Activation of nuclear factor-kappa B in human metastatic melanomacells and the effect of oxidative stress., Clin. Cancer Res., 5 (5), 1197--1202

202 Meierjohann, S., **2014**, Oxidative stress in melanocyte senescence and melanoma transformation., Eur. J. Cell Biol., 93 (1-2), 36--41

203 Hussein, M. R., **2006**, Tumour-associated macrophages and melanoma tumourigenesis: integrating the complexity., Int. J. Exp. Pathol., 87 (3), 163--176

204 Kern, P. A. *et al.*, **2001**, Adipose tissue tumor necrosis factor and interleukin-6 expression in human obesity and insulin resistance, Am J Physiol Endocrinol Metab, 280 (5), E745-751

205 Szlosarek, P. *et al.*, **2006**, Tumour necrosis factor-alpha as a tumour promoter, Eur J Cancer, 42 (6), 745-750

206 Ryu, T. Y. *et al.*, **2014**, Hyperglycemia as a risk factor for cancer progression, Diabetes & metabolism journal, 38 (5), 330-336

207 Carmeliet, P., **2000**, Mechanisms of angiogenesis and arteriogenesis, Nat Med, 6 (4), 389-395

208 Ferrara, N. & Gerber, H. P., **2001**, The role of vascular endothelial growth factor in angiogenesis, Acta Haematol, 106 (4), 148-156

209 Carmeliet, P., **2005**, VEGF as a key mediator of angiogenesis in cancer, Oncology, 69 Suppl 3 4-10

210 Costa, R. *et al.*, **2017**, Modulation of VEGF signaling in a mouse model of diabetes by xanthohumol and 8-prenylnaringenin: Unveiling the angiogenic paradox and metabolism interplay, Mol Nutr Food Res, 61 (4), 1-27

211 De Bock, K. *et al.*, **2013**, Role of endothelial cell metabolism in vessel sprouting, Cell Metab, 18 (5), 634-647

212 Yeh, W. L. *et al.*, **2008**, Enhancement of glucose transporter expression of brain endothelial cells by vascular endothelial growth factor derived from glioma exposed to hypoxia, Mol Pharmacol, 73 (1), 170-177

213 Atsumi, T. *et al.*, **2005**, Expression of inducible 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase/PFKFB3 isoforms in adipocytes and their potential role in glycolytic regulation, Diabetes, 54 (12), 3349-3357

214 Bando, H. *et al.*, **2005**, Phosphorylation of the 6-phosphofructo-2-kinase/fructose 2,6-bisphosphatase/PFKFB3 family of glycolytic regulators in human cancer, Clin Cancer Res, 11 (16), 5784-5792

215 Schoors, S. *et al.*, **2014**, Partial and transient reduction of glycolysis by PFKFB3 blockade reduces pathological angiogenesis, Cell Metab, 19 (1), 37-48

216 Incio, J. *et al.*, **2016**, Obesity-Induced Inflammation and Desmoplasia Promote Pancreatic Cancer Progression and Resistance to Chemotherapy, Cancer Discov, 6 (8), 852-869

217 Lu, P. *et al.*, **2012**, The extracellular matrix: a dynamic niche in cancer progression, J Cell Biol, 196 (4), 395-406

218 Park, J. *et al.*, **2013**, Inhibition of endotrophin, a cleavage product of collagen VI, confers cisplatin sensitivity to tumours, EMBO Mol Med, 5 (6), 935-948

219 Fukumura, D. *et al.*, **2016**, Obesity and Cancer: An Angiogenic and Inflammatory Link, Microcirculation, 23 (3), 191-206

220 Frantz, C. *et al.*, **2010**, The extracellular matrix at a glance, J Cell Sci, 123 (Pt 24), 4195-4200

221 Zhang, C. X. *et al.*, **2016**, Association between pretreatment Glasgow prognostic score and gastric cancer survival and clinicopathological features: a meta-analysis, Onco Targets Ther, 9 3883-3891

222 Parums, D. *et al.*, **1990**, JC70: a new monoclonal antibody that detects vascular endothelium associated antigen on routinely processed tissue sections, Journal of Clinical Pathology, 43 (9), 752-757

223 Attanoos, R. *et al.*, **2000**, Malignant vascular tumours of the pleura in “asbestos” workers and endothelial differentiation in malignant mesothelioma, Thorax, 55 (10), 860-863

224 Alexander-Sefre, F. *et al.*, **2003**, Detection of tumour lymphovascular space invasion using dual cytokeratin and CD31 immunohistochemistry, Journal of clinical pathology, 56 (10), 786-788

225 Ortiz-Hidalgo, C. *et al.*, **2000**, CD31 with strong membrane-based immunoreactivity in ductal carcinoma of the breast, Applied Immunohistochemistry & Molecular Morphology, 8 (4), 334-335

226 McKenney, J. K. *et al.*, **2001**, CD31 expression in intratumoral macrophages: a potential diagnostic pitfall, The American journal of surgical pathology, 25 (9), 1167-1173

227 Mantovani, A. *et al.*, **2008**, Cancer-related inflammation, Nature, 454 (7203), 436-444

228 Grivennikov, S. I. *et al.*, **2010**, Immunity, inflammation, and cancer, Cell, 140 (6), 883-899

229 Li, C. *et al.*, **2003**, Both high intratumoral microvessel density determined using CD105 antibody and elevated plasma levels of CD105 in colorectal cancer patients correlate with poor prognosis, Br J Cancer, 88 (9), 1424-1431